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## Autophagy Suppresses Tumorigenesis Through Elimination of p62

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### SUMMARY

Allelic loss of the essential autophagy gene *beclin1* occurs in human cancers and renders mice tumor-prone suggesting that autophagy is a tumor-suppression mechanism. While tumor cells utilize autophagy to survive metabolic stress, autophagy also mitigates the resulting cellular damage that may limit tumorigenesis. In response to stress, autophagy-defective tumor cells preferentially accumulate p62/SQSTM1 (p62), endoplasmic reticulum (ER) chaperones, damaged mitochondria, reactive oxygen species (ROS), and genome damage. Moreover, suppressing ROS or p62 accumulation prevented damage resulting from autophagy defects indicating that failure to regulate p62 caused oxidative stress. Importantly, sustained p62 expression resulting from autophagy defects was sufficient to alter NF- $\kappa$ B regulation and gene expression and to promote tumorigenesis. Thus defective autophagy is a mechanism for p62 upregulation commonly observed in human tumors that contributes directly to tumorigenesis likely by perturbing the signal transduction adaptor function of p62 controlling pathways critical for oncogenesis.

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Supplemental data

Supplemental data include supplemental experimental procedures, three figures, and three tables.

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## Keywords

autophagy; beclin1; atg5; genomic instability; p62; NF- $\kappa$ B; DNA damage; cancer

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## INTRODUCTION

Macroautophagy (autophagy) targets cellular proteins, protein aggregates and organelles for degradation in lysosomes (Levine and Kroemer, 2008). Autophagy is induced by starvation or stress where double membrane vesicles (autophagosomes) capture intracellular cargo, and then fuse with lysosomes and are degraded. This lysosome-mediated cellular self-digestion sustains cell metabolism during starvation and eliminates damaged proteins and organelles that accumulate during stress. In mice, autophagy enables survival to neonatal starvation by preventing energy depletion (Kuma et al., 2004). Brain-targeted autophagy-deficiency (*atg5<sup>-/-</sup>* or *atg7<sup>-/-</sup>*) causes damaged mitochondria and polyubiquitin-containing protein aggregate accumulation, and neuronal degeneration (Hara et al., 2006; Komatsu et al., 2006). Liver-targeted autophagy deficiency (*atg7<sup>-/-</sup>*) similarly results in protein aggregate accumulation, hepatocyte cell death and liver injury (Komatsu et al., 2005). These findings support a prosurvival role for autophagy in sustaining energy homeostasis and maintaining protein and organelle quality control by eliminating damaged proteins and organelles during stress and aging (Levine and Kroemer, 2008).

Autophagy is induced by and localizes to hypoxic tumor regions where it supports cell survival (Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007; Mathew et al., 2007b). Paradoxically, autophagy defects due to allelic loss *beclin1* or constitutive activation of the autophagy-suppressing PI-3 kinase/mTOR pathway are common in human tumors (Levine and Kroemer, 2008). How loss of autophagy survival promotes tumor growth is being gradually reconciled (Jin and White, 2007, 2008; Mathew et al., 2007a).

Analogous to a wound-healing response, chronic cell death in response to stress and induction of inflammation and cytokine production may provide a non-cell-autonomous mechanism by which autophagy defects promote tumorigenesis (Degenhardt et al., 2006). Autophagy-defective tumor cells also display elevated genome damage with stress, suggesting that damage mitigation by autophagy is a cell-autonomous mechanism of tumor suppression (Karantza-Wadsworth et al., 2007; Mathew et al., 2007a; Mathew et al., 2007b). Possible non-mutually exclusive mechanisms by which autophagy limits cellular damage include maintenance of energy homeostasis, reduction of oxidative stress, and elimination of damaged proteins and organelles.

The importance of autophagy in cellular garbage disposal is clear, as autophagy is the only known means for turnover of large cellular structures such as organelles and protein aggregates (Levine and Kroemer, 2008). How organelles are recognized and directed to autophagosomes for degradation is not known, but may involve organelle-specific processes such as mitophagy and ER-phagy (Bernales et al., 2006; Kim et al., 2007). Damaged proteins that accumulate during stress can be refolded, ubiquitinated and degraded by the proteasome pathway, or aggregated and degraded by autophagy. To direct damaged or unfolded proteins to the autophagy pathway, p62 binds to polyubiquitinated proteins and aggregates by oligomerization, and binds to Atg8/LC3 on the autophagosome membrane to target aggregates to autophagosomes for degradation (Figure 1A) (Pankiv et al., 2007; Wooten et al., 2008). Protein aggregation may be a protective mechanism to limit cellular exposure to toxic proteins through sequestration, and an efficient packaging and delivery mechanism that collects and directs damaged proteins to autophagosomes. Liver-specific autophagy defects in mice cause accumulation of p62 aggregates, oxidative stress and p62-dependent hepatocyte cell death

(Komatsu et al., 2007). Thus, the inability to eliminate p62 through autophagy can be toxic to normal tissues, but whether this is related to the tumor suppression by autophagy was not known.

We report here that metabolic stress caused autophagy-defective tumor cells to preferentially accumulate p62, ER chaperones and protein disulphide isomerases (PDIs), indicative of protein quality control failure. Moreover, autophagy defects caused accumulation of damaged mitochondria, elevated oxidative stress and DNA damage response activation, which were attributed directly to persistence of p62. Cytotoxic effects due to defective autophagy were suppressed by ROS scavengers or by p62 elimination, indicating that persistence of p62 and oxidative stress caused cellular damage. This failure of autophagy-defective cells to eliminate p62 was sufficient to alter NF- $\kappa$ B regulation and gene expression and to promote tumorigenesis, indicating that autophagy suppresses tumorigenesis by limiting p62 accumulation. As p62 is commonly upregulated in human tumors (Zatloukal et al., 2007), this is due to defective autophagy and plays a causal role in cancer.

## RESULTS

### Autophagy-defective tumor cells accumulate p62 in response to stress

To address the role of autophagy-dependant protein quality control in tumor suppression, we assessed p62 modulation during metabolic stress and recovery in autophagy-competent (*beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup>) and -defective (*beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup>) immortalized baby mouse kidney (iBMK) cells. Cells were engineered to express Bcl-2, as assessment of autophagy is facilitated in an apoptosis-defective background and expression of Bcl-2 is functionally equivalent to loss of Bax and Bak in the context of autophagy modulation and tumorigenesis (Degenhardt et al., 2006; Lum et al., 2005; Nelson et al., 2004). Under normal growth conditions, endogenous p62 levels were low by indirect immunofluorescence (IF) in *beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup> cells and slightly elevated in autophagy-defective iBMK cells (Figure 1B). Following 7 days of metabolic stress p62 was induced in *beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup> cells and further elevated in autophagy-defective cells in a punctate pattern indicative of aggregation. In *beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup> cells p62 was eliminated within 24 hr of recovery, whereas p62 persisted in autophagy-defective cells often in aggregates (Figure 1B). Higher p62 levels were observed in autophagy-deficient *atg5*<sup>-/-</sup> as compared to *atg5*<sup>+/+</sup> iBMK cells stably expressing myc-tagged p62 (myc-p62) (Figure 1C). Thus, metabolic stress-induced p62 accumulation required autophagy for elimination, consistent with the absence of p62 gene induction in *beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup> tumors (see below).

### Autophagy-defective tumor cells accumulate damaged mitochondria, ER chaperones and PDIs

Apoptosis-deficient *atg5*<sup>+/+</sup> iBMK cells respond to prolonged stress by undergoing progressive autophagy, yielding cells less than one-third their original size (Degenhardt et al., 2006) with well-preserved mitochondria (M) and ER (E) (Figures 2A and 2C). In contrast, Bcl-2-expressing *atg5*<sup>-/-</sup> (Figure 2B) and *beclin1*<sup>+/-</sup> (data not shown) iBMK cells contained mitochondria with structural abnormalities (M) and abnormal cytoplasmic structures (A) resembling protein aggregates (Figures 2B and 2D), consistent with p62 accumulation (Figure 1B). Thus, autophagy functions to prevent accumulation of damaged organelles and protein aggregates during metabolic stress.

Since tumor cells with defective autophagy displayed failure of protein quality control, we performed two-dimensional difference in gel electrophoresis (2-DIGE) coupled with mass spectrometry to characterize the cellular proteome. Autophagy-competent, apoptosis-defective (*bax*<sup>-/-</sup>/*bak*<sup>-/-</sup>) D3 iBMK cells manage long-term metabolic stress by activating autophagy

(Degenhardt et al., 2006) and induced ER chaperones (glucose-regulated proteins 170 [GRP170 and GRp78] and calreticulin), PDIs, metabolism and mitochondrial proteins (Table S1). Some of these proteins (TPI-1, PGK-1, PK-3 and GPDH) are targets of hypoxia inducible factor-1  $\alpha$  (HIF-1 $\alpha$ ) indicative of metabolic adaptation (Table S1), and HIF-1 $\alpha$  is induced in iBMK cells by metabolic stress in vitro and in tumors in vivo (Nelson et al., 2004). Similarly, Bcl-2-expressing autophagy-competent *beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup> iBMK cells induced ER chaperones GRp170, GRp78, calreticulin and calnexin indicating that metabolic stress response was independent of the means of apoptosis inactivation (Figures 2C and 2D, Table S1). To determine if autophagy status altered this stress response, Bcl-2-expressing, apoptosis and autophagy-defective (*beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup>) iBMK cells were similarly examined. Autophagy-defective cells displayed preferential upregulation of ER chaperones compared to *beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup> cells (Figures 2E and 2F, Table S1). Allelic loss of *beclin1* was associated with a marked, differential increase in GRp170, GRp78, calreticulin and calnexin while *atg5*<sup>-/-</sup> cells showed differential increase in GRp170, GRp78 and calnexin compared to autophagy-competent controls (Table S1). GRp170 levels were induced by 3- to 9-fold in autophagy-competent (D3, *beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup>) cells whereas induction was 20-fold in autophagy-deficient (*beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup>) cells under metabolic stress (Figure 2F, Table S1). A similar but less striking differential increase in GRp78 was also observed in *beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup> cells (Table S1). PDI family members (PDI and PDI-P4H $\beta$ ) instrumental in folding proteins in the ER were induced under metabolic stress and were further elevated by autophagy-deficiency (Figures 2E and 2F, Table S1). The lack of induction of calreticulin and PDI-P4H $\beta$  by metabolic stress in *atg5*<sup>-/-</sup> iBMK cells (Table S1) may be due to their increased susceptibility to metabolic stress (Mathew et al., 2007b). Interestingly, levels of cytoskeletal- and protein synthesis-related proteins were repressed with stress in all cell lines (Table S1). This enhanced induction of the protein folding machinery in the autophagy-deficient cells under stress suggests a role for autophagy in mitigating ER stress by eliminating unfolded proteins through lysosomal degradation.

As individual proteins can be represented by multiple spots in 2-DIGE, complicating estimation of protein levels by spot volume ratios, quantitation was validated by Western blotting. p62 and ER chaperones GRp170, GRp78, calnexin and PDI, showed higher induction in *beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup> compared to *beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup> cells under stress (Figure 3A) consistent with p62 IF and proteomic analysis. As with p62, elevated or persistent levels of these proteins were more evident in *atg5*<sup>-/-</sup> and *beclin1*<sup>+/-</sup> cells (Figure 3A), supporting that autophagy defects elevated demand for protein folding under metabolic stress that persists during recovery.

### Autophagy defects cause sensitivity to ER stress

Since autophagy defects upregulated protein folding regulators, we compared the sensitivity of *beclin1* cells to pharmacological induction of ER stress. Tunicamycin induces ER stress by inhibiting protein glycosylation and allelic loss of *beclin1* increased sensitivity to this drug (Figure 3B). To investigate if autophagy deficiency also elevated the burden on the ubiquitin-proteasome system, sensitivity to the proteasome inhibitor epoxomicin was assessed. Autophagy-defects sensitized to proteasome inhibition, and metabolic stress increased this sensitivity (Figures 3C and 3D) suggesting an elevated dependency of autophagy-defective cells on proteasome pathway. Thus, autophagy maintains protein quality control cooperatively with the ubiquitin-proteasome pathway, consistent with suppression of proteasome function activating autophagy, the inhibition of which promotes cell death (Ding et al., 2007).

Electron Microscopy (EM) revealed preferential accumulation of morphologically abnormal mitochondria in autophagy-deficient cells (Figures 2A–2D) and stressed *atg5*<sup>-/-</sup> cells showed aberrant regulation of mitochondrial proteins such as aconitase (ACO2) (Figures 2E and 2F)



consistent with mitochondrial deterioration. ACO2 is susceptible to mitochondrial oxidative stress and upon oxidation is either stabilized or degraded (Fariss et al., 2005). Autophagy-deficient cells showed increased (*beclin1*<sup>+/-</sup>) or reduced (*atg5*<sup>-/-</sup>) levels of ACO2 after 7 days of metabolic stress compared to autophagy-competent controls (Figure 3E). Furthermore, LON, which degrades the oxidized form of ACO2 (Fariss et al., 2005), increased under metabolic stress, as did other oxidative stress markers. The accelerated deterioration of mitochondria in *atg5*<sup>-/-</sup> compared to *beclin1*<sup>+/-</sup> cells may account for the reduction in ACO2, superoxide dismutase (SOD2) and peroxiredoxin (PRDX3) at 7 days of stress (Figure 3E). Thus autophagy defects are associated with accumulation of damaged mitochondria under stress.

### Defects in autophagy upregulate p62 and ER chaperones in tumors

To assess whether the differential accumulation of p62, ER chaperones and PDI was also a feature of autophagy defects in tumors, Bcl-2-expressing *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup>, and *beclin1*<sup>+/+</sup> and *beclin1*<sup>+/-</sup> iBMK tumor allografts, and spontaneous tumors from *beclin1*<sup>+/-</sup> mice were examined. As in iBMK cells with allelic loss of *beclin1* (Degenhardt et al., 2006; Mathew et al., 2007b), deficiency in *atg5* increased tumorigenesis and cooperated with defects in apoptosis to accelerate tumor growth (Figure 3F and G). Bcl-2-expressing *atg5*<sup>-/-</sup> tumors displayed elevated p62, GRp170, GRp78, calnexin, and PDI compared to *atg5*<sup>+/+</sup> tumors by Western blotting (Figure 3H) as did Bcl-2-expressing *beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup> tumors by immunohistochemistry (IHC) (data not shown). p62 was not transcriptionally upregulated in *beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup> tumors as determined in the gene expression analysis (p62 mRNA expression varied between 0.9- and 1.3-fold among wild type and autophagy-defective tumors) (data not shown). Upregulation of ubiquitinated proteins, although common in tissues of autophagy-defective mice (Hara et al., 2006; Komatsu et al., 2006), was not striking in *atg5*<sup>-/-</sup> tumors (Figure 3H). Additionally, tissues (lung, heart, and liver) and spontaneous lung and liver tumors from 1.5 year-old *beclin1*<sup>+/-</sup> mice showed significant accumulation of p62 and GRp170 when compared with age-matched tissues from *beclin1*<sup>+/+</sup> littermates (Figures 3I and 3J). Thus, allelic loss of *beclin1* and impaired autophagy caused elevated ER chaperone levels as a compensation mechanism and this phenotype is manifested in tissues and spontaneous tumors.

While p62 and ER chaperone upregulation are common in human tumors and are associated with poor prognosis, the cause is unknown (Ni and Lee, 2007; Zatloukal et al., 2007). Human hepatocellular carcinoma (HCC) is associated with p62 accumulation in Mallory-Denk bodies, and *beclin1*<sup>+/-</sup> mice display p62 accumulation in liver in association with steatohepatitis and spontaneous liver tumors (Figure 3J) (Komatsu et al., 2007; Qu et al., 2003; Yue et al., 2003), suggesting that autophagy defects may play a prominent role in HCC etiology. Indeed, liver and spontaneous liver tumors from *beclin1*<sup>+/-</sup> mice also showed higher levels of p62, GRp170 and DNA damage response activation ( $\gamma$ -H2AX) compared to normal liver tissue from age-matched *beclin1*<sup>+/+</sup> mice (Figure 3J). p62, GRp170 and  $\gamma$ -H2AX were examined in a panel of human liver and HCCs in a tissue microarray (TMA). p62, GRp170, and  $\gamma$ -H2AX were upregulated with high frequency in HCC compared to normal liver (Figure 3K). Thus, accumulation of p62 and GRp170 in human tumors may be biomarkers for defective autophagy manifesting as accumulation of unfolded proteins associated with activation of the DNA damage response. Moreover, failure of protein and organelle quality control caused by autophagy defects may cause oxidative stress that is genotoxic.

### Autophagy mitigates oxidative stress and progression to aneuploidy

Activation of the DNA damage response is a hallmark of oxidative stress caused by ROS. Protein re-folding in the ER by PDIs can elevate oxidative stress through redox reactions involving free radicals (Tu and Weissman, 2004), and mitochondrial stress and damage can

also be a source of ROS (Fariss et al., 2005) in autophagy-deficient cells. Since autophagy deficiency causes accumulation of damaged mitochondria and the oxidative protein folding machinery and activated DNA damage response, we examined ROS levels in *beclin1*<sup>+/+</sup> and *beclin1*<sup>+/-</sup> cells. Under normal growth conditions ROS levels were slightly elevated in the *beclin1*<sup>+/-</sup> iBMK cells compared to the *beclin1*<sup>+/+</sup> cells, however, 5 days of metabolic stress caused a marked ROS increase in *beclin1*<sup>+/-</sup> cells (Figures 4A and 4B). During recovery, *beclin1*<sup>+/-</sup> iBMK cells showed increased ROS that persisted for 24 hr compared to *beclin1*<sup>+/+</sup> cells (Figures 4A and 4B). Thus allelic loss of *beclin1* was associated with elevated ROS production with stress.

To determine if the elevated ROS in autophagy-deficient cells contributes to cellular damage, the stress response without and with the ROS scavenger, N-acetyl cysteine (NAC) was examined. Following 5 days of metabolic stress, *beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup> iBMK cells showed increased susceptibility to stress compared to control cells (Figure 4C and S2) (Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007; Mathew et al., 2007b). The presence of NAC during metabolic stress improved survival, and this protective effect was more profound in *beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup> cells, suggesting that elevated ROS contribute to increased susceptibility of autophagy-defective cells to stress (Figure 4C and S2). This enhanced survival provided by NAC was associated with decreased p62 accumulation during metabolic stress in the *beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup> iBMK cells (Figure 4D and S2), suggesting that ROS-mediated oxidative stress leads to protein damage and accumulation of p62. A feature of genomic instability associated with autophagy defects is the accelerated progression to aneuploidy (Mathew et al., 2007b). To examine the role of increased ROS levels on genomic instability, we monitored the DNA content of early passage diploid *beclin1*<sup>+/+</sup> and *beclin1*<sup>+/-</sup> iBMK cells without and with NAC. *beclin1*<sup>+/+</sup> cells maintained diploidy after 40 passages and the presence of NAC had no effect. In contrast, *beclin1*<sup>+/-</sup> cells showed accelerated progression to aneuploidy by passages 18 and 39 (Figure 4E), and NAC delayed this progression (Figure 4E), indicating a causative role for basal ROS-mediated oxidative stress in progression to aneuploidy associated with autophagy defects. Thus, metabolic stress causes p62 accumulation mediated in part by elevated ROS production and the failure to suppress ROS in autophagy-deficient cells is associated with genomic instability.

### p62 accumulation activates the DNA damage response

Since autophagy deficiency leads to accumulation of p62, oxidative stress and accelerated progression to aneuploidy, we examined if p62 accumulation was sufficient to induce ROS and the DNA damage response. Transiently expressed p62-EGFP formed aggregates (Figure S1B) and elevated ROS in the autophagy-deficient (*beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup>) but not in *beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup> iBMK cells, whereas EGFP expression alone did not (Figures 5A and S1A). Transient p62-EGFP expression was also associated with DNA damage response activation ( $\gamma$ -H2AX positive nuclear foci) in autophagy-deficient (*beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup>) cells compared to *beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup> cells (Figures S1B and S1C). To monitor accumulation and clearance of p62, apoptosis-deficient *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> iBMK cells engineered to stably express either EGFP or p62-EGFP were subjected to metabolic stress followed by recovery. As with endogenous p62 (Figure 1B) and myc-p62 (Figure 1C), p62-EGFP-expressing *atg5*<sup>-/-</sup> iBMK cells displayed p62 aggregates that were induced by metabolic stress and persisted in recovery which *atg5*<sup>+/+</sup> cells were able to eliminate (Figures 5B and 5C). Consistent with transient expression (Figure S1B), stable p62-EGFP expression activated the DNA damage response during stress and recovery as indicated by  $\gamma$ -H2AX positive nuclear foci. While the focal pattern of  $\gamma$ -H2AX staining characteristic of DNA double strand breaks (Balajee and Geard, 2004) was clear in majority of the  $\gamma$ -H2AX positive nuclei, there were also nuclei showing uniform  $\gamma$ -H2AX staining indicating variations in the levels of DNA damage. *atg5*<sup>-/-</sup> iBMK cells expressing EGFP showed markedly elevated levels of supernumerary

centrosomes compared to *atg5<sup>+/+</sup>* cells under normal conditions, but more strikingly so following stress and recovery. Expression of p62-EGFP increased centrosome abnormalities and multi-polar divisions, which were more dramatic under stress and recovery in *atg5<sup>-/-</sup>* compared to *atg5<sup>+/+</sup>* cells (Figure S1E and S1F). Thus p62 accumulation was sufficient for ROS and DNA damage response induction under metabolic stress leading to cell division abnormalities and genomic instability in autophagy-defective cells. Indeed, RNAi-mediated knockdown of p62 during metabolic stress reduced DNA damage induction in autophagy-deficient *beclin1<sup>+/-</sup>* and *atg5<sup>-/-</sup>* iBMK cells, further suggesting that impaired p62 elimination was the cause of DNA damage response activation (Figure 5D–5F).

### p62 promotes tumorigenesis of autophagy-defective cells

Since p62 accumulates in tissues and tumors from autophagy-deficient mice and in human cancers, we tested if p62 directly contributed to tumorigenesis. Apoptosis-deficient *atg5<sup>+/+</sup>* and *atg5<sup>-/-</sup>* iBMK cell lines expressing EGFP or p62-EGFP (Figure 6A) were assessed for their tumorigenic potential. p62-EGFP expression in *atg5<sup>+/+</sup>* cell lines did not substantially increase tumor growth (Figure 6B). In contrast, p62-EGFP expression in *atg5<sup>-/-</sup>* cells, dramatically increased tumor growth compared to EGFP-expressing *atg5<sup>-/-</sup>* controls (Figure 6B). In contrast to EGFP-expressing *atg5<sup>-/-</sup>* tumors displaying diffuse cytoplasmic EGFP localization and uniformly sized nuclei with occasional appearance of tumor giant cells, p62-EGFP-expressing *atg5<sup>-/-</sup>* tumors displayed dramatic p62 aggregate accumulation and numerous pleomorphic tumor cells with heterochromatic, giant nuclei by H&E staining indicative of polyploidy and aneuploidy (Figure 6D). Persistent p62 accumulation in p62-EGFP-expressing *atg5<sup>-/-</sup>* tumors was also associated with elevated DNA damage response induction ( $\gamma$ -H2AX) compared to EGFP-expressing *atg5<sup>-/-</sup>* tumors (Figure 6D), suggesting that inability to clear p62 through autophagy promoted tumor growth and elevated DNA damage and genomic instability.

In addition to its role in binding and targeting polyubiquitinated proteins to autophagosomes for lysosomal degradation, p62 also has a role as an adaptor protein regulating signal transduction in the RANKL, TrkA, and aPKC pathways through interactions with TRAF6 and RIP1 that can regulate NF- $\kappa$ B (Moscat et al., 2007; Wooten et al., 2008). To address the possibility that deregulation of p62 in autophagy defective cells altered cancer signaling pathways, global patterns of gene expression were analyzed in *atg5<sup>-/-</sup>* EGFP- and EGFP-p62-expressing iBMK tumors (Figure 6). Of the 14,000 genes assessed, 893 genes ( $p=0.05$ ) displayed differential expression in EGFP- compared with EGFP-p62-expressing tumors and were further subjected to Ingenuity Pathway Analysis (IPA) and Gene Set Enrichment Analysis (GSEA) (Figure 7A, supplemental Tables S2 and S3). Both analyses indicated down-regulation of host defense pathways including antigen presentation, Toll-like receptor and Natural Killer (NK) cell mediated cytotoxicity pathways in p62-EGFP- compared to EGFP-expressing tumors (Figure 7A and supplemental Table S2). Interaction map analysis indicated that a commonality in the pathways suppressed by p62 gain-of-function was the NF- $\kappa$ B pathway, and indeed NF- $\kappa$ B target genes were down regulated in p62-EGFP compared to EGFP-expressing tumors (Table S3).

Allelic loss of *beclin1* causes steatohepatitis, Mallory body formation (p62 aggregates), and HCC (Fig. 4E), which phenocopies hepatocyte-targeted deficiency in NF- $\kappa$ B activators IKK- $\beta$  or NEMO (Luedde et al., 2007; Maeda et al., 2005). To test the hypothesis that p62 gain-of-function was responsible for the suppression of NF- $\kappa$ B, IL-6-luciferase reporter assays were performed in autophagy-competent (*beclin1<sup>+/+</sup>* and *atg5<sup>+/+</sup>*) and autophagy-deficient (*beclin1<sup>+/-</sup>* and *atg5<sup>-/-</sup>*) iBMK cells in response to TNF- $\alpha$ . Despite similar basal levels, autophagy-deficient cells showed reduced NF- $\kappa$ B activation in response to TNF- $\alpha$ , which was further inhibited by p62 expression (Figures 7B, 7C and S3) suggesting that p62 accumulation

impaired NF- $\kappa$ B activation. In liver, deficient NF- $\kappa$ B canonical pathway activation leads to impaired hepatocyte survival, Kupffer cell activation and hepatomitogen (II-6, TNF- $\alpha$ , hepatocyte growth factor) production leading to non-canonical NF- $\kappa$ B pathway activation and compensatory proliferation of healthy hepatocytes and to HCC. To test if defective autophagy and p62 accumulation caused the same phenotype, nuclear localization of p50 NF- $\kappa$ B indicative of activation was assessed in liver tissue from age-matched *beclin1*<sup>+/+</sup> and *beclin1*<sup>+/-</sup> mice. Nuclear p50 and active caspase-3 were observed in many *beclin1*<sup>+/-</sup> liver and liver tumor hepatocytes but not in *beclin1*<sup>+/+</sup> livers (Figure 7D). Similarly, p65 NF- $\kappa$ B was frequently nuclear in *beclin1*<sup>+/-</sup> liver tumor hepatocytes but was not nuclear in *beclin1*<sup>+/+</sup> liver tissue (Figure 7E). Importantly, p62 accumulation in hepatocytes was heterogenous (Figure 3J). *beclin1*<sup>+/-</sup> hepatocytes that accumulated high p62 did not display nuclear p65 whereas those with less p62 showed nuclear localization of p65 (Figure 7E). This suggests that as in the IKK $\beta$ - and NEMO-deficient hepatocytes, defective autophagy and deregulation of p62 was associated with suppression of the canonical NF- $\kappa$ B pathway, impaired survival and hepatomitogen-driven oncogenesis through the non-canonical pathway. Indeed, *beclin1*<sup>+/-</sup> livers and liver tumors displayed increased TNF- $\alpha$  production (Figure 7D).

## DISCUSSION

Accumulation of p62 in response to metabolic stress is a striking phenotype of autophagy-defective tumor cells, suggesting defective protein quality control may contribute to tumorigenesis and that autophagy is the main mechanism by which tumor cells turnover p62. Moreover, the failure of autophagy-defective tumor cells to eliminate p62 was sufficient for tumorigenesis. Unlike brain tissue (Hara et al., 2006; Komatsu et al., 2006), there was no accumulation of polyubiquitinated proteins in autophagy-defective tumor cells. There may be tissue-specific differences in autophagy-mediated protein elimination or dilution of polyubiquitinated proteins through cell proliferation in tumor cells may prevent their accumulation, which is not possible in post-mitotic neurons. Alternatively, proteasome-mediated turnover of polyubiquitinated proteins may be elevated in tumor cells compared to neuronal tissues. Indeed, autophagy defects sensitized cancer cells to proteasome inhibitors, suggesting a compensatory function of the two protein degradation pathways.

The persistence of p62 and accumulation of ER chaperones and the oxidative protein folding machinery in autophagy-deficient cells and tumors indicated a defect in the management of protein turnover. The inability to degrade damaged or misfolded proteins through autophagy may increase the burden on the ER protein folding machinery necessitating its upregulation. Both p62 and GRp170 were dramatically upregulated in *beclin1*<sup>+/-</sup> tissues as well as in spontaneous tumors, indicating that coping with unfolded proteins may be a biomarker for impaired autophagy that precedes tumor initiation. ER chaperone and PDI upregulation are common in human tumors (Goplen et al., 2006; Ni and Lee, 2007), and increased GRp170 expression is associated with poor prognosis in breast cancer (Tamatani et al., 2001). Although the cause of ER chaperone accumulation in tumors was not known, chaperones are stress-responsive and provide a protective function by suppressing the accumulation of unfolded proteins that may be an important compensatory mechanism for autophagy-defective cells. Protein folding is a source of oxidative stress (Tu and Weissman, 2004), particularly when cells are overburdened with damaged and unfolded proteins, in concordance with increased ROS in stressed *beclin1*<sup>+/-</sup> cells.

Stressed autophagy-defective tumor cells accumulate damaged mitochondria as a potential additional source of oxidative stress. This accumulation of unfolded protein and protein aggregates and the persistence of damaged mitochondria may collectively lead to elevated ROS production in autophagy-defective cells. As ROS scavengers partially suppressed p62 accumulation and cell death in stressed *beclin1*<sup>+/-</sup> tumor cells, the elevated oxidative stress

may contribute to p62 induction, cell damage and death. Although oxidative stress and DNA damage arise through multiple genotoxic events (Halazonetis et al., 2008), stress-mediated p62 accumulation in autophagy-defective cells was sufficient for ROS and DNA damage response induction that was prevented by knockdown of p62, establishing that that elevated oxidative stress was attributable directly to p62 accumulation. Enforced p62 expression induced ROS, suggesting a possible amplification loop where oxidative stress induces p62 accumulation, which in turn amplifies ROS generation. Thus, the inability of autophagy-defective tumor cells to eliminate p62 contributes to oxidative stress and likely to DNA damage. These observations are strikingly similar to the rescue of oxidative stress toxicity caused by *atg7* deficiency with p62 deficiency in mouse liver (Komatsu et al., 2007). In normal tissues, toxicity due to p62 accumulation resulting from autophagy defect may trigger cell death, whereas in checkpoint-defective tumor cells this instead may also result in enhancement of mutations, genome instability and tumor progression.

*atg5*<sup>-/-</sup> tumors displayed pronounced p62 and p62 aggregate accumulation and this p62 expression was sufficient to activate the DNA damage response and to enhance tumor growth. p62 expression, p62 aggregates, and Mallory-Denk bodies containing p62 are common in steatosis and in HCC and other cancers (Zatloukal et al., 2007). Defects in autophagy may be a mechanism for sustained p62 accumulation and formation of Mallory-Denk bodies. As such, p62 accumulation is not merely a histologic marker for certain cancers, but rather, directly contributes to tumor growth. While the prevalence of autophagy defects in HCC is not yet known, mutations such as *pten* loss that constitutively activates the PI3-kinase pathway and mTOR that inhibits autophagy are common (Wong and Ng, 2008). Interestingly, *pten* (Watanabe et al., 2005), *beclin1*, or *atg7* deficiency (Komatsu et al., 2007; Qu et al., 2003; Yue et al., 2003) produce liver steatosis in mice suggesting that suppression of autophagy and the resulting steatosis can lead to HCC.

How persistent p62 promotes oxidative stress and tumorigenesis appears to be related to its role as an adaptor protein regulating receptor signaling and the activation of NF- $\kappa$ B. p62 is also required for efficient oncogene activation *in vitro* and p62 deficiency suppresses spontaneous lung tumorigenesis by *K-ras* (Duran et al., 2008). Thus, p62 had been identified as an oncoprotein in both loss- (Duran et al., 2008) and gain-of-function situations (Figure 6). p62 gain-of-function caused by defective autophagy altered NF- $\kappa$ B signal transduction pathways that regulate host defense. Whether p62 is upregulated and either sequestered in non-functional aggregates inhibiting signal transduction as indicated here, or is retained in an active state enhancing signal transduction, may be cell type- or stress-specific. In liver, defects in NF- $\kappa$ B canonical pathway activation promote tumorigenesis by stimulation of inflammation and activation of the non-canonical NF- $\kappa$ B pathway. However, this function of NF- $\kappa$ B may be tissue specific, and as NF- $\kappa$ B signaling also regulates anti-oxidant defense, suppression of NF- $\kappa$ B by p62 may explain increased oxidative stress in other autophagy-defective tissues. In conclusion, defects in autophagy promote a failure of protein and organelle quality control in tumors this leads to p62 accumulation resulting in perturbation of gene expression, increased oxidative stress, genome damage and tumorigenesis (Figure 7F). As p62 upregulation is common in liver tissues of individuals at risk and hepatocellular carcinomas in patients, this suggests that facilitating the clearance of p62 by promoting autophagy may be a strategy for cancer chemoprevention.

## EXPERIMENTAL PROCEDURES

### Generation of stable cell lines and culture conditions

*atg5*<sup>+/+</sup>, *atg5*<sup>+/-</sup>, *atg5*<sup>-/-</sup>, *beclin1*<sup>+/+</sup> and *beclin1*<sup>+/-</sup> iBMK cell lines were described previously (Degenhardt et al., 2006; Degenhardt et al., 2002; Mathew et al., 2008; Mathew et al., 2007b). Bcl-2-expressing *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> iBMK cells, were engineered to stably express



Myc-tagged p62 (pcDNA3-myc-p62), EGFP (pEGFPC1) or p62-EGFP (pEGFPC1-p62) (Rodriguez et al., 2006), or were co-transfected with pcDNA3-Zeo by electroporation as described previously (Nelson et al., 2004). Independent clones were selected in zeocin (500µg/mL) and expanded as stable cell lines in normal culture conditions (DMEM, 10% FBS, 1% Pen Strep (Invitrogen, Carlsband, CA) at 38.5°C and 8.5% CO<sub>2</sub>) (Mathew et al., 2008). To induce metabolic stress, cells were placed in glucose-free DMEM (Invitrogen) containing 10% FBS and incubated with a defined gas mixture containing 1% oxygen, 5% CO<sub>2</sub> and 94% N<sub>2</sub> (GTS-Welco, Allentown, PA) (Nelson et al., 2004). NAC (Sigma-Aldrich, St. Louis, MO) was used at a concentration of 1mM.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

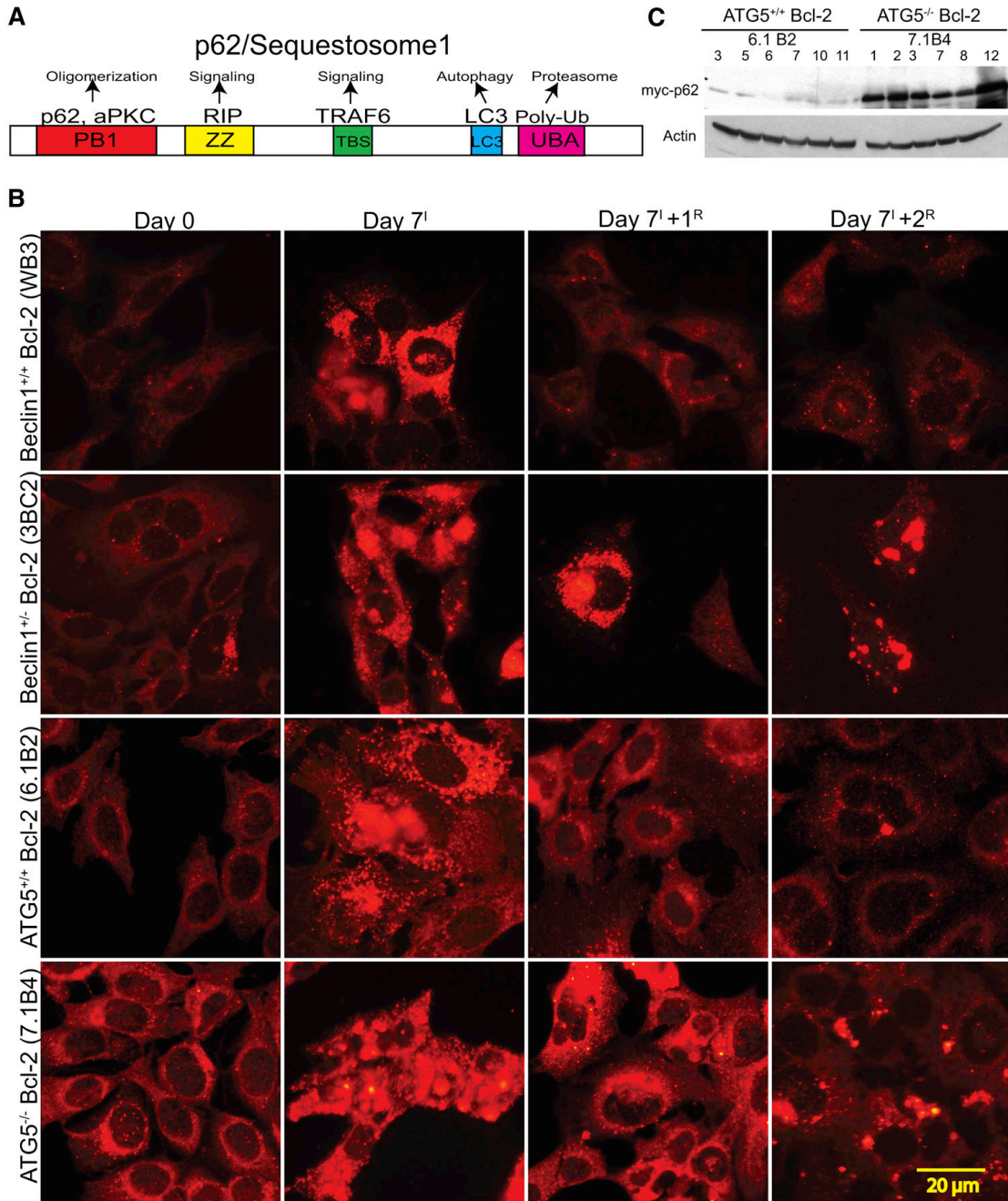
We thank Drs. Heintz, Yue and Jin for providing *beclin1<sup>+/+</sup>* and *beclin1<sup>+/-</sup>* mice, Dr. Mizushima for providing *atg5<sup>+/+</sup>* and *atg5<sup>-/-</sup>* mice, Dr. Zimmermann for GRp170 antibody and Dr. Moscat for myc-p62 and p62-EGFP plasmids. This work was supported by grants from the NIH (R37 CA53370 and RO1 CA130893) to E.W., (K99CA133181) to V.K.W., and DOD (W81XWH06-1-0514 and W81XWH05) to E.W. and R.S.D.

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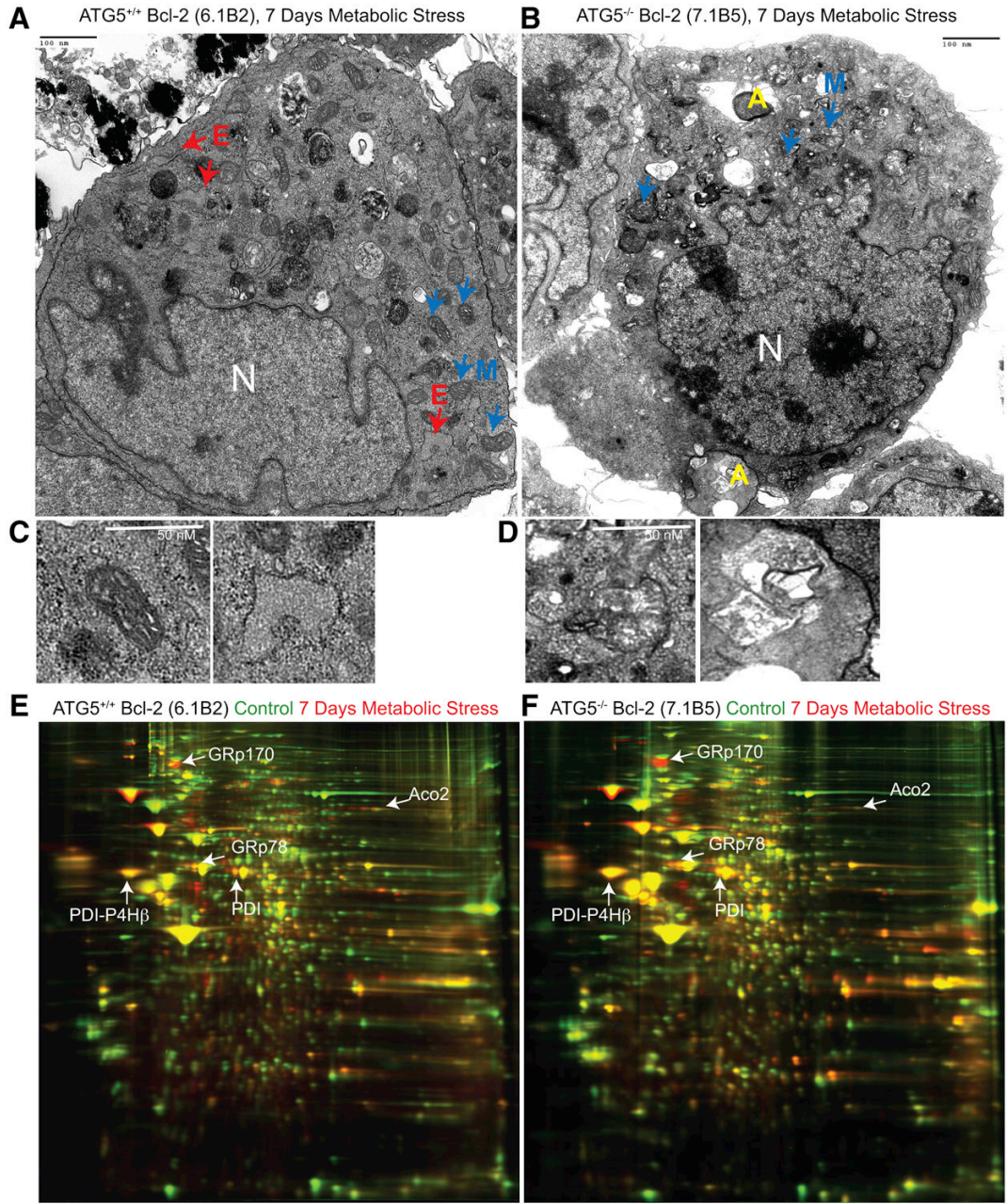
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**Figure 1. Elevated and persistent p62 in autophagy-defective tumor cells under metabolic stress**  
 (A) Domain organization of p62 illustrating the Phox and Bem1p (PB1) oligomerization domain (p62 and atypical Protein Kinase C [aPKC]), the Zinc finger (ZZ) Rip 1 binding domain, the TRAF6 binding site (TBS), the microtubule associated protein light chain 3 (LC3) domain (LC3/ATG8 binding), and the ubiquitin-associated (UBA) (poly-ubiquitin binding).  
 (B) IF of endogenous p62 showing accumulation and persistence of p62 in autophagy-defective cells under normal growth conditions (Day 0), 7 days of metabolic stress (Day 7<sup>I</sup>), and 1 (Day 7+1<sup>R</sup>) and 2 (Day 7+2<sup>R</sup>) days of recovery.  
 (C) Autophagy-defective cells express constitutively higher levels of exogenous Myc-p62. Six independent cell lines of Bcl-2-expressing *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> iBMK cells stably expressing

Myc-tagged p62 were evaluated for p62 expression levels by Western blotting with an antibody to Myc tag.





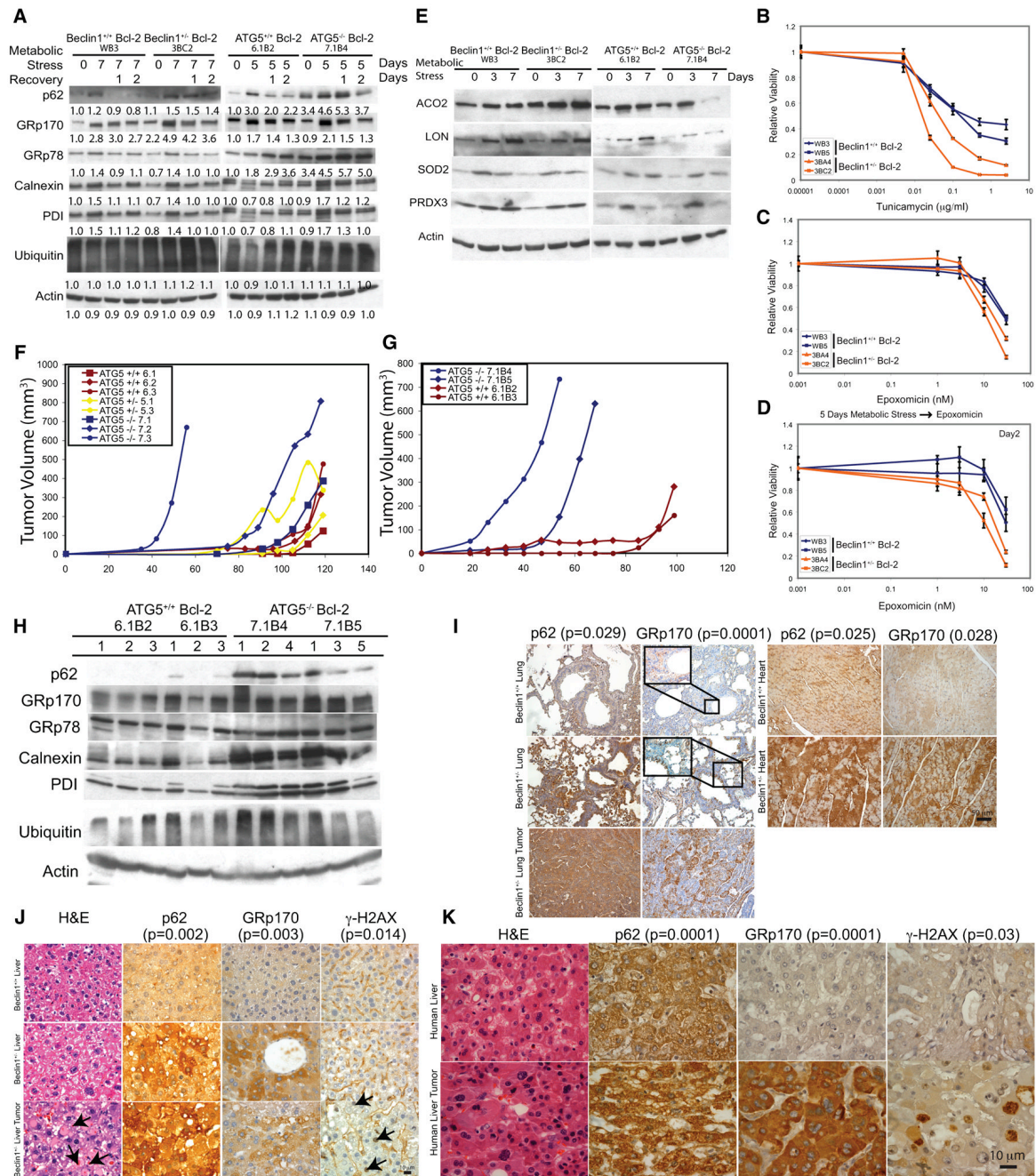
**Figure 2. Metabolic stress promotes organelle damage and ER chaperones and PDI upregulation in autophagy-deficient cells**

(A–D) Representative electron micrographs of Bcl-2-expressing *atg5*<sup>+/+</sup> (A) or *atg5*<sup>-/-</sup> (B) cells following 7 days of metabolic stress. Bcl-2 expressing *atg5*<sup>+/+</sup> iBMK cell (A) showing mitochondria (M, blue arrows and C, left panel), ER (E, red arrows and C, right panel), mutant cell in (B) showing mitochondria (M, blue arrows and D, left panel), and protein aggregates (A, yellow and D, right panel).

(E and F) 2-DIGE gels showing differential regulation of ER chaperones (GRp170, GRp78), PDI, PDI-P4H $\beta$  and ACO2 in Bcl-2-expressing *atg5*<sup>-/-</sup> iBMK cells in response to metabolic stress (7 days). Total protein from unstressed or metabolically stressed (7 days) Bcl-2-expressing *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> cell lines were labeled with Cy3 (Green-unstressed) or Cy5

(Red-stressed) and analyzed by 2-DIGE. Images show 2-DIGE gels with proteins that are induced (Red), repressed (Green) or unchanged (Yellow) under metabolic stress. Protein spots (n=106) that were differentially expressed were selected and identified by mass spectrometry.





**Figure 3. Autophagy defects promote ER stress and elevated DNA damage response in tumors in vivo**

(A) Western blots showing levels of p62, ER chaperones, PDI and ubiquitin in Bcl-2-expressing *beclin1*<sup>+/+</sup>, *beclin1*<sup>+/-</sup>, *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> iBMK cells following 5 or 7 days of metabolic stress, and 1 and 2 days of recovery. Values below the bands represent relative signal intensities compared to the basal protein level in the first lane in each group (untreated Bcl-2-expressing *beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup> control).

(B–D) Allelic loss of *beclin1* sensitizes cells selectively to ER stress and proteasome inhibition. MTT assays showing sensitivity of Bcl-2-expressing *beclin1*<sup>+/+</sup> (Blue) and *beclin1*<sup>+/-</sup> (Red) iBMK cells in response to increasing concentrations of (B) tunicamycin (3 days), or (C)

epoxomycin (2 days), and (D) following 5 days of metabolic stress. Data are presented as mean  $\pm$  SD.

(E) Autophagy deficiency causes mitochondrial damage. Western blots showing levels of mitochondrial proteins (ACO2, LON, SOD2 and PRDX3) in Bcl-2-expressing *beclin1*<sup>+/+</sup>, *beclin1*<sup>+/-</sup>, *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> iBMK cells, following metabolic stress.

(F) Deficiency in *atg5* in iBMK cells promotes tumorigenesis. Tumor allograft growth of *atg5*<sup>+/+</sup> (red), *atg5*<sup>+/-</sup> (yellow) and *atg5*<sup>-/-</sup> (blue) iBMK cell lines in nude mice.

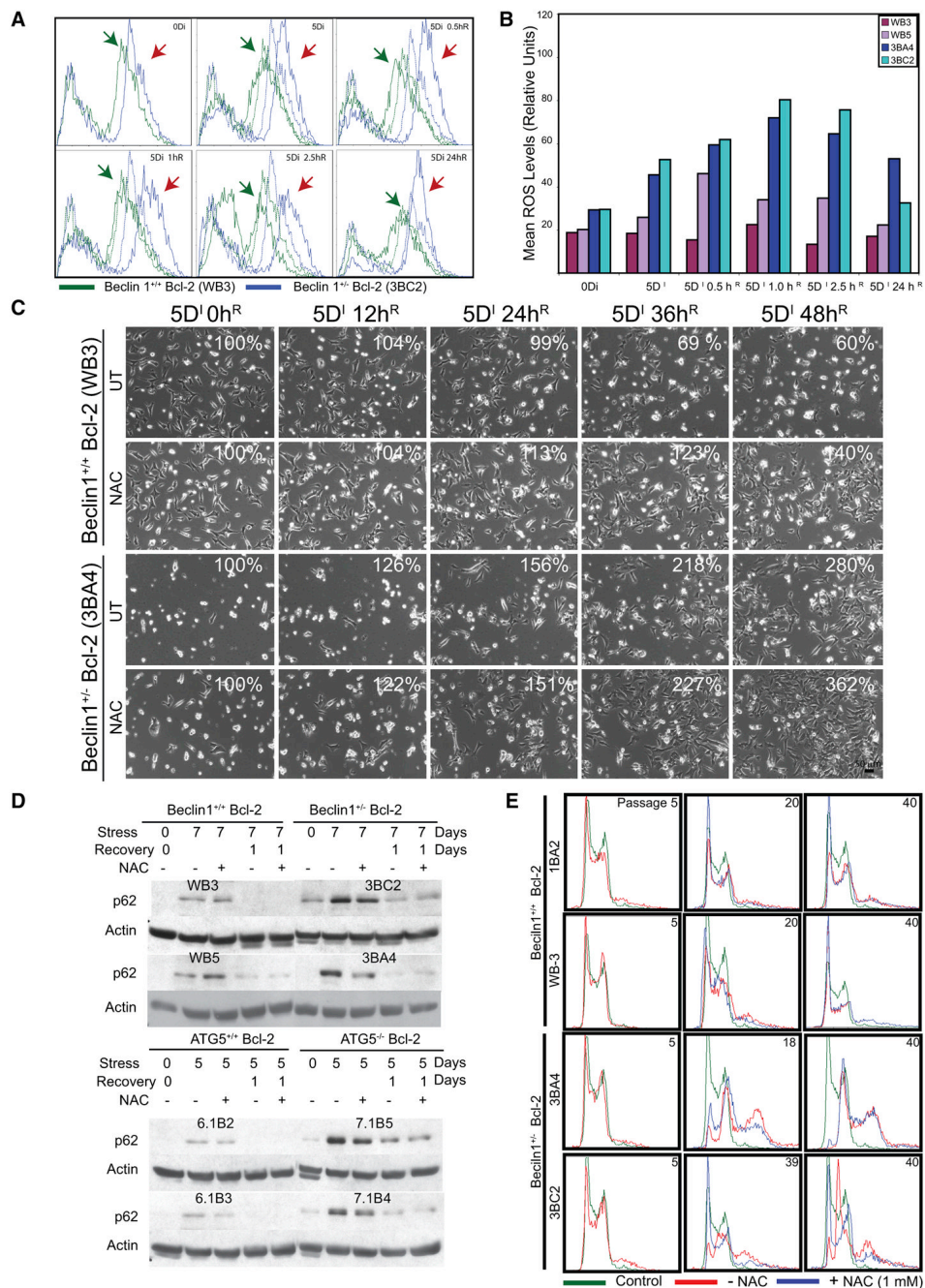
(G) Deficiency in *atg5* cooperates with defective apoptosis enhances tumor growth. Tumor allograft growth of Bcl-2-expressing *atg5*<sup>+/+</sup> (red), and *atg5*<sup>-/-</sup> (blue) iBMK cell lines in nude mice.

(H) Western blot showing elevated p62 and ER chaperones and PDI in Bcl-2-expressing *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> iBMK tumors in

(I) Elevated p62 and GRp170 levels in lung and heart tissues and spontaneous lung tumors from *beclin1*<sup>+/-</sup> mice. Lung, heart and spontaneous lung tumor sections from two 1.5-year-old *beclin1*<sup>+/+</sup> and four 1.5-year-old *beclin1*<sup>+/-</sup> mice were stained for p62 and GRp170 by IHC. Sections were independently scored and analyzed by Students t-test (lung) or Mann-Whitney test (heart) and a  $p < 0.05$  was considered statistically significant.

(J) Elevated p62 and GRp170 in liver tissue and p62, Mallory-Denk bodies (H&E, arrows) and  $\gamma$ -H2AX (arrows) in spontaneous liver tumors from *beclin1*<sup>+/-</sup> mice (1.5 years). Sections were independently scored and analyzed by Students t-test for significance ( $p < 0.05$ ).

(K) Elevated p62, GRp170, and  $\gamma$ -H2AX positive nuclei in human HCC. Representative images from a human liver TMA (46 samples), showing H&E, p62, GRp170 and  $\gamma$ -H2AX accumulation in HCCs. Representative images from a normal human liver TMA (14 samples) are shown for comparison. Sections were independently scored and analyzed by Students t-test for significance for significance ( $p < 0.05$ ).



**Figure 4. Metabolic stress promotes elevated ROS production and chromosomal instability in autophagy-deficient cells**

(A) Autophagy-deficiency leads to elevated ROS production. Overlays show ROS levels in Bcl-2-expressing *beclin1*<sup>+/+</sup> (Green) and *beclin1*<sup>+/-</sup> iBMK cells (Blue) (x-axis, log scale) under normal growth (ODi) and at 0.5, 1, 2.5 and 24 hr (*beclin1*<sup>+/+</sup>, green arrows and *beclin1*<sup>+/-</sup>, red arrows) during recovery (0.5–24hR) from 5 days of metabolic stress (5Di) by flow-cytometry using the ROS sensor DCF-DA. The ROS levels in untreated cells are shown in dotted lines for comparison.

(B) Representative histogram from three independent experiments measuring the mean ROS levels in Bcl-2-expressing *beclin1*<sup>+/+</sup> and *beclin1*<sup>+/-</sup> iBMK cells shown in (A).



(C) ROS scavenging partially rescues the susceptibility to metabolic stress and recovery due to allelic loss of *beclin1*. Representative time-lapse images of Bcl-2-expressing *beclin1*<sup>+/+</sup> and *beclin1*<sup>+/-</sup> iBMK cells during recovery following 5 days of metabolic stress in presence (NAC) and absence (UT) of the ROS scavenger NAC (1mM) (relative percentage of adherent cells compared to time 0 is shown).

(D) ROS scavenging suppresses p62 accumulation in autophagy-deficient (*beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup>) cells. Western blot analysis of p62 levels in Bcl-2-expressing *beclin1*<sup>+/+</sup>, *beclin1*<sup>+/-</sup>, *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> iBMK cell lines following 0 or 7 days of metabolic stress followed by 1 day recovery without and with NAC.

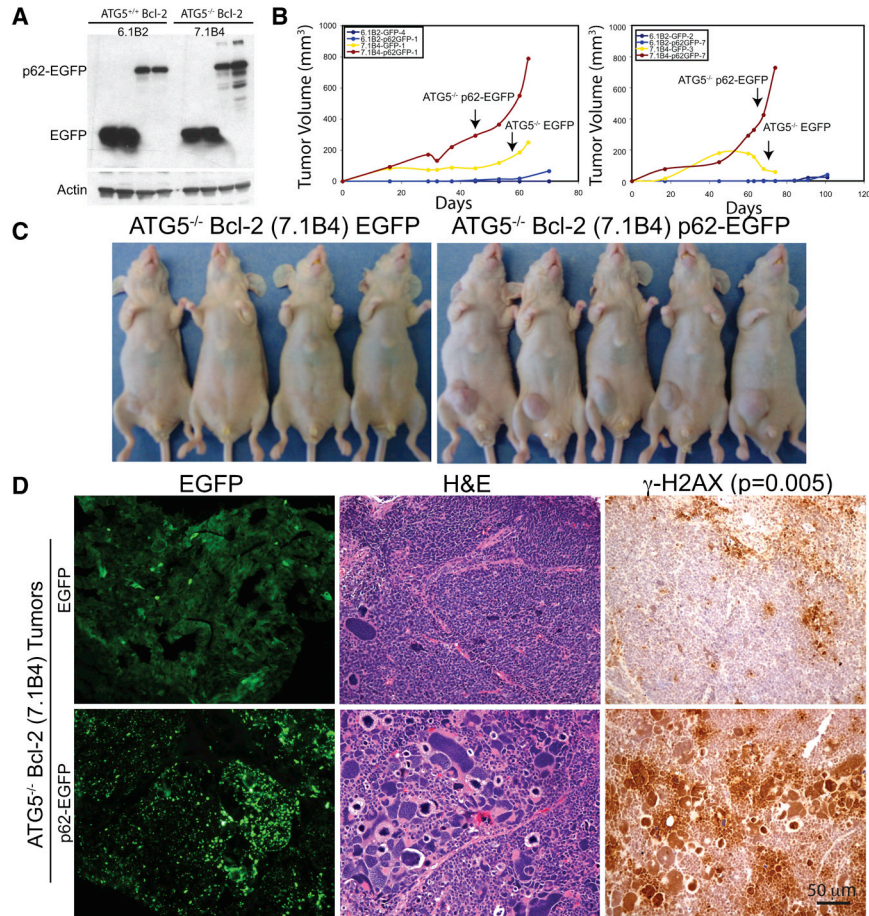
(E) ROS scavenging limits progression to aneuploidy associated with allelic loss of *beclin1*. Flow-cytometry analysis of DNA content in diploid, Bcl-2-expressing *beclin1*<sup>+/+</sup> and *beclin1*<sup>+/-</sup> iBMK cells grown in presence (blue) or absence (red) of the ROS scavenger NAC (1mM). Numbers represent passage numbers at which ploidy was determined.



(D) Western blots of RNAi-knockdown of p62. Bcl-2-expressing wild-type (*beclin1*<sup>+/+</sup> and *atg5*<sup>+/+</sup>) and autophagy-defective (*beclin1*<sup>+/-</sup> and *atg5*<sup>-/-</sup>) iBMK cells were transfected with either Lamin- or p62-siRNA and subjected to metabolic stress for 0, 24, 48 or 72 hr (24, 48, 72 and 96 hr post-transfection, respectively) and analyzed for p62 levels.

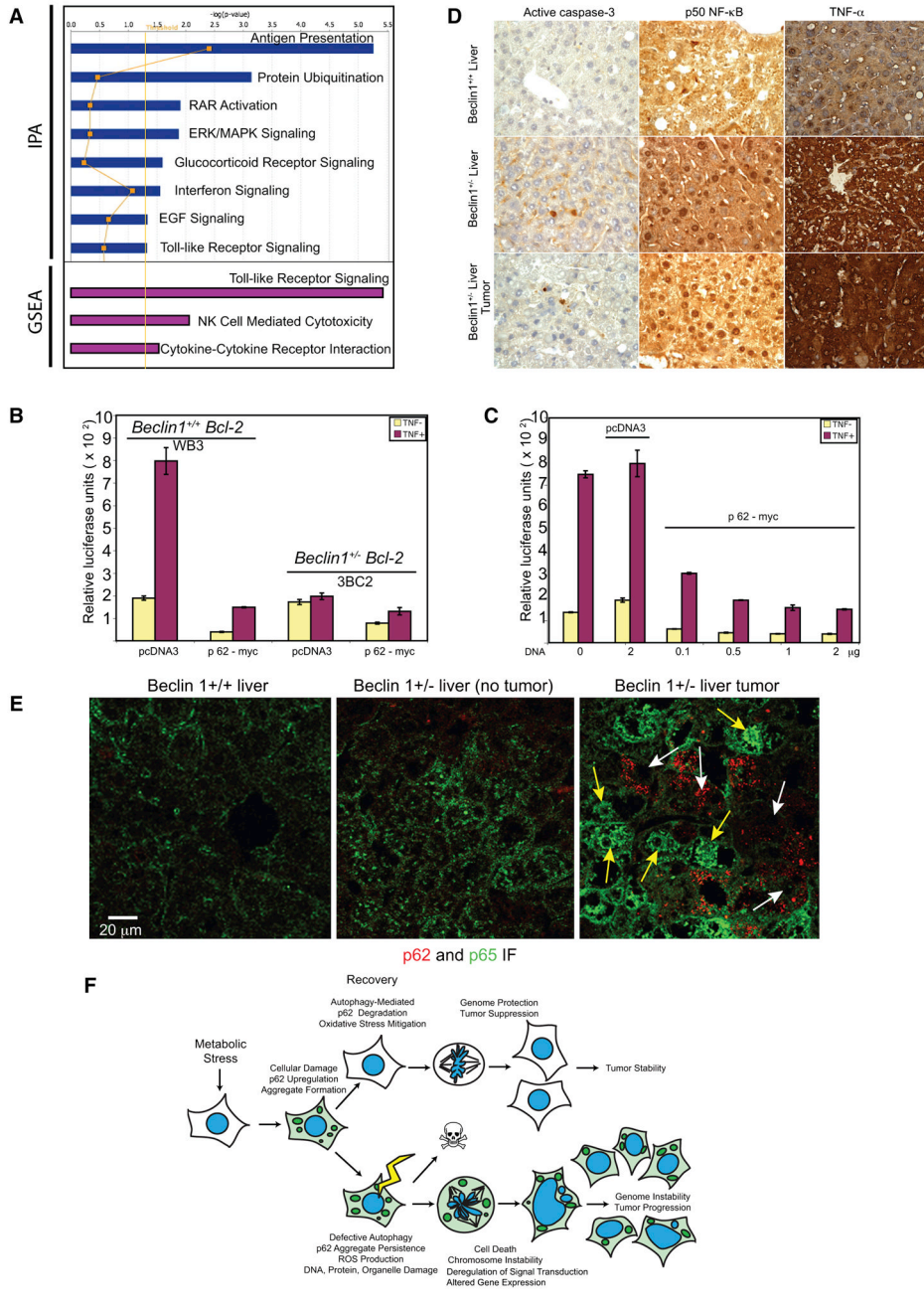
(E) p62 accumulation in autophagy-defective cells is responsible for activation of the DNA damage response. Cells in (D) were evaluated for  $\gamma$ -H2AX positive nuclear foci.

(F) Quantitation of percentage cells with  $\gamma$ -H2AX positive nuclei from the data shown in (E). Data from two hundred cells are presented as mean  $\pm$  SD.



**Figure 6. p62 expression cooperates with autophagy-deficiency to promote tumor growth**  
 (A) Western blot for EGFP, in Bcl-2-expressing *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> iBMK cells stably expressing EGFP or p62-EGFP.  
 (B) Tumor growth of cell lines in (A) showing enhanced tumor growth in p62-EGFP expressing *atg5*<sup>-/-</sup> tumors (red) compared to that of the control vector (yellow).  
 (C) Panel showing tumor-bearing mice injected with p62-EGFP- (right panel), and EGFP-expressing (left panel) *atg5*<sup>-/-</sup> iBMK cells from (B), at day 74 post-injection.  
 (D) Tumors from p62-EGFP expressing *atg5*<sup>-/-</sup> cells are associated with p62 aggregates and polymorphic and  $\gamma$ -H2AX positive nuclei. Representative photomicrographs of frozen tumor sections (left), and paraffin embedded sections stained by H&E (middle) or IHC for  $\gamma$ -H2AX (right) [Students t-test for significance for significance ( $p < 0.05$ )] in tumors from *atg5*<sup>-/-</sup> cells shown in (C).





**Figure 7. Accumulation of p62 in autophagy-defective cells alters signal transduction pathways involved in host and anti-oxidant defense**

(A) Pathways ( $p=0.05$ ; yellow line) suppressed in tumors from *atg5*<sup>-/-</sup> iBMK cells expressing p62-EGFP compared to those expressing EGFP analyzed by IPA (blue bars) and by GSEA (purple bars).

(B) Luciferase-reporter assays in *Bcl-2* expressing wild-type (*beclin1*<sup>+/+</sup>) and autophagy-defective (*beclin1*<sup>+/-</sup>) iBMK cells showing suppression of IL-6-Luciferase reporter (NF-κB activity) in autophagy-defective cells by p62.



(C) Luciferase-reporter assays in Bcl-2 expressing *beclin1*<sup>+/+</sup> iBMK (WB3) cells showing that over-expression of p62 is sufficient to suppress NF-κB transcriptional activity in a concentration dependant manner.

(D) Impaired hepatocyte survival, activation of NF-κB and cytokine production in *beclin1*<sup>+/-</sup> liver and *beclin1*<sup>+/-</sup> liver tumors. IHC staining showing elevated levels apoptotic cell death (active caspase-3), NF-κB activation (nuclear p50), and cytokine production (TNF-α) in liver tissue and spontaneous liver tumors from *beclin1*<sup>-/-</sup> mice.

(E) Defective autophagy and accumulation of p62 suppress NF-κB activation and promotes HCC. Representative photomicrographs of frozen liver sections from *beclin1*<sup>+/+</sup> and *beclin1*<sup>+/-</sup> and spontaneous HCC from *beclin1*<sup>+/-</sup> mice, immunostained for p62 (red) and p65 NF-κB (green) and analyzed by confocal microscopy. Note that those *beclin1*<sup>+/-</sup> hepatocytes that accumulate p62 do not display nuclear p65 (yellow arrows) whereas those without p62 display nuclear localization of p65 indicative of NF-κB activation.

(F) A model for the role of autophagy as a tumor suppressor mechanism by limiting p62 accumulation.