Oncogenic Ras-Induced Expression of Noxa and Beclin-1 Promotes Autophagic Cell Death and Limits Clonogenic Survival

Mohamed Elgendy,1 Clare Sheridan,1 Gabriela Brumatti,1,2 and Seamus J. Martin1,*

1Molecular Cell Biology Laboratory, Department of Genetics, The Smurfit Institute, Trinity College, Dublin 2, Ireland
2Present address: Children’s Cancer Centre, Murdoch Children’s Research Institute, Flemington Road, Parkville, Victoria 3052, Australia
*Correspondence: martinsj@tcd.ie
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SUMMARY

Deregulated oncogenes such as MYC and RAS are typically insufficient to transform cells on their own due to the activation of pathways that restrain proliferation. Previous studies have shown that oncogenic H-Ras can induce proliferative arrest or senescence, depending on the cellular context. Here, we show that deregulated H-Ras activity can also lead to caspase-independent cell death with features of autophagy. Ras-induced autophagy was associated with upregulation of the BH3-only protein Noxa as well as the autophagy regulator Beclin-1. Silencing of Noxa or Beclin-1 expression reduced Ras-induced autophagy and increased clonogenic survival. Ras-induced autophagy was also inhibited by coexpression of Bcl-2 family members that inhibit Beclin-1 function. Ras-induced autophagy was associated with Noxa-mediated displacement of the BH3-only subset of the Bcl-2 family. However, oncogenic Ras-induced Noxa expression did not result in apoptosis but promoted extensive autophagy followed by cell death. Because ablation of Noxa or Beclin-1 expression attenuated Ras-induced autophagy and increased cell survival, we propose that Ras-induced autophagic cell death restrains the transforming potential of deregulated Ras signals.

INTRODUCTION

Complete oncogenic transformation is a relatively rare event, due to a variety of mechanisms that operate to limit the proliferation and survival of transformed cells. Deregulated oncogenes such as MYC, RAS, and BRAF can increase rates of cellular proliferation, but typically require cooperating oncogenes or inactivation of tumor suppressor genes such as P53 or RB to fully transform cells (Land et al., 1983; Serrano et al., 1997). In the absence of cooperating mutations, deregulated oncogene activation typically leads to cell-cycle arrest, premature senescence, apoptosis, or other modes of cell death, depending on the intensity of oncogene expression or the cellular context (Serrano et al., 1997; Nicke et al., 2005; Sarkisian et al., 2007; Overmeyer et al., 2008). These responses most likely act as fail-safe measures to limit the oncogenic potential of activating mutations in proliferation-inducing genes. Sporadic reports have also suggested that oncogenic Ras can promote cell death that is associated with features of autophagy, but it is unclear whether oncogenic Ras-induced autophagy facilitates or opposes cell survival. Similarly, the molecular mechanisms of Ras-induced cell death remain obscure (Joneson and Bar-Sagi, 1999; Chi et al., 1999; Kitakane et al., 2002; Young et al., 2009).

To explore the mechanism of Ras-initiated cell death, we used a tetracycline-inducible H-RasV12 human ovarian epithelial (HOSE) cell line, previously characterized by Hancock and colleagues (Nicke et al., 2005). Upon induction of RasV12 expression, HOSE cells undergo irreversible proliferative arrest and display a marked reduction in the ability to form colonies (Nicke et al., 2005). Using this system, we have found that induction of H-RasV12 expression induced cell death, which was associated with a strong upregulation of Noxa—a member of the BH3-only subset of the Bcl-2 family. However, oncogenic Ras-induced Noxa expression did not result in apoptosis but promoted extensive autophagy followed by cell death. Because ablation of Noxa or Beclin-1 expression attenuated Ras-induced autophagy and increased cell survival, we propose that Ras-induced autophagic cell death restrains the transforming potential of deregulated Ras signals.

RESULTS

H-RasV12 Expression Results in Proliferative Arrest Followed by Cell Death

In the absence of doxycycline (Dox), HOSE-RasV12 cells proliferated normally, but cell division rapidly declined 4–5 days after induction of H-RasV12 expression (Figure 1A). Furthermore, peak cell numbers in Dox-treated HOSE-RasV12 cultures steadily decreased over the ensuing 3–4 days, suggesting that cell death was taking place under these conditions (Figure 1A). Inspection of Dox-treated HOSE-RasV12 cells after 5–7 days highlighted dramatic differences in the morphology of RasV12-expressing cells as compared with noninduced controls (Figure 1B). Dox-treated cells became highly vacuolated within 3–5 days of treatment and thereafter adopted a progressively “ragged” appearance, followed by detachment from the plate (Figure 1B). Propidium iodide staining confirmed that many H-RasV12-expressing cells died within 5–7 days of induction (Figure 1C). We also assessed the ability of H-RasV12-expressing cells to form colonies 21 days after Dox treatment, which revealed
a dramatic loss in colony-forming potential as compared with controls (Figure 1D). These data suggest that expression of oncogenic Ras, in the absence of other cotransforming genes, can promote proliferative arrest followed by cell death.

We also transiently expressed H-RasV12 in MCF-7 cells as well as primary HUVECs and again observed cell death as a consequence of deregulated Ras expression, but not as a consequence of expression of a dominant-negative RasN17 construct (Figures S1A and S1B).

of HOSE-H-RasV12 cells, Ras expression levels were increased, and this was associated with increases in phospho-ERK levels, as expected (Figure 1E). We also detected increased levels of the CDK inhibitor p21WAF1/CIP1 upon induction of H-Ras, as previously reported (Nicke et al., 2005). Under the same conditions, we also observed robust upregulation of the BH3-only protein, Noxa, while expression of the other BH3-only proteins examined either was unaffected or declined upon Dox treatment (Figure 1F). The effect of deregulated H-RasV12 expression on
Noxa was dose dependent, with increasing concentrations of Dox leading to increases in detectable expression of oncogenic Ras and phospho-ERK and with concomitant increases in Noxa expression (Figure 1G). Dox treatment of wild-type HOSE cells failed to induce Noxa expression (Figure S1C) or induce cell death (Figure 1C), confirming that the increased expression of Noxa and cell death observed under these conditions was Ras dependent.

**RasV12-Induced Expression of Noxa Is ERK Dependent but p53 Independent**

To ask whether Ras-induced expression of Noxa was ERK dependent, we cultured HOSE-RasV12 cells in the presence or absence of two different inhibitors of the upstream ERK-activating kinase, MEK, both of which dramatically inhibited H-RasV12-induced expression of Noxa (Figure 1H) as well as oncogenic Ras-induced cell death (Figure S1D). Because p53 has been implicated in the transcriptional upregulation of Noxa in certain situations, we also explored whether this transcription factor was required for Ras-induced Noxa upregulation. However, robust Ras-induced Noxa expression was still observed in p53 null Saos-2 cells (Figure 1I); furthermore, knockdown of endogenous p53 in HOSE-RasV12 cells failed to inhibit Ras-induced Noxa expression or associated cell death (Figure S1E).

Transient expression of H-RasV12 in HEK293T cells as well as HeLa cells also led to selective upregulation of Noxa expression, which was abolished by the MEK inhibitor U0126, confirming that oncogenic Ras-mediated regulation of Noxa is ERK dependent (Figures S1F–S1H).

**Ras-Induced Cell Death Is Not Associated with Caspase Activation or Other Features of Apoptosis**

Because Ras-induced cell death was associated with upregulation of Noxa, this suggested that these cells might be undergoing apoptosis through activation of the mitochondrial pathway to caspase activation (Strasser, 2005). However, our initial observations did not reveal morphologic features typical of apoptosis in cells undergoing Ras-induced cell death, with such cells displaying features more consistent with necrosis (Figure 1B). We also failed to detect any evidence of caspase activation or proteolysis of caspase substrates in association with oncogenic Ras-induced cell death (Figure 2A). Furthermore, Ras-induced cell death was not associated with chromatin hypercondensation or fragmentation of nuclei (Figure 2B), which are highly characteristic features of apoptosis (Kroemer and Martin, 2005). In sharp contrast, when HOSE-RasV12 cells were treated with a known proapoptotic drug (actinomycin D), caspase activation, nuclear hypercondensation/fragmentation, and extensive membrane blebbing typical of apoptosis were readily detected (Figures 2A and 2B). Furthermore, incubation of Dox-treated HOSE-RasV12 cells with the poly-caspase inhibitor zVAD-fmk failed to block cell death to any significant degree (Figures 2C and 2D) and also failed to alter the morphology of RasV12-induced cell death (Figures 2D and 2E). In contrast, zVAD-fmk significantly delayed cell death in response to actinomycin D treatment and also blocked the characteristic features of apoptosis in these cells (Figures 2C–2E). Collectively, these data suggested that Ras-induced cell death occurs via a mechanism other than apoptosis, in agreement with previous studies (Chi et al., 1999; Kitamura et al., 2002).

**Oncogenic Ras-Induced Cell Death Displays Features Characteristic of Autophagy**

As noted earlier, upon induction of H-RasV12 expression, HOSE cells became progressively vacuolated within 3–4 days of Ras activation, suggestive of macroautophagy (Figures 3A and 3B). Furthermore, the abundant vacuolated cells present in Ras-expressing cells also stained positive for acridine orange (Figure 3C), which is taken up preferentially by acidic lysosomes. Although primarily a cell survival modality in response to stress, prolonged or excessive autophagy can result in cell death (Shimizu et al., 2004; Kroemer and Levine, 2008), which can be modulated by members of the Bcl-2 family (Shimizu et al., 2004; Pattingre et al., 2005). Thus, we explored whether Ras-induced cell death was associated with other features of autophagy.

Microtubule-associated protein 1A/1B-light chain 3 (LC3) is normally expressed in a diffuse pattern in healthy cells. During autophagy, autophagosomes engulf bulk cytoplasmic constituents, including cytosolic proteins and organelles. During this process, the cytosolic form of LC3 (LC3I) is conjugated to phosphatidylethanolamine to form LC3II, which is recruited to autophagosomal membranes, resulting in a more punctate distribution pattern (Mizushima et al., 2010). Thus, we explored whether GFP-tagged LC3 converted from a diffuse to a punctate pattern upon Dox treatment of HOSE-RasV12 cells, and this was indeed found to be the case (Figures 3D and 3E), with cells displaying punctate punctate GFP-LC3 staining becoming very abundant within 3–5 days of H-RasV12 expression, whereas such cells did not increase above basal levels in control cultures (Figures 3D and 3E). Furthermore, the phosphatidylethanolamine-conjugated form of endogenous LC3, LC3II, was also readily detected upon induction of Ras expression but barely detectable in control cultures (Figure 3F). p62, a protein involved in the coupling of protein substrates to autophagosomes that typically becomes degraded during the process, was also decreased upon oncogenic Ras expression, which is consistent with enhanced autophagic flux (Figure 3F).

Increased detection of autophagic markers, such as GFP-LC3 puncta, can result either from increased autophagy or, conversely, from inhibition of ongoing autophagy (i.e., decreased autophagic flux). To discriminate between these two possibilities, we inhibited the late stages of autophagy using the lysosomal protease inhibitors E64 and pepstatin to determine whether oncogenic Ras stimulated increased autophagy or decreased ongoing autophagic flux. As Figure 3G illustrates, E64/pepstatin treatment of Dox-treated HOSE-RasV12 cells led to the stabilization of p62, suggesting that Ras expression was indeed stimulating rather than inhibiting autophagy. Furthermore, RasV12-induced LC3II levels were also further increased by inhibiting lysosomal activity (Figure 3G), again suggesting that oncogenic Ras enhances autophagic flux.

To explore whether oncogenic RasV12 could also induce autophagy in other cell types, we transfected MCF-7 cells as well as primary HUVECs with H-RasV12, and we also observed...
Figure 2. Ras-Induced Cell Death Is Caspase Independent

(A) HOSE-RasV12 cells were either left untreated or were treated with Dox (100 ng/ml) for the indicated times or with actinomycin D (Act D; 2 μM) for 24 hr as a positive control for apoptosis. At the indicated time points, cell lysates were analyzed by immunoblotting for the indicated proteins.

(B) HOSE-RasV12 cells were treated as in (A), and nuclei were visualized by Hoechst staining.

(C and D) HOSE-RasV12 cells were either left untreated or treated with Dox (100 ng/ml) or actinomycin D (2 μM) in the presence or absence of the poly-caspase inhibitor zVAD-fmk (50 μM). Cells were harvested at the indicated time points and analyzed for PI uptake by flow cytometry. The percentage of PI-positive cells in each treatment is indicated in the top right corner of each panel. Results are representative of at least three independent experiments. Error bars represent the mean ± SEM of a representative experiment.

(E) Phase contrast and Hoechst-stained images of cells treated as in (C). Images were taken 5 days after Dox treatment or 24 hr after Act D treatment. ***p < 0.005 by Student’s t test.
dramatic increases in the numbers of cells with GFP-LC3 puncta upon expression of constitutively active RasV12, but not with a dominant-negative RasN17 construct (Figures S2A and S2B). Moreover, expression of RasV12 in both of these cell types was also associated with upregulation of Noxa and accumulation of somes in mammalian cells (Liang et al., 1999; Pattingre et al., 2005). Because many autophagic triggers upregulate Beclin-1, we next explored whether oncogenic Ras could also lead to increased expression of this autophagy regulator. As Figure 4A illustrates, Dox treatment of HOSE-RasV12 cells was associated with a marked increase in endogenous Beclin-1 protein levels. Upregulation of endogenous Beclin-1 was also observed upon transient expression of constitutively active H-RasV12 in MCF-7 cells and primary HUVECs (Figures S2C and S2D). Furthermore, transient expression of constitutively active H-RasV12 in HeLa cells also led to marked induction of Beclin-1 (Figure 4B). In contrast, expression of a dominant-negative RasN17 mutant neither induced features of autophagy in HeLa cells (Figure 4C) nor induced expression of Beclin-1 (Figure 4B). Similar to Noxa, Ras-induced Beclin-1 expression was also ERK dependent and could be inhibited by the MEK inhibitor U0126, but was unaffected by inhibitors of PI3K, JNK, and p38MAPK (Figure S3).
Ras-Induced Cell Death Is Inhibited by Knockdown of Beclin-1, Atg5, or Atg7

The preceding experiments suggested that oncogenic RasV12 promotes autophagy, followed by cell death. At least two scenarios could explain these observations. Ras-induced autophagy could be cytoprotective, operating in parallel to Ras-induced signals that instigate cell death; in this scenario, inhibition of autophagy would be expected to accelerate Ras-induced cell death. In the alternative scenario, prolonged Ras-induced autophagy could eventually lead to cell death; in this situation, inhibition of autophagy would be expected to diminish Ras-induced cell death and to promote long-term cell survival.

To ask whether inhibition of autophagy in this context blocked Ras-induced cell death, we used a previously validated shRNA construct targeted against Beclin-1 mRNA (Pattingre
et al., 2005) to knockdown expression of this protein in HOSE-RasV12 cells (Figure 4D). As Figures 4E and 4F illustrate, knockdown of Beclin-1 expression markedly attenuated Ras-induced cellular degeneration as well as vacuolation. More importantly, Ras-induced cell death was also dramatically attenuated as a consequence of Beclin-1 knockdown (Figure 4G), strongly suggesting that Ras-induced autophagy is cytotoxic rather than cytoprotective. We also silenced expression of another essential autophagy gene, Atg5 (Figure 4D), and this had essentially identical effects, with a marked attenuation of Ras-induced vacuolation (Figure 4F) and cell death (Figure 4G).

To further validate these observations, we used two different siRNAs targeted against Beclin-1 (Figure S4A), as well as Atg-7 (Figure S4B), all of which attenuated Ras-induced cell death in HOSE cells, as compared with a control siRNA oligonucleotide (Figure S4C). Furthermore, RasV12-induced death of MCF cells and HUVECs was also significantly attenuated through knockdown of Beclin-1, Atg5, or Atg7 (Figures S4D and S4E).

Importantly, Beclin-1 knockdown also resulted in long-term survival of RasV12-expressing HOSE cells, as these cells displayed greatly enhanced colony formation upon knockdown of Beclin-1 (Figure 4H).

Collectively, these data suggest that expression of oncogenic Ras induces autophagic cell death, which contributes to the dramatic reduction in clonogenic survival that is seen upon expression of this oncogene in the absence of cooperating oncogenic mutations (Figures 1D and 4H).

**Noxa Is Required for Ras-Induced Autophagy and Cell Death**

Beclin-1 is known to interact with several members of the antiapoptotic subset of the Bcl-2 family, and Beclin-1-dependent autophagy can be repressed by Bcl-2 and its close relatives (Pattingre et al., 2005; Ciechomska et al., 2009). Because BH3-only proteins can, in turn, neutralize the activity of the Bcl-2 prosurvival proteins, we wondered whether Ras-induced Noxa expression (Figures 1F–1H) was involved in promoting Ras-induced autophagy and subsequent cell death. To explore this, we knocked down Noxa in HOSE-RasV12 cells using an shRNA construct targeted against this transcript. As controls, we also knocked down expression of other BH3-only proteins—Puma, Bad, Bim, and Bmf—using similar shRNAs.

As Figure 5A illustrates, silencing of Noxa expression had a marked inhibitory effect on the appearance of cells with LC3-GFP puncta upon Dox treatment of HOSE-RasV12 cells, whereas knockdown of most other BH3-only proteins had little effect.

The Ras-induced accumulation of the lipidated form of LC3 (LC3II) and degradation of p62 were also inhibited upon silencing of Noxa expression (Figure 5A, bottom panel). However, knockdown of Puma also consistently diminished Ras-induced LC3-GFP puncta formation, albeit to a lesser extent than attenuation of Noxa expression (Figure 5A). Moreover, knockdown of Noxa also dramatically attenuated Ras-induced morphologic changes in HOSE cells (Figure 5B) and Ras-induced cell death (Figure 5C), with knockdown of other BH3-only proteins having little effect on these endpoints—once again, with the exception of Puma, which displayed less significant but reproducible inhibitory effects (Figures 5B and 5C).

Similarly, knockdown of Noxa in MCF-7 cells and HUVECs also attenuated Ras-induced cell death in these cell types (Figures S4D and S4E).

We also examined the role of Noxa in long-term clonogenic assays and, consistent with the cell viability data (Figure 5C), observed that knockdown of this BH3-only protein in HOSE-RasV12 cells greatly enhanced Ras-induced colony formation (Figure 5D). Once again, knockdown of other BH3-only proteins, with the exception of Puma, had little effect in terms of permitting oncogenic Ras-induced colony formation (Figure 5D).

To explore the requirement of Noxa for Ras-induced autophagy further, we monitored the appearance of LC3-GFP puncta in wild-type versus NOXA−/− MEFs upon transient expression of oncogenic H-RasV12. As Figures 5E and 5F illustrate, whereas H-RasV12 readily induced LC3-GFP puncta formation in wild-type MEFs, NOXA−/− MEFs were resistant to the induction of LC3-GFP puncta by oncogenic Ras. However, re-expression of Noxa in NOXA−/− MEFs resulted in a substantial increase in LC3-GFP puncta formation upon transient expression of H-RasV12 in these cells (Figures 5E and 5F).

**Prosurvival Bcl-2 Family Members Block Ras-Induced Autophagy and Cell Death**

The preceding results demonstrated that Noxa and Beclin-1 were required for Ras-induced autophagy and associated cell death. Because both Noxa and Beclin-1 can interact with members of the prosurvival subset of the Bcl-2 family (Bcl-2, Bcl-xL, Mcl-1, A1, Bcl-b), we wondered whether Ras-induced autophagy and associated cell death could be inhibited through ectopic expression of the latter proteins. Although Noxa preferentially binds to Mcl-1 and A1, cell death due to ectopic expression of Noxa can be blocked by essentially all members of the prosurvival subset of the Bcl-2 family, albeit to different degrees (Figure 6A). Thus, we also explored the effects of overexpression of the prosurvival Bcl-2 family members on Ras-induced autophagy and cell death. As Figure 6B illustrates, transient overexpression of several Bcl-2 family proteins significantly inhibited the accumulation of LC3-GFP puncta in H-RasV12-expressing HOSE cells and also suppressed Ras-induced vacuolation (Figure 6C) and cell death, as assessed by morphological criteria (Figure 6D) as well as by propidium iodide uptake (Figure 6E). However, the most robust effects on Ras-induced cell death were seen with A1 and Mcl-1, both of which are preferential Noxa-interacting proteins at endogenous levels (Chen et al., 2005). To ask whether prosurvival Bcl-2 family proteins could also confer clonogenic survival of RasV12-expressing cells, we conducted colony-formation assays. As Figure 6F shows, in agreement with the short-term cell viability results, A1 and Mcl-1 afforded the greatest protection from RasV12-induced loss of clonogenicity, with both of which are preferential Noxa-interacting proteins at endogenous levels (Chen et al., 2005). To ask whether prosurvival Bcl-2 family proteins could also confer clonogenic survival of RasV12-expressing cells, we conducted colony-formation assays. As Figure 6F shows, in agreement with the short-term cell viability results, A1 and Mcl-1 afforded the greatest protection from RasV12-induced loss of clonogenicity, with significant protection also seen upon overexpression of Bcl-2, Bcl-xL, and Bcl-b.

Consistent with their ability to interact with and regulate the activity of Beclin-1 as well as Noxa, several members of the Bcl-2 family can cooperate with oncogenic Ras to promote long-term clonogenic survival through inhibiting Ras-induced autophagic cell death.
Ras-Induced Noxa Displaces Beclin-1 from Mcl-1

An obvious mechanistic explanation of our observations is that oncogenic Ras-induced Noxa could promote autophagy by displacing one or more members of the Bcl-2 family, such as Mcl-1, from Beclin-1. Indeed, recent studies have reported that BH3-only proteins, or BH3 mimetics, can promote Beclin-1-dependent autophagy through disrupting the interaction between Beclin-1 and other members of the Bcl-2 family (Maiuri et al., 2007; Erlich et al., 2007). We initially tested the scenario through ectopic expression of Beclin-1 in MCF-7 cells in the presence or absence of Mcl-1.

Using this model, we found that Mcl-1 can also suppress Beclin-1-dependent autophagy, as indicated by the ratio of diffuse to punctate LC3-GFP staining (Figures 7A and 7B). Furthermore, coexpression of Noxa substantially reversed this protection (Figures 7C and 7D). These results indicate that Noxa substantially enhances the ability of Ras to promote autophagy. Overexpression of Beclin-1 in MCF-7 cells promotes autophagy that can be suppressed through coexpression of Bcl-2 or Bcl-xL (Pattingre et al., 2005).

Figure 5. Noxa Is Required for Ras-Induced Autophagy and Cell Death

(A) HOSE-RasV12 cells were transfected with plasmids encoding GFP-tagged LC3 along with control shRNA or shRNA plasmids targeting the indicated BH3-only proteins. Forty-eight hours after transfection, cells were treated with or without Dox (100 ng/ml) for a further 7 days. Cells were then analyzed for PI uptake by flow cytometry (top panel), and cell lysates were immunoblotted for the indicated proteins (bottom panel). Results represent triplicate counts of 100 cells per treatment and are representative of three independent experiments. (B) Representative phase-contrast images of HOSE-RasV12 cells treated as in (A). (C) HOSE-RasV12 cells were transfected as in (A), followed 48 hr later by treatment with or without Dox (100 ng/ml) for a further 7 days. Cells were then analyzed for PI uptake by flow cytometry (top panel). Results are representative of at least three independent experiments. Error bars represent mean ± SEM. Knockdown of BH3-only proteins was assessed by immunoblotting 72 hr after transfection with the indicated shRNAs (bottom panel). (D) HOSE-RasV12 cells, transfected as in (A), were subjected to clonogenic survival assays as described in Experimental Procedures. Colony numbers were scored 3 weeks after plating. Results are representative of at least three independent experiments. Error bars represent the mean ± SEM. For all panels, ***p < 0.005, **p < 0.005, *p < 0.01, *p < 0.5 by Student’s t test. (E and F) Wild-type (WT) or NOXA−/− MEFs were transfected with plasmids encoding GFP-tagged LC3 along with H-RasV12 plasmid, alone or in combination with Noxa or Bad expression plasmids. Forty-eight hours after transfection, cells exhibiting punctate GFP-LC3 distribution were scored (E), and representative images of cells in each treatment were acquired by confocal microscopy (F). Results represent triplicate counts of 100 cells per treatment and are representative of three independent experiments. See also Figure S4.
Figure 6. Prosurvival Members of the Bcl-2 Family Inhibit Ras-Induced Cell Death and Loss of Colony-Forming Potential
(A) HeLa cells were transfected with a GFP reporter plasmid (100 ng per well of a 6-well plate) in combination with empty vector (400 ng) or with a Noxa expression plasmid (400 ng), alone or in combination with expression plasmids encoding the indicated Bcl-2 family proteins. Cell death in the GFP-positive population was scored after 24 hr based on morphological criteria. Results represent the mean ± SEM of triplicate counts of 100 cells per treatment and are representative of three independent experiments.

(B) HOSE-RasV12 cells were transfected with empty vector or expression plasmids encoding the indicated Bcl-2 family proteins along with GFP-tagged LC3 expression plasmid. Forty-eight hours after transfection, cells were treated with or without Dox (100 ng/ml) for a further 5 days, and cells exhibiting punctate GFP-LC3 distribution were then scored. Results represent triplicate count of 100 cells per treatment and are representative of three independent experiments. Error bars represent the mean ± SEM.

(C) HOSE-RasV12 cells treated as in (B) were scored at the indicated time points for cells exhibiting cytoplasmic vacuolation.

(D) Representative images of cells treated as described in (B) were taken 7 days after Dox treatment.

(E) HOSE-RasV12 cells were transfected as in (B), followed 48 hr later by treatment with or without Dox (100 ng/ml) for a further 7 days. Cells were then harvested and analyzed for PI uptake by flow cytometry. The percentage of PI-positive cells, as defined by the M1 gate (bottom panels), is indicated in the top right.
However, upon induction of Ras expression by Dox treatment, Mcl-1 binding to Beclin-1 diminished drastically by day 5 (Figure 7C). We also observed displacement of Bcl-xL from Beclin-1 at the same time point (Figure 7C). Conversely, when endogenous Mcl-1 was immunoprecipitated, associated Beclin-1 was readily detected prior to Ras expression (Figure 7D, top panel); however, upon expression of Ras, the levels of Beclin-1 bound to Mcl-1 greatly diminished, despite total cellular amounts of Beclin-1 increasing during this time (Figure 7D, bottom panel). We also explored the behavior of endogenous Noxa under the same conditions and found that whereas little Noxa coprecipitated with Mcl-1 prior to Ras activation, the levels of Mcl-1-bound Noxa greatly increased upon Ras activation (Figure 7D).

Because Mcl-1 degradation correlated with the upregulation of Noxa in HOSE-RasV12 cells (Figures 1F and 7D, bottom panel), we wondered whether these events might be causally related as a consequence of Noxa-induced degradation of Mcl-1, as previously reported (Willis et al., 2005). To address this, we monitored Mcl-1 levels in HOSE-RasV12 cells under conditions where Noxa upregulation was antagonized using an shNoxa construct. As Figure 7E illustrates, when Ras-induced Noxa upregulation was inhibited, Mcl-1 protein levels remained stable, in contrast to both control shRNA and shBad-treated cells. Consistent with this, immunoprecipitation of Beclin-1 under similar conditions revealed that Ras-induced displacement of Mcl-1 from Beclin-1 was much less efficient where Noxa had been knocked down (Figure 7F). Noxa also appeared to contribute to displacement of Bcl-xL from Beclin-1, either directly or indirectly, as these complexes were also stabilized upon Noxa knockdown (Figure 7F). Knockdown of Puma had a more modest effect on stabilization of Mcl-1/Beclin-1 complexes, but impacted to a greater degree on Beclin-1/Bcl-xL complexes (Figure 7F).

Taken together, these data suggest a model where Ras-induced expression of Noxa promotes autophagy by displacing Mcl-1 and perhaps other members of the Bcl-2 family, such as Bcl-xL, from Beclin-1 (Figure 7G). Puma also contributes to the disruption of Beclin-1/Bcl-2 family complexes, although activation of this BH3-only protein does not appear to be obviously Ras regulated. Beclin-1 also accumulates dramatically upon deregulated Ras expression. In this situation, autophagy leads to caspase-independent cell death that can be reversed through ablating expression of Noxa, Beclin-1, Atg5, or Atg7 or through ectopic expression of prosurvival members of the Bcl-2 family that preferentially bind Noxa and/or Beclin-1.

**DISCUSSION**

Here, we have demonstrated that expression of oncogenic RasV12 in the absence of cotransforming oncogenes leads to extensive autophagy, which culminates in cell death. Ras-induced expression of Noxa as well as Beclin-1 was required for induction of autophagy and cell death in this context. Noxa-dependent autophagy was associated with displacement and degradation of the Bcl-2 family member, Mcl-1, from Beclin-1. Thus, autophagic cell death appears to represent one of the mechanisms that limit the oncogenic potential of Ras, which is consistent with observations that beclin-1 haploinsufficiency can predispose to tumor development (Qu et al., 2003; Yue et al., 2003).

**Noxa-Dependent Induction of Autophagy**

The observation that Noxa, a member of the BH3-only family of proteins, can promote a nonapoptotic cell death pathway is initially counterintuitive. However, Noxa is one of the least potent effectors of Bax/Bak activation and activation of the apoptosome pathway to apoptosis (Kuwana et al., 2005). Moreover, targeted inactivation of the NOXA gene has little effect on numerous proapoptotic signals (Villunger et al., 2003), suggesting that this BH3-only protein may play a more prominent role in other contexts. In addition to suppressing opening of the Bax/Bak channel, several members of the Bcl-2 family are constitutively bound to Beclin-1 and in this capacity act as suppressors of autophagy (Pattingre et al., 2005; Erlich et al., 2007). Thus, increased expression/stabilization of BH3-only proteins, in addition to their well-known role in promoting apoptosis, may also promote autophagy in certain contexts (Maiuri et al., 2007; Rashmi et al., 2008). Either outcome (i.e., apoptosis or autophagy) is likely to depend on the particular BH3-only protein that is upregulated/activated, the capacity of individual BH3-only proteins to engage the required range of prosurvival Bcl-2 family members to promote apoptosis, as well as the particular context. In this regard, Noxa is well known to engage a limited spectrum of prosurvival Bcl-2 proteins, displaying greatest affinity for Mcl-1 and A1, but little binding to other members of the Bcl-2 family at endogenous levels (Chen et al., 2005).

**Autophagy versus Autophagic Cell Death**

The link between autophagy and cell death has been controversial (Kroemer and Levine, 2008). On the one hand, autophagy undoubtedly enhances cell survival in response to nutrient deprivation, organellar damage, or other forms of stress by enabling the recycling of bulk cytoplasmic constituents and organelles, which are subsequently degraded in autophagosomes (Mizushima et al., 2010). On the other hand, prolonged or sustained autophagy can result in cell death and has been observed in several contexts, including involution of insect salivary glands (Berry and Baehrecke, 2007), as a result of enforced expression of Beclin-1 (Pattingre et al., 2005), in BAX/BAK null cells challenged with stimuli that would otherwise promote apoptosis (Shimizu et al., 2004), and in response to certain agents capable of elevating cytosolic free calcium levels (Hayer-Hansen et al., 2007). Therefore, although restrained autophagy may operate as a survival mechanism in many contexts, autophagy may be capable of promoting cell death in situations where it is excessive or prolonged.
Figure 7. Ras-Induced Noxa Displaces Prosurvival Bcl-2 Proteins from Beclin-1 to Promote Autophagy

(A) MCF-7 cells were transfected with the indicated combinations of expression plasmids encoding Mcl-1, Noxa, and Beclin-1 along with an expression plasmid encoding GFP-tagged LC3. Forty-eight hours after transfection, cells were starved in EBSS for 4 hr at 37°C, and cells exhibiting punctate GFP-LC3 distribution were scored. Results represent triplicate counts of 100 cells per treatment and are representative of three independent experiments. ***p < 0.005, **p < 0.01, *p < 0.5 by Student’s t test.

(B) Representative images of MCF-7 treated as in (A).

(C) HOSE-RasV12 cells, treated with or without Dox (100 ng/ml) for the indicated times, were harvested, followed by immunoprecipitation with control Ig or a monoclonal antibody specific for Beclin-1. Immune complexes were analyzed by immunoblotting for the indicated proteins.

(D) Top panel: HOSE-RasV12 cells were treated as in (C), followed by immunoprecipitation of endogenous Mcl-1 and probing for the indicated proteins. Bottom panel: immunoblot analysis of the indicated proteins in total cell lysates used for immunoprecipitations in (C) and (D).

(E) HOSE-RasV12 cells were transfected with control shRNA plasmid or shRNA plasmids targeted against Bad or Noxa. Cells were then treated with or without Dox (100 ng/ml). At the indicated time points, cell lysates were generated and were analyzed by immunoblotting for the indicated proteins, with actin as a loading control. Results are representative of two independent experiments.
The role of autophagy in oncogenesis has also been debated, with some reports indicating a role for this process in tumor persistence, while other data suggest that autophagy limits initial tumor development (Liang et al., 1999; Qu et al., 2003). In support of the latter view, certain autophagy regulators such as Beclin-1 have been found to act as haploinsufficient tumor suppressors, and genetic inactivation of a single beclin-1 allele results in enhanced rates of tumor formation (Liang et al., 1999; Qu et al., 2003). Thus, along with proliferative senescence, autophagic cell death may be one of the effectors mechanisms that act as a safeguard against excessive or unscheduled proliferative signals.

**Ras-Induced Cell Death Outcomes May Be Context Dependent**

Previous studies have also noted that deregulated Ras activation can induce nonapoptotic cell death in various cell types, including glioblastoma and neuroblastoma, via mechanisms that remained obscure (Chi et al., 1999; Overmeyer et al., 2008). However, whether this nonapoptotic cell death represents autophagy (Chi et al., 1999), or extensive macropinocytosis followed by simple necrosis (Overmeyer et al., 2008), has been debated. The reasons underlying the cell type differences in Ras-induced cell death outcomes remain unclear. However, the mode of cell death triggered by deregulated oncogenes is likely to be related to the presence of other mutations in transformed cells as well as cell-lineage-related issues that may place a limit on autophagic or apoptotic responses to such events.

**Threshold Effects of Ras Activation**

Previous studies have demonstrated that oncogenic Ras activation is associated with features of senescence in primary untransformed fibroblasts (Serrano et al., 1997). A recent study has also shown that Ras-induced autophagy precedes the senescent phenotype (Young et al., 2009), although it was not clear from this study whether cells displaying features of autophagy and those displaying features of senescence were derived from the same subpopulation. The outcome of deregulated Ras signals, whether senescence or autophagic cell death, may be related to the intensity of Ras signaling. Indeed, where this issue has been explicitly investigated using a titratable oncogenic H-RasV12, it has been found that low-intensity Ras activation leads to proliferation and eventual tumor formation, whereas high-intensity Ras activation leads to senescence induction (Sarkisian et al., 2007). This issue has also been investigated in human neuroblastoma tumors, where Ras positivity is correlated with spontaneous regression of such tumors and a favorable prognostic outcome (Kitanaka et al., 2002). Moreover, enforced expression of RasV12 in neuroblastoma cell lines was also found to induce caspase-independent cell death, exhibiting features of autophagic degeneration (Kitanaka et al., 2002). Therefore, signaling thresholds may be the critical factor that dictates whether a cell responds to Ras activation by entering the cell cycle, undergoing transient or sustained proliferative arrest (i.e., premature senescence), or undergoing autophagic cell death.

**Concluding Remarks**

In conclusion, we have shown here that deregulated H-Ras activity can lead to caspase-independent cell death with features of autophagy. Thus, in addition to premature cellular senescence, Ras-induced autophagic cell death is likely to represent an additional mechanism that serves to limit the oncogenic potential of deregulated Ras signals.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following antibodies were used: anti-Pan Ras, anti-Noxa, anti-Bim, and anti-p21 (Calbiochem, Darmstadt, Germany); anti-Bcl2, anti-Bcl-XL, anti-Bid, anti-p16, anti-p23, anti-p62, anti-Caspase-3, and anti-Caspase-7 (BD Biosciences, Oxford); anti-Mcl-1 (Santa Cruz Biotechnology; Santa Cruz, CA); anti-Puma (Prosci, Poway, CA); anti-Akt5, anti-LC3, and anti-Beclin1 (Novus, Cambridge); anti-Aktin (MP Biomedicals, Illkirch, France); and anti-phospho ERK, anti-phospho p70S6K, and anti-Bad (Cell Signaling, Bray, Ireland). Unless otherwise indicated, all other reagents were purchased from Sigma (Arklow, Ireland).

**Transfection**

HeLa and MCF-7 cells were transfected using GeneJuice reagent (Merck, Darmstadt, Germany) according to standard procedures. HOSE-RasV12 cells, IMR-90 fibroblasts, HUVECs, and MEFs were electroporated using Amaxa Nucleofection procedures (Cologne, Germany). Briefly, cells were trypsinized, pelleted, and resuspended in nucleofection buffer. Cell suspension (100 µl) was added to the plasmid mixture, transferred to a nucleofection cuvette, immediately electroporated using program T-020, and seeded in a 10 cm dish containing 8 ml of prewarmed medium.

**Quantification of Cell Death**

Cells were harvested by trypsinization, washed in PBS (pH 7.2), and then stained with propidium iodide (10 µg/ml) added immediately prior to analysis. Cell fluorescence was then measured on a flow cytometer (FACSCalibur; Becton Dickinson, CA) and analyzed using CellQuest software.

**For further Experimental Procedures, see the Supplemental Information available online.**

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.molcel.2011.02.009.

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