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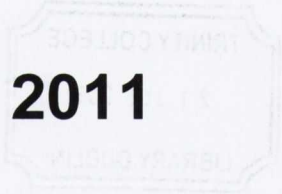
Investigation of the Role of the Bcl-2 Family Proteins in Ras-Induced Transformation

Thesis submitted to Trinity College Dublin for the degree of Doctor of Philosophy



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ACKNOWLEDGEMENTS

I am very grateful to my father and my mother. Without their support I would not have achieved anything. A big thank-you to my sisters Manal, Hala and Heba and their families for the warm feelings and support all along the journey.

Thanks also to my small family, Helena, Layla and Karim who without them, this thesis would probably have been slightly better.

To my beloved homeland Egypt.

I would also like to thank the people in the lab: Prof. Seamus Martin, Dr Clare Sheridan, Dr Susan Logue and Dr Sean Cullen who trained me and helped me with their valuable advices. Many thanks are due also to the other members of the lab, past and present, for all their help and support.

I am also grateful to my teachers in Ain Shams University and in Ghent University-Belgium for giving me the first chance.

Finally, I am grateful to Marie Curie Research Training Network ApopTrain and Science Foundation Ireland for funding this work.

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Summary

RAS is a proto-oncogene that is found mutated in 25% of human tumours. Deregulated Ras expression is typically insufficient to transform cells as it triggers anti-proliferative responses that altogether limit tumorigenesis. Co-operating mutations are thus required to allow cells to evade such responses and to proceed to full transformation. Mutational activation of Ras has been shown to induce cell cycle arrest and senescence. Here we show that deregulated H-Ras expression can also induce autophagy and autophagy-associated cell death. Expression of oncogenic H-Ras induced autophagy markers including cytoplasmic vacuolation, LC3I-to-LC3II conversion and p62 degradation, GFP-LC3 puncta formation, LC3 localization to the vacuoles membranes and positive staining for the acidotropic agents, monodansyl cadaverine and acridine orange. Ras-induced autophagy was followed by cell death that we found was caspase-independent and was morphologically distinct from apoptosis.
Inhibition of autophagy by silencing Atg5, Atg7 or Beclin-1 diminished Ras-induced autophagy and cell death indicating that autophagy mediates cell death rather than playing a pro-survival function in this context. Furthermore, ablation of Beclin-1 enhanced clonogenic survival of cells expressing oncogenic Ras. Ras-induced autophagy and the subsequent cell death may thus serve to restrain transformation and may represent another level of innate tumour suppression.

Importantly, we show that Ras-induced autophagy and cell death require ERK-dependent upregulation of Beclin-1 and the pro-apoptotic BH3-only protein Noxa through a mechanism that involves ATF4. Ablation of Beclin-1 or Noxa or the overexpression of the anti-apoptotic members of the Bcl-2 family proteins, especially A1 and Mcl-1, diminished Ras-induced autophagy and cell death and enhanced clonogenic survival.

We have also observed that oncogenic Ras expression results in downregulation of Mcl-1. Ras-induced Mcl-1 downregulation contributed to the induction of

autophagy and cell death as we found that ablation of Mcl-1 sensitizes the cells to Ras-induced autophagy and cell death.

Noxa has previously been shown to bind Mcl-1 and enhance its degradation by the proteasome; we tested whether Noxa upregulation contributes to Mcl-1 downregulation upon oncoegnic Ras expression. Noxa knockdown stabilized Mcl-1 in cells expressing oncogenic Ras suggesting that indeed Noxa plays a role in this context.

Our results strongly suggest a model in which Beclin-1 and Noxa are upregulated in response to H-Ras activation. Upregulated Noxa then binds Mcl-1 displacing it from the interaction with Beclin-1 and setting Beclin-1 free. Upregulated and unsequestered Beclin-1 then promotes autophagy which when it becomes excessive and prolonged eventually culminates in cell death.

Our results show the novel involvement of the pro-apoptotic protein Noxa in this form of caspase-independent cell death triggered by activation of H-Ras. It also suggests a novel role for Noxa and Beclin-1 in restraining Ras-induced transformation.

Interestingly, we observed that two melanoma cell lines that harbour endogenous Ras mutations express constitutively high levels of Beclin-1 and Noxa compared to normal melanocytes and two other cells lines HeLa and HOSE. Endogenous Noxa and Beclin-1 expression in these cells was under the control of ERK as inhibition of MEK diminished the basal levels of these proteins. Moreover, we found that these melanoma cell lines also expressed comparably high levels of Mcl-1, which we found to be crucial for their survival possibly by inhibiting Noxa/Beclin-1 driven autophagy downstream of Ras. Ablation of Mcl-1 in these melanoma cell lines restored Ras-induced autophagy that was followed by massive spontaneous cell death that resembled cell death observed in other cell lines upon expression of oncogenic Ras. Cell death induced by ablation of Mcl-1

in these melanoma cell lines was attenuated when Beclin-1 was simultaneously knocked down together with Mcl-1. Ras-induced autophagic cell death may thus represent a new alternative therapeutic option that may prove to be useful for targeting tumors harbouring Ras mutations. However this remains to be tested.

ABBREVIATIONS

A1	Bcl-2 related protein-A1
Act D	Actinomycin D
AMPK	Adenosine monophosphate-activated protein kinase
AP1	Activator protein 1
ASK1	Apoptosis signal-regulating kinase 1
ATF	Activating transcription factor
Atg	Autophagy related gene
ATP	Adenosine triphosphate
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist killer 1
Bax	Bcl-2-associated x protein
Beclin-1	coiled-coil, myosin-like BCL2 interacting protein
Bcl-2	B-cell lymphoma 2
Bcl-b	Bcl-2 related gene b
Bcl-w	Bcl-2 related gene w
Bcl-xL	Bcl-2 related gene x-long isoform
BH	Bcl-2 homology domain
Bid	BH3-interacting domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting protein Bim
Bmf	Bcl-2 modifying factor
BRCA1	Breast cancer gene 1
BSA	Bovine serum albumin
Caspase	Cysteinyl aspartate-specific protease
CDK	Cyclin-dependent kinase
DAPK	Death-associated protein kinase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
eIF2 alpha	Eukaryotic translation initiation factor 2, subunit 1 alpha

Egl-1	Egg laying defective 1
Elk-1	Ets Like gene1
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated protein kinase
FOXO3a	Forkhead Box03a
Fra 1	Fos-related antigen 1
GDAP1	Ganglioside-induced differentiation-associated protein 1
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
H-Ras	Harvey rat sarcoma viral oncogene homolog
HEK	Human embryonic kidney cells
Hif-1α	Hypoxia-inducible factor 1-alpha subunit
HMGB1	High mobility group box 1
HOSE	Human Ovarian Surface Epithelium
Hrk	Harakiri
Hsc70	Heat shock cognate protein 70 kDa
HUVEC	Human Umbilical Vein Endothelial cell
IL	Interleukin
INK4	Inhibitor of cyclin-dependent kinase 4
IP	Immunoprecipitation
IRE1	Inositol requiring enzyme 1
JAK	Janus kinase
JNK	c-jun kinase
K-Ras	Kirsten rat sarcoma viral oncogene homolog
LB	Luria-Bertani broth
m-TOR	Mammalian target of Rapamycin
MAP1LC3B	Microtubule-associated proteins 1A/1B light chain 3B
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia 1
MEK	MAPK/ERK kinase
MNK	MAPK-interacting kinase 1

MOMP	Mitochondrial outer membrane permeabilisation
N-Ras	Neuroblastoma RAS viral oncogene homolog
NHEM	Normal Human Epidermal Melanocytes
NfkB	Nuclear factor kappa-light-chain enhancer of activated B cells
PAGE	Polyacrylamide gel
PBS	Phosphate buffered saline
PERK	Protein kinase RNA (PKR)-like ER kinase
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
Puma	p53-upregulated modulator of apoptosis
Ras	Rat sarcoma viral oncogene homolog
Rb	Retinoblastoma protein
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RSK	Ribosomal protein S6 kinase
SAHF	Senescence-associated heterochromatin foci
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siRNA	Small interfering Ribonucleic acid
shRNA	Short hairpin Ribonucleic acid
STAT	Signal transducer and activator of transcription
TBST	Tris buffered saline with tween 20
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
UFP	Unfolded protein response
UVRAG	UV radiation resistance-associated gene protein
ZVAD.fmk	Benzylocarbonyl-valine(V) alanine(A) aspartic acid(D)-(OMe)-fluoromethylketone

PUBLICATIONS

Elgendy M., Sheridan C., Brumati G. and Martin S.J. Oncogenic Ras-Induced Expression of Noxa and Beclin-1 Promotes Autophagic Cell Death and Limits Clonogenic Survival.In press. *Molecular Cell* (2011), 42(1), 23 – 35.

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Brumati G., Sheridan C., Elgendy M. , Brunet M. and Martin S.J. 2010. An ERK-dependent pathway to Noxa expression regulates apoptosis by platinum-based chemotherapeutic drugs. *Oncogene* 29, 6428-6441

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Chapter I

Introduction

1.1 The *RAS* proto-oncogenes

The *RAS* proto-oncogenes encode low molecular weight guanine nucleotide-binding proteins that play essential roles in the control of cellular growth and differentiation. Ras communicates signals from outside of the cell to the nucleus. Activating mutations in *RAS* gene can thus lead to inappropriate signalling inside the cell. RAS is found mutated in 25-30 % of all human tumours (Bos, 1989) which makes it an important oncogene and a subject of intensive investigation.

Mammalian genomes encode three RAS genes that give rise to four gene products: N-Ras, H-Ras, K-Ras4A, and K-Ras4B. The K-Ras4A and K-Ras4B isoforms result from alternative splicing of the KRAS gene (Omerovic et al., 2007; Li et al., 2004; Rosseland et al., 2007). Ras proteins predominantly sit on the inner leaflet of the plasma membrane and are switched on when a wide range of cell surface receptors become activated. Ras proteins cycle between inactive GDP bound and active GTP-bound states (Figure 1.1). Normally, these proteins are tightly regulated by guanine nucleotide exchange factors (GEFs) that cause the exchange of GDP for GTP to produce the active, GTP-bound state, and by GTPase-activating proteins (GAPs) that catalyse hydrolysis of bound GTP to turn off Ras activity and return Ras to its resting state (Figure 1.2). When activated, Ras signals through a broad range of downstream targets that regulate cell survival and proliferation (Buday *et al.*, 2008).

In this chapter we review Ras signalling and the cellular responses to mutational activation of Ras.

1.1.1 Ras signalling

Active, GTP-bound Ras associates with a wide diversity of effector targets that transmit signals downstream (Figure 1.2). To date, the Raf serine/threonine kinases (Raf-1, A-Raf and B-Raf), phosphoinositide 3-kinases (PI3Ks) and the Ral small GTPases represent the best-characterized downstream effectors of Ras.

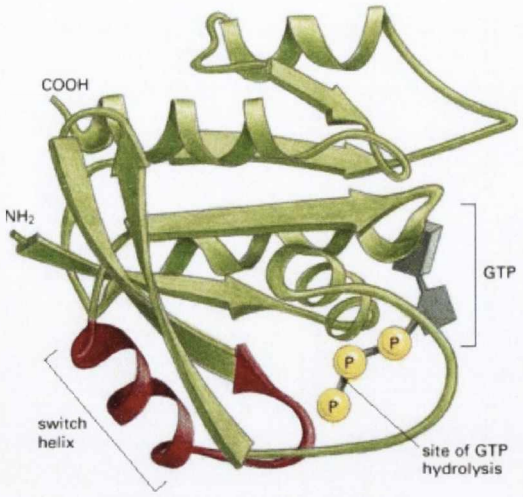


Figure 1.1 Structure of Ras protein

3D structure of Ras protein showing the site of GTP hydrolysis.

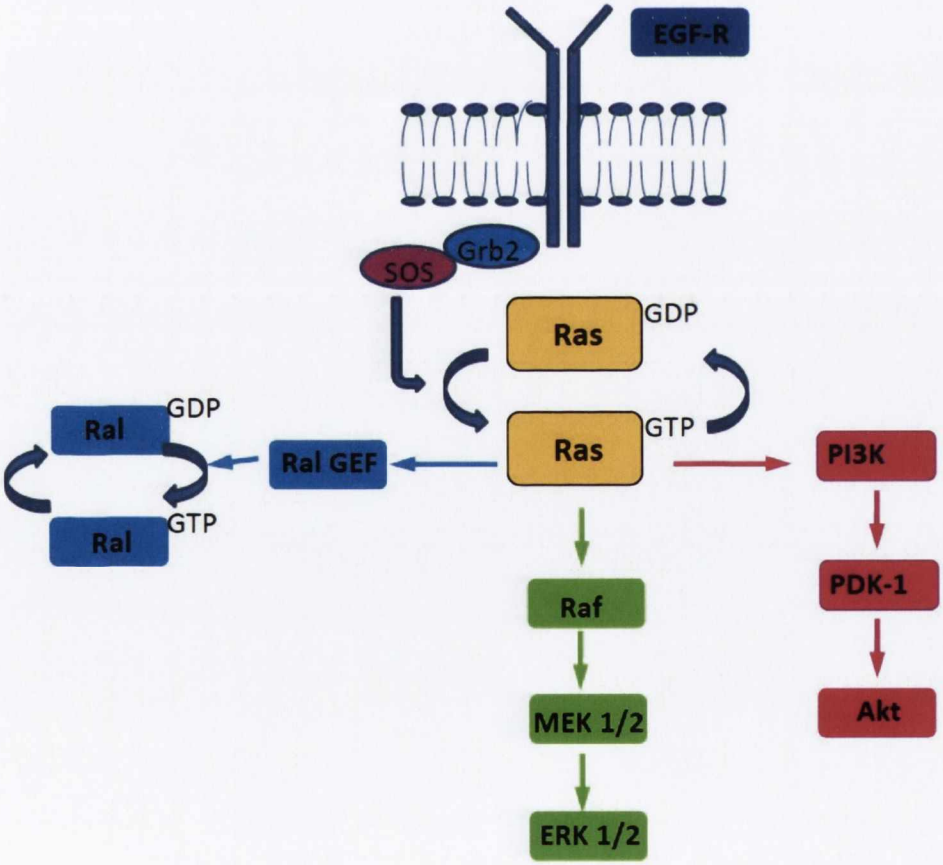


Figure 1.2 Ras signalling

Active, GTP-bound Ras signals through several pathways. Raf/MEK/ERK, PI3K/Akt and Ral small GTPases are the best characterized effectors pathways downstream of Ras

Upon activation by Ras, Raf phosphorylates and activates the MEK1/2 dual-specificity kinases, and MEKs in turn phosphorylate and activate the extracellular signal-regulated kinases ERK1/2. Activated ERKs translocate into the nucleus where they phosphorylate and activate various transcription factors (e.g. Elk1, C-Fos, c-Jun) as well as other targets (Davis, 1995). Ras activation of the Raf serine/threonine kinases and the subsequent activation of the mitogen-activated protein kinases (MAPKs) remains a key signalling pathway that is important for many facets of Ras biology (Campbell *et al.*, 1998).

Phosphoinositide 3-kinases (PI3Ks) have emerged as the next best-understood effectors of Ras. Ras interacts with the p110 catalytic subunit of PI3K to stimulate its lipid kinase activity. PI3K phosphorylation of the D3 position of phosphatidylinositides converts, for example, PtdIns(4,5)P2 (PIP2) to PtdIns(3,4,5)P3 (PIP3) (Ramjaun *et al.*, 2007). One well established target of PI3K-mediated production of PtdIns(3,4,5)P3 is Akt/PKB (protein kinase B), which in turn phosphorylates numerous target proteins. Activated Ras causes elevated PtdIns(3,4,5)P3 and Akt/PKB activities (Castellano *et al.*, 2010). An important function of the Akt/PKB pathway is promotion of cell survival (Ramjaun *et al.*, 2007). PI3K can also activate the Rac GTPase, and this Rho family protein is an important mediator of oncogenic Ras transformation (Feig *et al.*, 1996).

In addition, Ras activates a family of GDP–GTP exchange factors (GEFs) for the Ral small GTPases (RalGDS, RGL and RGL2/Rlf) that represent the third-best-understood family of Ras effectors. Activated Ras promotes RalGDS activation of Ral (Wolthuis *et al.*, 1999). Although the activation of Ral is one of their actions, Ral-independent activities of RalGDS have also been described (Feig *et al.*, 1996). Ras-related small GTPases. RalBP1 (also called RLIP1/RIP1) is a GTPase-activating protein and negative regulator of the Cdc42 and Rac GTPases. Ral can also interact with phospholipase D1 (PLD1) to augment its activation by the Arf small GTPase. PLD1 catalyses the conversion of phosphatidylcholine (PC) to phosphatidic acid (PA). Akt and Rac facilitate Ras

activation of the NF-kB transcription factor, which serves an anti-apoptotic role in Ras function (Venkatesan *et al.*, 2010).

1.1.2 The Role of Ras in cancer

In many tumors, oncogenic mutations, particularly at positions 12, 13 or 61, cause Ras to become insensitive to negative regulation by GAP and to remain constitutively GTP bound and active. In other tumors, deregulated signalling from upstream activators or downstream negative regulators can also result in excessive levels of Ras-GTP (Boss, 1988). The *RAS* gene is found mutated in about 25- 30% of all human cancers. These mutations can be found in a variety of tumor types. The highest incidences are found in adenocarcinomas of the pancreas (90%), the colon (50%), and the lung (30%); in thyroid tumors (50%); and in myeloid leukemia (30%) (Bos, 1989).

1.1.3 Therapeutic strategies targeting Ras

Targeting Ras for therapeutic intervention is complicated by the diversity of its downstream targets and the presence of crosstalk between different effector pathways. Ras targeting is further complicated by cell type specificity and the conflicting signals elicited by the same signalling pathway in different contexts. This all raises doubts about the effectiveness of targeting a single target or even a pathway downstream of Ras. Simultaneous inhibition of both PI3K/Akt and Raf/MEK/ERK was found to have a synergistic killing effect on cancer cells with mutated Ras, which gives a rationale for combinational therapy as a more effective approach for therapeutic targeting of Ras (Kawaguchi *et al.*, 2007). Understanding how our natural defences react to mutational activation of Ras and how anti-tumorigenic mechanisms have evolved to react to such tumorigenic threats can teach us much. Mimicking or restoring those responses that might have been disabled by a co-operating oncogenic event along the cumulative process of cellular transformation can help us develop more efficient and safer therapeutic strategies.

1.1.4 Anti-proliferative responses triggered by mutational activation of *RAS*

Aberrant proliferative signals induced by gain of function mutations in oncogene paradoxically trigger anti-proliferative responses such as senescence or cell death. Such anti-proliferative responses represent an innate tumor suppressive mechanism installed by evolution to constrain the tumorigenic potential arising from frequent DNA errors and mitogenic mutations. As no single pathway can confer a net growth advantage, a single oncogenic lesion is usually insufficient on its own to impart a full tumorigenic phenotype unless synergized by other co-transforming mutation(s) that uncouple aberrant proliferative signals from the restraining anti-proliferative responses. Only when such cooperative lesions allow cells to evade innate tumor suppression, can a fully tumorigenic phenotype finally be acquired. The connecting pathways between oncogenic lesion and the subsequent anti-proliferative responses are finely coordinated by a series of sensors, transducers and effectors. They can act in several steps along one or alternative pathways to maximize the perquisites to evade such responses and to ensure that the pre-neoplastic cell is efficiently trapped in a growth cul-de-sac or eliminated by a suicide program. Those responses collectively limit the incidence of tumorigenesis and make cancer rather a rare incidence considering the potential of its occurrence. Studying such anti-proliferative responses to oncogenic activation can give us insight into the anti-tumorigenic strategies our cells are equipped with and could be exploited for more efficient rational therapeutic targeting of oncogenes such as Ras.

Mutational activation of *RAS* has been suggested to induce several anti-transformative responses including senescence and two types of programmed cell death; apoptosis and autophagic cell death. Here we review those responses in some details.

1.1.5 Ras-induced senescence

(I) Senescence: an overview

Senescence is a state of irreversible permanent cell cycle arrest that can be distinguished from a reversible form of cell cycle arrest called quiescence by morphological changes and gene expression pattern that senescent cells exhibit. Replicative senescence is reached after numerous cell cycles due to telomeres shortening. However cellular senescence can be induced prematurely in primary human and rodent cells in response to oncogene activation such as oncogenic Ras expression, DNA damage or oxidative stress (Leikam *et al.*, 2008).

Cells usually exhibit gross morphological changes when entering the senescence stage. These changes vary greatly depending on the cell line and the initiating factors that lead to senescence. For instance, while many cell lines become more flat when senescing, other cell lines look spindlier or even rounded. Senescent cells sometimes acquire a multinucleated phenotype (Leikam *et al.*, 2008). Senescent cell also show cytoplasmic vacuolization in certain circumstances (Nicke *et al.*, 2005). However, the nature of these vacuoles and their relevance to the senescence phenotype has not been studied in details.

One of the important morphological changes that accompany senescence is the dramatic changes in chromatin organization to form heterochromatin foci that were coined the name senescence-associated heterochromatin foci (SAHF). Formation of SAHF has been reported to be dependent on retinoblastoma protein (Rb) and to be associated with stabilizing the cells in the senescence state (Narita *et al.*, 2003). However the formation of SAHF per se does not seem to be essential for the establishment of the senescence as not all senescent cells show profound SAHF morphology. Senescent cells also exhibit high β-galactosidase activity that, together with SAHF in some cell lines, have been exploited as classical markers of senescence.

(II) Mechanisms of Ras-induced senescence

Little is known about the precise molecular mechanism of induction of senescence program or the factors that lock the cells permanently in the senescence state upon oncogenic Ras expression. However, the Raf/MEK/ERK pathway is generally thought to be the main effector of senescence induction downstream of Ras (Cagnol *et al.*, 2010). Ras/Raf/ERK pathway-induced senescence relies on a set of cell cycle regulators such as p16/INK4A, pRb, p53, p21 and p19/ARF, which are typically associated with the induction of the senescence program. The execution of the senescence program seems to take place through two axes: ARF-p53 and $p16^{INK4A}$-pRb axes. p53 and pRb then promote senescence by controlling a number of effectors, including p21, PML and various chromatin-modifying factors. The two axes could cooperate, serve redundant functions or be preferentially induced depending on cell linage specificity. For instance while senescence in human fibroblasts and some rodent haematopoietic cells replies mainly on p16, in MEFs p16 seems to be dispensable. In the latter cells, ARF-p53 seems to be the main mediator of Ras-induced senescence (Chesnokova *et al.*, 2007).

1.1.6 Regulation of cell death by Ras

Mutational activation of oncogenes can also lead to cell death. c-myc expression was shown to evoke an apoptotic response (Thompson, 1998). Oncogenic Ras expression has also been described to induce cell death in certain contexts (Kitanaka *et al.*, 2007; Chi *et al.*, 1999). Like senescence, cell death represents another main anti-proliferative response triggered by the expression of oncogenic Ras. While our understanding of how Ras induces cell death is far from complete, it is not surprising that evolution has installed programs by which cells that represent a tumorigenic threat commit themselves to suicide to terminate the threat as a failsafe mechanism when the damage is beyond their ability to repair. A cell's decision whether to senesce or die is context-dependent and may rely on many factors such as intensity and duration of Ras expression or whether or not some checkpoints (e.g. p21 checkpoint) are intact (Lantinis *et al.*, 1997).

Oncogenic Ras has been described to evoke at least two types of cell death programs that are functionally, mechanistically and morphologically distinct: apoptosis and autophagy-associated cell death (also known as type II programmed cell death). Here we briefly review how oncogenic Ras expression can evoke these two types of programmed cell death.

(I) Regulation of apoptosis by Ras

Evading apoptosis is an essential perquisite for transformation. Ras has been described to provoke anti- as well as pro- apoptotic signals. The balance between those conflicting signals is what ultimately dictates the final outcome. Ras-mediated protection against apoptosis is thought to be mainly through the activation of the pro-survival PI3K/Akt pathway. In contrast, Raf/MEK/ERK pathway can be anti- or pro- apoptotic perhaps depending on the degree of activation or the cellular context.

A- Anti-apoptotic roles of PI3K/Akt pathway

Akt protects against apoptosis by phosphorylating a number of apoptosis regulators, the most important of which is the BH3-only protein Bad. Akt-mediated phosphorylation of Bad prevents the latter from interacting with and inactivating the anti-apoptotic proteins Bcl-xL and Bcl-2 (Datta *et al.*, 1997). Additionally, Akt has also been suggested to phosphorylate other targets which inactivate caspase-9 to inhibit its protease activity (Fujita *et al.*, 1999); inactivate the AFX forkhead transcription factor, to prevent AFX from entering nucleus, thereby inhibiting gene expression of the death-activating Fas ligand; and activate IKK to cause inactivation of IkB, leading to NF-kB release and its stimulation of expression of anti-apoptotic genes (Brownawell *et al.*, 2001). PI3K also causes activation of Rac, and Rac can activate NF-kB by production of reactive oxygen species.

B- Anti-apoptotic roles of Raf/MEK/ERK pathway

The Raf/MEK/ERK pathway can also exert anti-apoptotic actions. The Raf/MEK/ERK pathway was reported to protect neuronal cells from apoptosis induced by neurotrophic factor withdrawal (Xia *et al.*, 1995). In some cases, p53

is induced in response to Ras activation, which then results in cell cycle arrest or apoptosis. Ras can bypass p53-induced apoptosis by inducing its degradation via Mdm2 in a Raf-dependent manner (Ries *et al.*, 2000). Activation of the Raf/MEK/ERK pathway has also been shown to phosphorylate Bad on S112, which contributes to Bad inactivation and sequestration by 14-3-3 proteins (Fang *et al.*, 1999). The Raf/MEK/ERK pathway can also result in phosphorylation of Bim (Sheridan *et al.*, 2008) and its subsequent degradation by the proteasome. In addition, Raf may contribute to the pro-survival response exerted by Ras by downregulating transcription of Par-4, a pro-apoptotic transcriptional repressor in a MEK-dependent manner than can be mimicked by constitutively activating MEK or PKCδ but not PI3K (Nalca *et al.*, 1999). Raf-1 was also shown to interact with mammalian sterile 20-like kinase (MST-2), a kinase activated in response to pro-apoptotic agents such as Fas ligand and saturosporin. Interaction between Raf-1 and MST-2 results in inactivation of the latter by preventing its dimerization (O'Neill *et al.*, 2004). Raf-1-MST-2 complex can also contribute to apoptosis inhibition by interacting with ASK1 (Du *et al.*, 2004) ASK1 is a mediator of apoptosis in response to cytotoxic stress exerted by agents such as TNF, Fas and ROS. Raf/MEK/ERK pathway has also been described to phopsphorylate caspase 9 on residue T125 which leads to its inactivation (Allan *et al.*, 2003)

C- Pro-apoptotic roles of Ras/MEK/ERK pathway

Conversely, mutational activation of *RAS* can elicit pro-apoptotic signals mainly through the Raf/MEK|/ERK pathway. Under stress conditions such as loss of matrix adhesion (Nikiforov *et al.*, 1996), TNF treatment (Trent *et al.*, 1996) or expression of activated Ras oncogene can result in or augment apoptosis. Overexpression of oncogenic Ras alone in fibroblasts has been described to induce apoptosis even in the absence of other stress factors (Fukasawa *et al.*, 1997). Ras-induced apoptosis has been described in other cell types as well. Overexpression of activated Ras in Jurkat cells induced apoptosis through MEK/ERK-mediated increased expression of Fas ligand and dominant negative Ras inhibited FasL expression in response to antigen receptor stimulation

suggestive for an autocrine mechanism of Ras-induced apoptosis, at least in this context (Latinis *et al.*, 1997). Raf/MEK/ERK pathway also potentiates the activation of death receptors such as Fas, DR4 or DR5. MEK/ERK pathway has also been implicated in mediating apoptosis in response to DNA-damaging chemotherapeutic agents such as cisplatin (Brumati *et al.*, submitted).

D- Crosstalk between different Ras effectors in the regulation of apoptosis

Regulation of apoptosis by Ras is even further complicated by the presence of crosstalk and feedback loops between Ras effector pathways. An example that illustrates crosstalk between PI3K/Akt and Raf pathways is the protective role of Ras in IL-3-depnedent haematopoietic cells against apoptosis induced by of IL-3 withdrawal. While Ras-induced protection is mediated through Raf/MEK/ERK pathway, Akt seems to be still required for full protection as constitutively active Akt synergizes with Raf (von Gise *et al.*, 2001). PI3K/Akt pathway can also play a role in the negative regulation of the Raf/MEK/ERK through Akt-related kinase SGK. Akt can also regulate the activity of Raf kinases themselves by direct phosphorylation of Raf-1 at Ser259 (Zimmermann and Moelling, 1999) and B-Raf at Ser364, Ser428 and Thr439 resulting in diminishing their kinase activity (Guan *et al.*, 2000). Conversely, Raf/MEK/ERK can indirectly regulate PI3K/Akt pathway in some cell lines by activating mTOR, which is a downstream target of Akt but can also inhibit Akt by a negative feedback loop (Martelli *et al.*, 2007; Courtois-Cox *et al.*, 2006).

(II) Ras-induced caspase-independent cell death

A few sporadic reports have linked Ras to another form of cell death that is distinct from apoptosis and instead is associated with autophagy. The association between Ras activation and the induction of autophagy was first suggested by Chi and co-workers (Chi *et al.*, 1999). In the light of the observation that mutations in the *RAS* gene are common in many human cancers but quite rare in glioblastomas and gastric cancer (Bos, 1989), Chi and co-workers investigated the reason behind this and demonstrated that expression of oncogenic H-Ras in human glioma cell U251 and U343 and gastric

cell lines MKN-1 and TMK-1 triggers a caspase-independent cell death that was distinct from apoptosis. Cell death in that case was preceded by massive cell vacuolation. Those vacuoles were found to be derived from lysosomes as staining for lysosomal markers LAMP -1, 2 and LGP-85 colocalized with the vacuolar membrane (Chi *et al.*, 1999). Those results suggested for the first time that expression of H-Ras could trigger a non-apoptotic cell death with autophagic features, which constrains the clonogenic capability of the cells. This form of cell death induced by Ras was described to occur in vivo and to play an important role in the spontaneous regression of neuroblastoma (Kitanaka *et al.*, 2002). H-Ras expression is a biological marker that correlates with positive prognosis in neuroblastoma. Kitanaka and co-workers conducted immunohistochemical analysis of neuroblastoma tissues and demonstrated that neuroblastoma cells overexpressing Ras show morphological changes indicative of cell death. Degenerating neuroblastoma cells positive for Ras did not have morphological features of apoptosis and were negative for TUNEL and active caspase-3 staining but stained positive for PAS, a marker of autolysosome and generally showed autophagic features. Additionally, ectopic expression of active Ras in neuroblastoma cell lines in vitro induced autophagic degeneration in the absence of caspase activation similar to the tumor cell death observed in neuroblastoma tissues. The mechanism of Ras-induced autophagy and autophagic cell death however remained elusive. Several later studies pointed to a role for MEK/ERK pathway in mediating induction of autophagy downstream of Ras (Wang *et al.*; 2009, Shima *et al.*; 2007, Zhu *et al.*, 2007; Pattingre *et al.*, 2003). Ras-induced autophagy correlated with enhanced MEK/ERK activity and inhibition of MEK by UO126 diminished Ras-induced autophagy (Pattingre *et al.*, 2003). But how does MEK/ERK pathway contribute to autophagy induction? To answer this question Ogier-Denis and co-workers demonstrated that ERK-dependent phosphorylation of Ser 151 in the RGS domain of Gα-interacting protein (GAIP) in response to amino acid starvation contributes to ERK-induced autophagy in human colon cancer HT-29 cells. GAIP is a regulator of G protein signalling (RGS) that accelerates the rate of GTP hydrolysis by the alpha-subunit of the trimeric G(i3)

protein. Both proteins are part of a signalling pathway that controls lysosomal-autophagic catabolism. However a perhaps more important mechanism by which MEK/ERK pathway induces autophagy was revealed in a recent report, Wang and co-workers reported that a non-canonical MEK/ERK module which is positioned downstream of AMP-activated protein kinase (AMPK) and upstream of tuberous sclerosis complex (TSC) regulates autophagy by regulating the levels of Beclin-1 (Wang *et al.*, 2009).

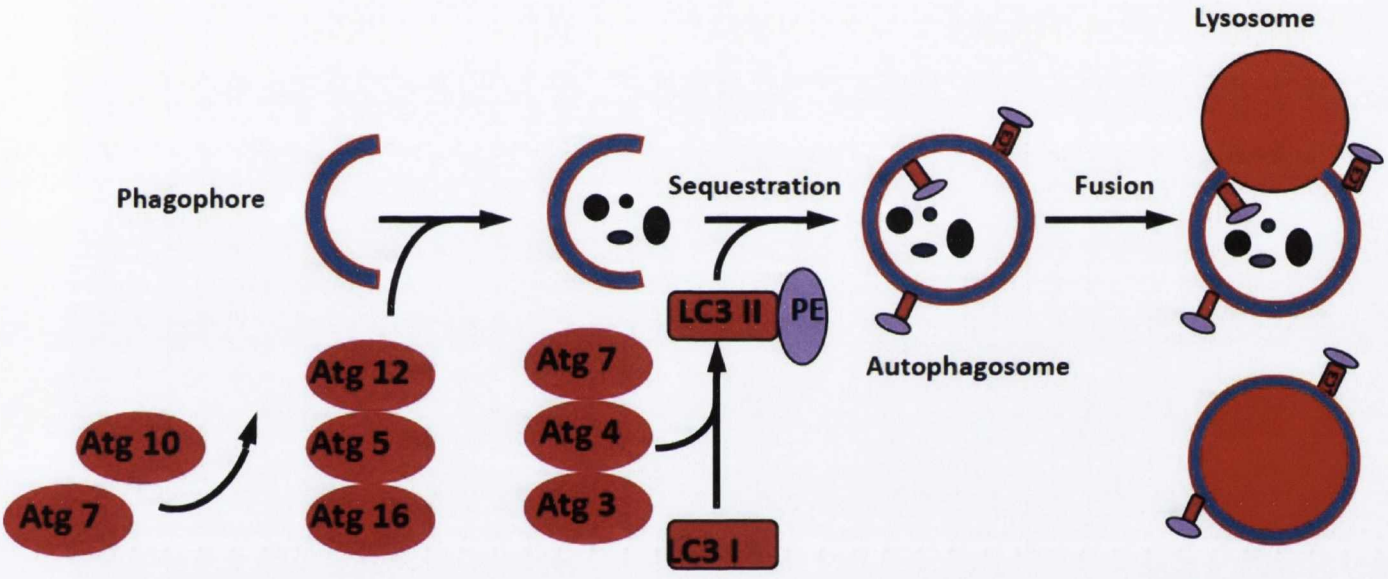
1.2 Autophagy and autophagy-associated cell death

1.2.1 Autophagy in mammalian cells: an overview

Macroautophagy (hereafter referred to as autophagy) is a recycling process by which cellular components are sequestered within the vesicular system and delivered to lysosomes for degradation. Autophagy was described in mammalian cells over 50 years ago. In 1974, Christian de Duve was jointly awarded Nobel Prize for Physiology or Medicine for his discovery of lysosomes and peroxisomes. It was not however until the last decade that the mechanism of autophagy in mammalian cells was largely unravelled thanks to discoveries of the mechanism of autophagy in yeast.

Elucidation of the molecular pathways that execute autophagy was largely achieved through genetic approaches using yeast mutants defective in autophagy. The main steps and much of the autophagy machinery is conserved between yeast and mammalian cells. Over 20 autophagy related genes (ATGs) have been discovered to date (Figure 1.3).

Briefly, the autophagy can be broken down to several subsequent steps. The first step is the initiation of autophagy where a selection of the components targeted for degradation or "the cargo" takes place. Very little is known about the recognition signalling that governs the selection process but it has been



igure 1.3 Steps of autophagy

asic steps of autophagy, the formation of double-membrane structures (phagophores) which engluf
ıe cargo materials. Lipidated form of LC3 (LC3II) localizes to the outer and inner membarnes of au-
ɔphagosomes. Cargo materials are then degraded when autophagosomes fuse with lysosomes.

suggested that selection is initiated by signals delivered from the cargo material itself (Shintani *et al.*, 2002). However, not all autophagic responses are selective and specific. In certain circumstances autophagy is non-selective probably in response to stronger overwhelming autophagy-inducing signals. A main difference between selective and non-selective autophagy is that initiating signals that drive vesicles membrane biogenesis come from the cargo materials in the first type and from direct activation of the autophagy machinery in the second. The next step in the process of autophagy is membrane biogenesis and the formation of crescent shaped- membranous structures that circle the materials destined for degradation. The origin of those membrane cistern has been a subject of intensive study with some studies suggesting that they originate from a unique compartment called pre-autophagosomal structure (Suzuki *et al.*, 2001; Kim *et al.*, 2002) and other more recent studies arguing that they originate from smooth endoplasmic reticulum (ER) cisternae (Bernales *et al.*, 2006; Hayashi-Nishino *et al.*, 2009) It, However, remains a matter of debate. The formed membrane structures then completely engulf the cargo materials to form what is known as "autophagosomes". An important event that accompanies the formation of autophagosomes and is exploited for the assessment of autophagic activities is the conversion of the cytosolic form of microtubules-associated protein 1A/1B-light chain 3 (LC3I) to the lipidated form LC3II. LC3 is a soluble protein with a molecular mass ~ 17KDa that is distributed ubiquitously in mammalian cells. Upon the induction of autophagy, LC3I is converted by site specific proteolysis and lipidation near the C-terminus by conjugation to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3II), which is recruited to autophagosomal membranes. Autophagosomes then fuse with lysosomes to form autolysosomes, and intra-autophagosomal components are degraded by lysosomal hydrolases. At the same time, LC3II in autolysosomal lumen is degraded.

1.2.2 Detection and quantification of autophagy

Several methods have been described for the detection and quantification of autophagy. Electron microscopy was the first and remains a classical way for detection of autophagy. It can provide reliable qualitative as well quantitative data. However, Electron microscopy is laborious and requires dedicated personnel and expensive equipment. LC3 was recently introduced as the first specific marker of autophagy. Detection of LC3I to LC3II conversion by western blotting, immunofluorescence staining for endogenous LC3 or the assessment of the change in distribution of flurophore-tagged LC3 e.g. GFP tagged LC3 from diffuse to punctuate morphology are widely used and generally accepted methods for the measurement of autophagy. However, LC3II is degraded in the lysosomal lumen upon the maturation of autophagosomes into autolysosomes. Disruption of this maturation step could lead to the inhibition of LC3II turnover by the autophagic flux and its subsequent accumulation. Increased LC3II levels therefore could mean enhanced autophagic activity or inhibited autophagic flux. A way to distinguish between the two scenarios is through the use of lysosomal enzymes inhibitor. If LC3II level is elevated in response to a mature and complete autophagic process then inhibiting LC3II degradation in the lysosome would be expected to result in further accumulation of LC3II compared to the situation when the same autophagy-inducing stimulus is applied in the absence of lysosomal enzymes inhibitors. Conversely, if the elevated LC3II level is a result of disruption of the autophagosomes to autolysosome maturation step i.e. inhibited autophagic flux, then the use of lysosomal enzyme inhibitors should not have any further effect as LC3II levels as the process is already disrupted at an earlier step.

Autophagy can also be assessed using acidotropic agents such as monodansyl cadaverine or acridine orange, which exhibit characteristic fluorescence in acidic compartments of the cell. However, it should be noted that such agents can only detect autophagy in a late stage as they only label the acidic autolysosomes but not the early-formed autophagosome. Although this may represent a limitation

for using this method, it can be useful for distinguishing mature autophagic response that leads to autolysosome formation, which stain positive for those dyes from disrupted autophagic flux where vacuolation represents accumulated autophagosomes that exclude those dyes.

Other methods for assessment of autophagy include immunofluorescence staining for autophagosome-lysosome colocalization and measurement of rate of protein degradation by labelling cellular proteins with radioactive amino acids then measure the time-dependent release of acid-soluble radioactivity from the labelled proteins.

1.2.3 Regulation of autophagy

As one would expect, a crucial process that controls cell survival or death such as autophagy is tightly controlled by a complex network of regulators. Autophagy is a dynamic multi-step process that can be positively or negatively modulated at several steps. Autophagy can be induced by internal or external stimuli. Nutrient deprivation is a well-known stimulus of autophagy and has been subjected to intensive studies. Most of our cumulative knowledge on autophagy regulation comes mainly from studies that used starvation to induce autophagy. In nutrient-rich conditions, autophagy is inhibited and kept in check through the activation of the kinase TOR/mTOR that is a well-characterized autophagy inhibitor that is activated by Akt downstream of class I PI3K. mTOR activation inhibits autophagy through many regulatory alternations in both transcription and translation that are presumably mediated by p70S6 kinase and the eukaryotic initiation factor 4E binding protein 1 (Dann *et al.*, 2007). Under nutrient deprivation conditions, mTOR is inhibited which relieves the inhibitory effect of mTOR on autophagy and leads ultimately to the induction of an autophagic response (Tamburini *et al.*, 2008). This is important for cell survival under low nutrient conditions by recycling proteins and organelles to supply the cell with nutrients until the nutrient supply becomes available again. Notably, class I and class III PI3K has opposite roles in the autophagy process. While the class I PI3K

is involved in the negative regulation of autophagy through mTOR pathway, class III PI3K promotes the sequestration of cytoplasmic materials that occurs during autophagy (Kroemer *et al.*, 2010).

The phosphatase and tensin homologue (PTEN) is another autophagy regulator. PTEN is a tumor suppressor protein that regulates autophagy by dephosphorylating and thus inhibiting class I PI3K (Park *et al.*, 2009).

Beclin-1 is another important regulator of autophagy. Beclin-1 was identified in 1998 as a Bcl-2 binding protein and was described later as the mammalian ortholog of Atg6. It was the first direct link between tumorigenesis and disruption of autophagy (Liang *et al.*, 1998). The BECN1 gene maps to a tumor susceptibility locus on human chromosome 17q12 and is found mono-allelically deleted in 40-75% of sporadic breast and ovarian cancers. Ectopic expression of Beclin-1 in MCF-7 cells which express no or very low basal levels of Beclin-1 mediated starvation induced autophagy (Liang *et al.*, 1999). Beclin-1 regulates autophagy by binding to class III PI3K to form a complex that has been suggested to play important roles in autophagosome formation and trafficking of lysosomal enzyme. Other proteins that form part of the Beclin-1/Class III PI3K complex such as Bif1 and UVRAG may also play a role in regulation of autophagy. Notably, UVRAG is mono-allelically deleted in high percentage of human colon cancers (Liang *et al.*, 2006) and deletion of *Bif1* in mice results in the development of spontaneous tumors (Takahashi *et al.*, 2007).

Interestingly, Nishida and co-workers described a form of alternative autophagy that is Atg5/Atg7 independent but Beclin-1 dependent type of autophagy (Nishida *et al.*, 2009).

Other molecules such as death-associated protein kinase (DAPK), eIF2α, GDP-bound form of the Gαi3 subunit and Bcl-2 family proteins have also been reported to play a role in the regulation of autophagy. DAPK interacts with the microtubules-associated protein MAP1B especially under amino acid starvation conditions to induce autophagy. Phosphorylation of eIF2α has also been suggested to induce autophagy in yeast and MEFs (Talloczy *et al.*, 2002).

Hydrolysis of GTP to GDP by GTPase-activating protein GAIP stimulated autophagy induced by GDP-bound form of the Gα i3 subunit. Bcl-2 family proteins have recently emerged as important regulators of autophagy as discussed elsewhere in this thesis.

1.2.4 Role of autophagy in cancer

Several autophagy-regulating genes are associated with cancer suggesting a close link between the two processes. Oncoproteins, including phosphatidylinositol 3-kinase, Akt and anti-apoptotic members of the Bcl-2 family suppress autophagy. Conversely, several tumor suppressor proteins (e.g., Atg4; beclin 1; Bif-1; BH3-only proteins; death-associated protein kinase 1; LKB1/STK11; PTEN; UVRAG) promote the autophagic pathway (Maiuri *et al.*, 2009). Taken together, those observations strongly suggest a role for autophagy in tumor suppression. Like apoptosis and senescence, evidence now suggests that excessive autophagy and subsequent cell death may represent another level of anti-transformative response to oncogenic activation. Overexpression of c-Myc mutant deficient in pro-apoptotic and oncogenic transformation activities caused an increase in autophagic activity in rat fibroblasts (Tsuneoka *et al.*, 2003). Similarly, H-Ras expression in glioma and gastric cell lines induced a caspase-independent cell death that was accompanied by signs of autophagic vacuolation. Autophagic cell death thus may act as a safeguard mechanism to limit tumorigenesis in these contexts. However due to the well-described pro-survival functions of autophagy whether or not the tumor suppressive role of autophagy is related to its ability to result in cell death remains controversial. Another possible explanation for the tumor-suppressive role of autophagy comes from the role of autophagy in the management of metabolic stress and the removal of damaged organelles. Mismanagement of metabolic stress or accumulation of damaged organelles in autophagy-deficient tumor cells can lead to genome damage and tumor progression.

1.2.5 Induction of autophagy as a therapeutic strategy

The emerging role of autophagy in tumor suppression gives a rationale for targeting autophagy as a tool to combat cancer. An even increasing number of anticancer agents are being realized to confer their cytotoxicity, at least in part, by inducing autophagy in tumor cells. Rapamycin is a classical inducer of autophagy with a clinically proven anticancer potential. Similarly, many other anticancer agents such SAHA (Hrzenjak *et al.*, 2008), Tamoxifen (Bursch *et al.*, 1996), temozolomide (Kanzawa *et al.*, 2004), arsenic trioxide (Kanzawa *et al.*, 2003) and obatoclax (Martin *et al.*, 2009) have been reported to exert their anti-cancer effect via inducing autophagic cell death.

1.3 Bcl-2 family proteins

1.3.1 Overview

Bcl-2 is the prototype of a family of proteins containing at least one Bcl-2 homology (BH) region. Bcl-2 family members can be divided into three groups that serve different functions in the regulation of apoptosis: anti-apoptotic family members proteins that contain four BH domains (Bcl-2, Bcl-xL, Bcl-w, Bcl-b, Mcl-1 and A1); pro-apoptotic multi-domain proteins (Bax and Bak) that contain three BH domains and pro-apoptotic BH3-only proteins (Noxa, Puma, Bad, Bim, Bmf, Bik, Hrk, Bid...etc) which as the name indicates contain a single BH domain (BH3). The anti-apoptotic Bcl-2 proteins have on their surface a hydrophobic cleft, the BH3 binding groove, through which they can interact with the pro-apoptotic BH3 only proteins. Anti-apoptotic Bcl-2 family members bind with greatly variable affinities to various BH3 only proteins depending on the amino acid composition of both the BH3 domain on the BH3-only proteins and the BH3-binding groove on the anti-apoptotic Bcl-2 family member (Chipuk *et al.*, 2010). Due to this variable affinity for binding, a BH3 only protein can disrupt the interaction between an anti-apoptotic Bcl-2 protein and another BH3 only protein with lower binding affinity which in turn can displace a third BH3 only protein

engaged in another interaction in a "dominos-like" chain interaction. It is therefore the balance between the pool of anti-apoptotic Bcl-2 family proteins and the pool of pro-apoptotic BH3-only proteins that dictates the final cellular outcome. Elevation in the level of one of either group of proteins can tip the balance towards a certain fate.

1.3.2 Role of Bcl-2 family proteins in the regulation of apoptosis

The Mitochondrial membrane is the main site of action of apoptosis regulation by Bcl-2 family proteins. The role of Bcl-2 family members in protecting against or mediating apoptosis is well-established. Briefly, anti-apoptotic Bcl-2 proteins reside at the mitochondria guarding them against mitochondrial membrane permeabilization (MOMP). BH3-only proteins can interact with anti-apoptotic Bcl-2 family members dissociating them from other BH3-only proteins or from Bax/Bak which in turn mediate MOMP (Chipuk *et al.*, 2010).

1.3.3 Role of Bcl-2 family proteins in the regulation of autophagy

Beclin-1, the mammalian ortholog of yeast Atg6 was first identified in 1998 as a binding partner for Bcl-2 from a mouse brain library in a yeast two-hybrid screen (Liang *et al.*, 1998). However it was not until 2005 that the importance of the interaction between Beclin-1 and Bcl-2 in autophagy regulation was revealed (Pattingre *et al.*, 2005). Bcl-2, Bcl-X, Mcl-1 and Bcl-w have been shown to bind Beclin-1 with varying affinities leading to inhibition of autophagy as a consequence of this interaction (Figure 1.4). Binding of Bcl-2 family proteins to Beclin-1 exerts inhibitory effect on autophagy by either disrupting the interaction between Beclin-1 and class 3 PI3K or just diminishing the activity of class 3 PI3K bound to Beclin-1 without necessarily displacing it from that interaction (Levine *et al.*, 2008). The ability of Bcl-2 family members to bind Beclin-1 raised the question if this interaction occurs through a BH3 domain on Beclin-1. Indeed, Beclin-1 has recently been identified as a novel BH3-only protein and was shown to contain a BH3 domain. Truncation mutations of (aa 112-159) region were initially shown to render Beclin-1 unable to bind Bcl-2 or Bcl-X. Subsequent

structural and functional analysis confirmed the presence of a BH3 domain (aa 112-123) in that region (Maiuri *et al.*, 2007). Beclin-1 mutants that cannot bind anti-apoptotic Bcl-2 proteins exhibit a gain-of-function phenotype and are more potent than wild type Beclin-1 at driving autophagy when transfected into cells. Similarly, mutations in anti-apoptotic Bcl-2 proteins that diminish their ability to bind BH3 domain diminish their ability to interact with Beclin-1 as shown by co-immunopreciptation and in vitro binding assays (Maiuri *et al.*, 2007). Anti-apoptotic Bcl-2 proteins thus function as anti-autophagy as well as anti-apoptotic. Conversely, BH3-only proteins Bad, Puma, Bik and Bim were shown to induce autophagy in certain contexts. Bad was shown to induce autophagy under starvation conditions by disrupting the interaction between Bcl-X and Beclin-1 and thus liberating Beclin-1 from that inhibitory interaction (Maiuri *et al.*, 2007). Similarly, the BH3 peptide ABT737 was shown to induce autophagy in cells that are resistant to the pro-apoptotic action of ABT-737 by displacing Bcl-2 and Bcl-X from interaction with Beclin-1. Additionally, Egl-1, the only BH3-only protein in *C. elegans* was shown to be an essential mediator of autophagy in this nematode. Starvation induced autophagic response is blunted in Egl-1-deficent embryo (Maiuri *et al.*, 2007).

1.3.4 The BH3-only protein Noxa

Noxa is one of the pro-apoptotic members of the Bcl-2 family proteins. *NOXA* cDNA was first cloned in 1990 from an adult T-cell leukemia (ATL) (Hijikata *et al.*, 1990). It was initially given the name ALT-derived PMA-responsive gene (APR) as the transcript was found to be induced by PMA. However, the function of that gene remained unknown for a decade till it was shown by Oda and co-workers to be a p53-responsive gene and a mediator of p53-induced apoptosis (Oda *et al.*, 2000). Noxa was shown later on to be induced by other p53-indpendent mechanisms in response to a variety of stimuli. The p53-related protein, p73 was shown to induce the transcription of Noxa (Filnterman *et al.*, 2005). Noxa was also found to be induced under hypoxic conditions. It was described to be a direct target of Hif-1α and a hypoxia-responsive element was identified in *NOXA*

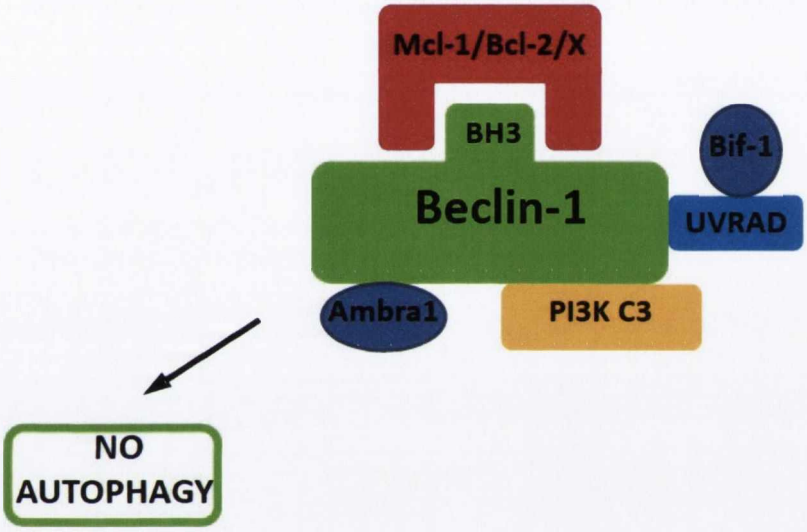


Figure 1.4 Regulation of autophagy by anti-apoptotic Bcl-2 family proteins

Anti-apoptotic Bcl-2 family proteins bind Beclin-1 and inhibit the ability of Beclin-1 to drive autophagy.

promoter (Kim *et al.*, 2004). In addition, myc-induced Noxa upregulation was described in response to proteasome inhibition (Nikiforov *et al.* 2007). Noxa upregulation was also observed in response to ER stress. ATF3 and ATF4 were implicated in mediating Noxa upregulation in these conditions (Wang et al., 2009). Interestingly, Noxa induction was also observed in response to oncogenic stress caused by E1A overexpression. E1A overexpression was also shown to induce Noxa in p53-deficient SAOS-2 cells (Flinterman *et al.*, 2005). Noxa upregulation in this condition may reflect an anti-transformative response suggestive for anti-tumorigenic role for Noxa in the context of oncogene activation. However, to date no direct link between deregulated expression of Noxa and tumor development has been established. *NOXA*$^{-/-}$ and even *NOXA*$^{-/-}$ *PUMA*$^{-/-}$ double- deficient animals develop normally and do not develop spontaneous tumor formation (Shibue *et al.*, 2003).

Noxa is one of the BH3-only proteins that exhibit weak pro-apoptotic potential and limited binding capability to anti-apoptotic members of the Bcl-2 family proteins. Noxa binds exclusively Mcl-1 and A1. The interaction between Noxa and Mcl-1 has important implications as it may target the latter to the proteasome for degradation. Elevated levels of Noxa therefore can tip the balance towards induction of apoptosis especially in cells that rely mainly on the anti-apoptotic functions of Mcl-1 for survival.

1.4 ER Stress

The endoplasmic reticulum (ER) is the site for synthesis, folding, modification, and trafficking of secretory and cell-surface proteins. As a major intracellular calcium storage compartment, the ER also plays a critical role toward maintenance of cellular calcium homeostasis. Disruption of these physiological functions by ER stress has been implicated in a wide variety of human diseases. ER stress occurs when the protein-folding capacity of the ER is exceeded. Stresses which perturb redox state, energy levels or calcium homeostasis trigger the accumulation of unfolded proteins within the ER, eliciting a stress response termed the unfolded-protein response (UPR).

1.4.1 Unfolded protein response

The UPR senses unfolded proteins in the ER lumen and transmits that information to the cell nucleus, where it drives a transcriptional program that is tailored to re-establish homeostasis. The unfolded protein response in mammalian cells is governed by three transmembrane ER stress sensors; PERK PKR-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6), which are kept in an inactive state by binding to the ER chaperone Grp78, preventing their oligomerization-induced activation. When ER homeostasis is perturbed, accumulating misfolded proteins become progressively bound to BiP, titrating away Grp78 from interaction with these transmembrane signalling proteins (Szegezdi *et al.*, 2006). Upon homodimerization these ER sensors activate a complex ER-to-nucleus signalling pathway that transmits information across the ER membrane to an extensive gene-expression program mediated by the activation of downstream transcription factors. Dissociation of GRP78 from PERK initiates the dimerization and autophosphorylation of the kinase and generates active PERK. Once activated, PERK phosphorylates eukaryotic initiation factor 2 (eIF2), which leads to inhibition of general protein translation relieving the protein burden on the stressed ER. Paradoxically, this translational shutdown leads to the selective translation of the transcription factor ATF4, a member of the bZIP family of transcription factors (Szegezdi *et al.*, 2006). The PERK-eIF2α-ATF4 axis regulates the expression of genes involved in amino acid biosynthesis and transport functions, antioxidant stress responses, and cell death. IRE1 is a type I transmembrane protein which undergoes oligomerization in ER stress. Oligomerization of IRE1 results in the activation of XBP1s that regulates the transcription of several genes involved in ER quality control mechanisms and ER/Golgi biogenesis. ATF6 is processed under ER stress conditions. Processed ATF6 moves to the nucleus where it forms active homodimers or dimerizes with other bZIP transcription factors like NF-Y (CAAT binding factor) as well as XBP1s

to regulate transcription from ATF/cAMP response elements (CREs) and ERSEs (Schroder, 2008).

1.4.2 ER stress and autophagy

Two degradation routes co-ordinate with the UPR to restore homeostasis: (i) retro-translocation of the unfolded polypeptide chain into the cytosol followed by ubiquitination and proteasomal degradation in a process termed ERAD (ii) targeting of parts of the ER to lysosomes or vacuoles in autophagy. ER stress is thus closely related to autophagy. Autophagy has been shown to counterbalance the ER expansion during UPR (Bernales *et al.*, 2006). ER provides materials for the formation of the isolation membranes (Hayashi-Nishino *et al.*, 2009). Recently some ER stress regulators have been implicated in the transcriptional regulation of autophagy regulating genes. PERK has been shown to mediate the transcriptional activation of LC3 and Atg5 in hypoxic conditions through a mechanism that involves the activation of ATF4 and its downstream target CHOP, respectively (Rouschop *et al.*, 2010). However little is known about other possible mechanisms and pathways that connect the two processes.

1.5 Aims of the study

RAS is an important proto-oncogene that has been a subject of numerous studies. Mutational activation of Ras triggers a wide range of cellular responses including cell cycle arrest and cellular senescence (Serrano *et al.*, 1997).

A few sporadic reports suggested that the expression of oncogenic Ras triggers a form of cell death that correlated with signs of autophagy (Chi *et al.*, 1999; Kitanaka *et al.*, 2002). However, the molecular mechanism of Ras-induced cell death and whether autophagy in this context mediates or opposes cell death remained elusive. We aimed in the first part of this study to characterize Ras-induced cell death and to get insight on the molecular mechanisms that regulate it. Furthermore, we aimed to unravel whether autophagy contributes or opposes Ras-induced cell death. We also studied the differential induction of autophagy by different Ras isoforms. Additionally, as several reports have clearly demonstrated that in certain contexts Ras triggers cell cycle arrest and senescence, we aimed to explore the interplay between Ras-induced cell death and senescence and to determine what factors may contribute to cell's decision to senesce or die.

In the second part of this study, we found that the Ras-induced cell death correlated with the upregulation of the pro-apoptotic protein Noxa. We aimed to explore the role of Noxa in mediating this form of non-apoptotic cell death triggered by oncogenic Ras. We also sought to investigate whether Noxa plays a role in restraining Ras-induced transformation. Furthermore, we dissected the pathway that leads to the induction of Noxa expression downstream of Ras and explored whether p53, the "classical" inducer of Noxa, plays a role in this process.

Finally, in the third part of the study, we explored the role of anti-apoptotic members of the Bcl-2 family members in regulating Ras-induced autophagy and cell death and whether they can co-operate with Ras to enhance transformation. We also investigated their involvement in regulating autophagy in other contexts

and aimed to impose our comprehensive understanding of the role of Bcl-2 in autophagy to get further insight on the regulation of Ras-induced autophagy and cell death and how to possibly exploit this understanding for therapeutic targeting of Ras.

Chapter II

Materials and Methods

2.1 Materials

2.1.1 General reagents

Doxycycline, Actinomycin D, E64d, pepstatin, puromycin, acridine orange and monodansyl cadaverine were purchased from Sigma Aldrich. UO126 was purchased from Cell Signalling, zVAD-fmk from Bachem, siRNA's were purchased from Ambion and DNA-related reagents were purchased from NEB biosciences.

2.1.2 Antibodies

Primary antibodies were purchased from the following suppliers:

α-Mcl-1 pAb	Santa Cruz Biotechnology
α-p62 mAb	Santa Cruz Biotechnology
α-p53 mAb	Dako
α-Puma pAb	Prosci
α-Bim pAb	Calbiochem
α-Pan Ras mAb	Calbiochem
α-NOXA mAb	Calbiochem
α-MDM-2 mAb	Calbiochem
α-p21 mAb from	Calbiochem
α-Atg7 from	Epitomics
α-ATF4 mAb	Abnova
α-P-ERK pAb	Cell Signalling
α-ERK pAb	Cell Signalling
α-P-p70S6K pAb	Cell Signalling
α-P-eI2F alpha pAb	Cell Signalling
α-BAD pAb	Cell Signalling
α-P-Akt pAb	Cell Signalling
α-Beclin-1 mAb	Novus
α-Atg5 pAb	Novus

α-Bmf mAb	Novus
α-Bcl-2 mAb	BD Biosciences
α-Bcl-xL pAb	BD Biosciences
α-Bid pAb	BD Biosciences
α-p16 mAb	BD Biosciences
α-caspase 3 mAb	BD Biosciences
α-p23 mAb	BD Biosciences
α-cyclin D1 mAb	BD Biosciences
α-p73 mAb	BD Biosciences
α-myc mAb	BD Biosciences
α-Grp78 mAb	BD Biosciences
α-caspase 7 mAb	BD Biosciences
α-Actin mAb	MPL Biomedicals.
α-LC3 pAB	MPL Biomedicals

Secondary peroxidase-conjugated antibodies:

Goat α-Rabbit IgG	Jackson Immuno Research Lab
Goat α-Mouse IgG	Jackson Immuno Research Lab
Goat α-Rabbit IgG light chain specific	Jackson Immuno Research Lab
Goat α-Mouse IgG light chain specific	Jackson Immuno Research Lab

2.1.3 siRNA oligos

siRNA oligos were purchased from Ambion Inc. and Dharmacon Inc. Sequences of siRNA oligos are as follows:

The non-silencing sequence TAAGGCTATGAGAGATAC

Atg 5 # 1 GCAACTCTGGATGGGATTG (sense)

Atg 5 # 2 CATCTGAGCTACCCGGATA (sense)

Atg7 # 1 CCAAAGUUCUUGAUCAAUA (target sequence)

Atg7 # 2 CAACAUCCCUGGUUACAAG (target sequence)

Beclin-1 # 1 GGAUGACAGUGAACAGUUA (target sequence)

Beclin-1 # 2 UAAGAUGGGUCUGAAAUUU (target sequence),
c-Fos # 1 TGGGTTCATTATTGGAATTAA (target sequence)
c-Fos # 2 GACCAATATTATAVTAAGAAA (target sequence)
c-Jun # 1 AAGAACGTGACAGATGAGCAG (target sequence)
c-Jun # 2 AAGAAGTGTCCGAGAACTAAA (target sequence)
Elk1 # 1 CTGCTGAGAGAGCAAGGCAAT (target sequence)
Elk-1 # 2 AGGGAGTCATCTCTTCCTATA (target sequence)
ATF4 GCCUAGGUCUCUUAGAUGATT (sense)
ATF6 GAACAGGGCUCAAAUUCUC (target sequence).

2.2 DNA-related protocols

2.2.1 Preparation of competent bacteria

To prepare E. coli DH5α competent bacteria, a well-defined DH5 α colony was picked from a freshly streaked LB agar plate and was added to a 3 ml starter culture. The culture was grown overnight at 37°C at 280 rpm in shaker incubator. 1 ml of the starter culture was added to 100 ml of LB (1% bacto tryptone, 0.5% yeast extract, 10 mM NaCl) and grown at 25°C at 280 rpm in shaker incubator till the culture reached an OD_{600} of 0.4-06. Then the culture was chilled on ice for 10 min followed by centrifugation at 800 g for 10 min at 4°C to pellet the bacteria. The bacterial pellet was resuspended gently in 10 ml of ice cold, sterile 0.1M $CaCl_2$ and incubated on ice for 5 min. The bacterial cells were repelleted at 800 g for 10 min at 4°C before resuspension in 4 ml of ice cold, sterile 0.1M $CaCl_2$. Finally, 280 μl of DMSO was added and incubated on ice for 10 min before swirling the suspension to mix, aliquoting into sterile vials and storing at -70 °C.

2.2.2 Transformation of competent bacteria

DH5α competent bacteria were thawed on ice. 1μl of plasmid prep was mixed with 50 μl of DH5α cells in a pre-chilled tube and incubated on ice for 30 min to allow the plasmid to coat the bacterial cells followed by heat-shock by incubation at 42°C water bath for 40 sec to induce the uptake of the plasmid. Cells were

then incubated on ice for 2 min to recover. Then 450 μl of LB was added to the bacterial cells and they were incubated at 37°C shaker incubator under low agitation (220 rpm) for 1 hr to allow expression of the antibiotic resistance gene. 50 -250 μl of the bacterial culture was then plated on LB agar plates containing the relevant antibiotic, distributed evenly and incubated overnight in 37°C incubator.

2.2.3 Plasmid DNA preparation

For plasmid mini-preparation, a well-defined colony was picked using a sterile tip and added to 3ml of LB containing 100 μg/ml of freshly added Amp. Culture was then incubated overnight at 37°C in a shaker incubator (280 rpm). Following overnight incubation 1 ml of the starter culture was centrifuged in a microfuge at top speed for 1 min. The supernatant was aspirated and the pellet was resuspended in 100 μl of P1 buffer (50mM Tris Hcl, pH 8, 10 mM EDTA) by vortexing. Bacteria were lysed by addition of 200 μl of P2 buffer (0.2M NaOH,1% SDS) and mixed gently and left to stand on ice for 1 min. 200 μl of P3 (3M KAc,pH 5.5) was added to cell lysate to precipitate genomic DNA and centrifuged at top speed in a microfuge for 15 min. The supernatant was isolated and transferred to a fresh tube. 0.7 volumes of isopropanolol were added to the supernatant and left to stand on bench for 20 min. The plasmid was pelleted by centrifugation in a microfuge at top speed for 10 min and the pellet was washed with 1 ml of 70% ice-cold ethanol. Plasmid was centrifuged again, the supernatant was discarded and the pellet was left to air-dry for 10 min on bench. Finally the DNA was resuspended in 10μl sterile distilled water then 5 min later 10 μl of TE (10 mM Tris, 1mM EDTA, pH 8) was added. Plasmid was visualized on 0.6% agarose gel.

Plasmid midi-preparations were prepared using Qiagen Tip-100 columns as per manufacturer's instructions. Briefly, plasmid-containing bacteria were inoculated into 50 ml of liquid media in a 250 ml Erlenmeyer flask and incubated overnight (15–18 hr) at 37 °C in a rotary shaker at 300 rpm. 40 ml of the overnight culture were transferred to a 50 ml screwcap centrifuge tube and spinned down by

centrifugation for 5 minutes at 5000 rpm. The supernatant was discarded and 5 ml of Cell Resuspension Solution was added to the cell pellet. The cells were vortexed to resuspend the pellet and 5 ml of Cell Lysis Solution were added and mixed by inverting the tube 6–8 times until the solution became viscous and somewhat clear. 5 ml of Neutralization Solution were added and mixed by inverting the tube 6–8 times. The mix was centrifuged for 10 minutes at 8000 rpm. The Quantum Prep matrix was resuspended by shaking vigorously. 1.0 ml of Quantum Prep matrix was added to the cleared lysate and swirled gently for 15–30 seconds to mix followed by centrifugation for 2 minutes at 8,000 rpm to pellet the matrix. The supernatant was poured off from the pelleted matrix and 125 ml of 95% ethanol were added to the wash buffer before first use. 10 ml of wash buffer were added to the matrix. The matrix was resuspended in the wash buffer by shaking and then centrifuged for 2 minutes at 8000 rpm. The wash buffer was poured from the pelleted matrix. 600µl of wash buffer (200 mM NaCl, 40 mMTris, 4 mM EDTA, pH 7.5 plus an equivalent amount of 95% ethanol) were added to the matrix and resuspend. The spin column was placed inside a 2 ml collection tube and the resuspended matrix was transferred from the previous step to the spin column. After three washing steps, the plasmids were eluted in 600µl TE solution. DNA was sterilized by filter-centrifuging in Spin-X tubes (Sigma), labelled and stored in +4°C fridge.

2.2.4 Agarose gel electrophoresis

Products of DNA mini- and midi-preparation were assessed by agarose gel electrophoresis. 0.6% agarose gel was prepared by dissolving agarose in TEA (40 mM Tris-HCl, pH 8, 1 mM EDTA) and cooking in a microwave briefly then cooling and solidifying in gel casting rigs. DNA was diluted using loading buffer and electrophoresed at 5-10 V/cm. Gels were then incubated in ethedium bromide and bands were visualized in UV light using a BioRad Gel Doc 2000.

2.2.5 DNA quantification

DNA was quantified on a Nanodrop by measuring the absorbance at 260 nm.

2.3 Cell Culture

2.3.1 Origin of the cell lines

HOSE control cells	Dr Julian Downward (Cancer Research UK- London)
HOSE-Ras ^{V12} cells	Dr Julian Downward (Cancer Research UK- London)
HUVECs	Promo Cell
NOXA$^{-/-}$ MEFs	Prof. Andreas Villunger, (Innsbruck Medical University)
counterpart WT MEFs	Prof. Andreas Villunger, (Innsbruck Medical University)
HeLa	Prof. D. Green (St. Jude Children's Hospital, Memphis)
IMR90 fibroblasts	Dr Kevin Ryan (Beatson Institute for Cancer Research, UK)
HEK293T	Prof. Inder Verma (Salk Institute for Biological studies, CA)

2.3.2 Cell lines maintenance

HOSE control and HOSE-RasV12 cells, SAOS-2 cells, MCF-7 cells, HUVECs, WT and *NOXA*$^{-/-}$ MEFs and IMR90 fibroblasts were all cultured in DMEM (Gibco) supplemented with 10% bovine calf serum (Sigma) and 2 mM L-glutamine (Gibco).. HeLa cells were grown in RPMI (Gibco) supplemented with 5% bovine calf serum (Sigma) and 2 mM L-glutamine (Gibco). Cells were cultured at 37°C and 5 % CO_2. Cells were passed every 2-3 days. Adherent cells were trypsinised with Trypsin-EDTA (0.25% trypsin, 1 mM EDTA, in Hank's balanced salt solution) (Gibco) at 37 °C. The trypsin was inactivated by addition of fresh medium with bovine calf serum. For the induction of H-Ras^{V12} expression in HOSE-Ras^{V12} cells, cells were cultured in a medium containing 1-1000ng/ml Doxycycline (Dox). Cell freezbacks were prepared by harvesting the cells by trypsinization followed by centrifugation. Cell pellet was resuspened in 0.5 ml freezback medium (45% serum in DMEM medium) and transferred immediately to -70 °C freezer. For long term storage, cell freezbacks were stored in liquid nitrogen tanks.

When needed, cells were thawed by incubation for 1-2 min in 37 °C water bath and then added immediately to 10 ml of medium followed by centrifugation at 1300 rpm for 5 min. Cell pellet was resuspneded in 10 ml of fresh medium and plated in 10 cm dishes in 37 °C incubator.

2.3.3 Tetracycline-inducible system

Two tetracycline operator sequences ($TetO_2$) have been inserted between the TATA box of the CMV promoter and the transcriptional start site. The $TetO_2$ sequence itself has no effect on expression. When the tetracycline repressor protein (TR) is present, it effectively binds the $TetO_2$ sites and blocks transcription initiation. Tetracycline added to the culture medium binds to, and changes the conformation of, the TR protein. This change causes the TR protein to release the $TetO_2$ sites, derepressing transcription from the CMV promoter. The result is high-level expression of the gene of interest. Expression levels can be modulated based on the tetracycline concentration and can be induced to levels that are achieved with constitutive CMV expression vectors.

2.4 Transfection of mammalian cells

2.4.1 Genejuice® method

HeLa cells and MCF-7 cells were transfected using Genejuice® reagent (Merk-Germany) according to manufacturer's protocol, briefly Genejuice® was added to RPMI in a 1-2:100 ratio. Genejuice®-RPMI mix was added dropwise to the plasmid combination in a 96 well plate and was allowed 15 min for the DNA-Genejuice® fine precipitate to form before it was added to cells plated in 6 well plates.

2.4.2 Nucleofection of mammalian cells using Amaxa method

HOSE-Ras.[12] cells, IMR-90 fibroblasts, HUVECs and MEFs were electroporated using Amaxa Nucleofection procedures. Briefly, cells were trypsinized, pelleted and resuspended in nuclefection buffer (see below). 100 μl of the cell suspension was added to plasmid mix, transferred to a nucleofection cuvette and immediately nucleofected using Amaxa nucleofector and the specific program for every cell line. 500μl of fresh medium was added immediately to the cells after

nucleofection and the nucleofection mix was seeded in a 10cm dish containing 8 ml of pre-warmed medium.

Nucleofection buffer composition:

- 5 mM KCl
-15 mM MgCl2
-20 mM HEPES
-150 mM Na2HPO4/ NaH2PO4 pH 7.2
-75 mM NaCl
-50 mM Tris HCl pH7.2
-10 mM Glucose

2.4.3 Calcium phosphate precipitation method

HEK293T cells were plated at a density of 1-2 x 10^6 cells per 10 cm dish 24h prior to transfection. Precipitates were prepared in a 24-well plate by mixing the DNA diluted to 200 µl in sterile dH_2O with 50 µl of filter-sterilized 2.5 M $CaCl_2$ to bring the total volume to 250 µl. DNA/$CaCl_2$ mix was added dropwise in each well to 250 µl of 2X HBS buffer (280mM NaCl, 10mM KCl, 1.5mM Na_2HPO_4, 12mM dextrose, 50 mM HEPES, pH 7.1.). The complexes were incubated at room temperature for 20-30 min to allow fine precipitates to form. Then the precipitates were added to the cells and incubated overnight to allow uptake of the plasmid precipitates.

2.5 PROTEIN ANALYSIS

2.5.1 Protein quantification by BioRad assay

The required volume of a 1:5 dilution of the BioRad reagent in ddH2O was prepared just before use. A series of protein standard dilutions (25 µg/mL -1 mg/mL of BSA in water) were prepared for calibrating the assay. 20 µl of each standard, or each unknown protein sample, or buffer only (as a blank) were taken and pipetted into an eppendorf. Then 980 µl of diluted Bio-Rad reagent were pipetted into each tube and mixed. Samples were incubated for 15 min prior to reading on a spectrophotometer. Samples were read at OD595 and unknown

protein concentrations were calculated by plotting a line of the BSA standards versus their OD595 readings and fitting the best line plot using CricketGraph.

2.5.2 SDS Polyacrylamide Gel Electrophoresis

Total cell lysates were prepared by lysing cells or cell pellets in SDS-PAGE lysis buffer (100mM Tris-HCl pH6.8, 4%SDS, 20% glycerol, 0.1% bromophenol blue and 5% β-mercaptoethanol added immediately before use) followed by heating at 94 °C for 7 min. Lysates were then electrophoresed on 8-15% SDS-PAGE gels. Gels were run at 50 V (stacking gel) / 75 V (separation gel) on Protean III apparatus (BioRad) and at 60 V / 90 V on Criterion apparatus (BioRad).

2.5.3 Western Immunoblotting

Gels were transferred onto nitrocellulose and probed with the appropriate primary antibody followed by the corresponding secondary antibodies diluted 1:1000. For reprobing blots with different antibodies, the signal was attenuated by incubating the blots in 0.2M NaOH for 5 min followed by washing with TBST for 30 min.

2.6 Immunoprecipitation (IP)

HOSE-RasV12 cells were treated as indicated in each experiment. Cells were harvested by trypsinization, washed with ice cold PBS pH 7.2 and lysed in 800 μl per1x107 cells of IP lysis buffer (150mM NaCl, 50mM Tris-HCl, pH 8.1%, 1% NP-40, 1mM PMSF, 10μg/ml leupeptin, 1μg/ml aprotinin). Cell lysates were precleared by incubation for 2 hours with pre-immuned serum and 20 μl protein G agarose beads under constant rotation at 4°C Beads were precipitated by centrifuging at 2000g for 2 min at 4°C. Supernatants were isolated and incubated overnight with 1 μg of the indicated antibody or a pre-immuned serum as control and 20 μl protein G agarose under constant rotation at 4°C. Beads were washed 5 times in ice cold lysis buffer containing 0.1% NP-40 .Beads were finally collected by centrifugation at 5000g for 2 min and brought up in SDS-PAGE lysis buffer.

2.7 Microscopy

2.7.1 Light microscopy

Cells were visualized at 4-40 x magnification power under an Olympus BX51 microscope.

2.7.2 Confocal microscopy

Cells were plated on coverslips and allowed 48 h to attach then transfected with various plasmid combinations or alternatively cells were plated on coverslips immediately after nucleofection and left for 48-72 h. Cells were then washed in PBS pH 7.2, fixed with 3.7% paraformaldehyde in PBS for 10 min and washed twice with PBS pH 7.2. Cells were mounted with Slow Fade (Invitrogen) and observed on a laser scanning microscope (Olympus FV1000) using a 488 nm Argon laser (green fluorescence), a 543 nm HeNe laser (red fluorescence) and a 405 nm LD laser (Hoechst). Confocal images were acquired with Fluoview 1000 V.1 application software and images were processed with Adobe InDesign.

2.8 Senescence-associated β-galactosidase (SA- β-Gal) assay

Cells were washed twice with PBS pH 7.2, then fixed with 0.5% glutaraldehyde in PBS and then washed three times with PBS pH 7.2 supplemented with 1 mM MgCl2. Cells were stained for 4–6 h with X-Gal solution (1 mg ml–1 X-Gal, 0.12 mM K3Fe[CN]6, 0.12 mM K4Fe[CN]6, 1 mM MgCl2 in PBS at pH 6.0). β-gal positive cells (blue-stained) were counted using Olympus microscope under bright phase.

2.9 Induction of apoptosis

As a positive control for apoptotic cell death, cells were induced to undergo apoptosis by treatment with 2 μM Actinomycin D (Act D). Act D was added directly to the cells plated at 10^5 cells/ well in 6-well plates. Culture was incubated overnight. Apoptosis induction was confirmed by examination of morphological features of apoptosis (cell shrinking, detachment, membrane blebbing and chromatin condensation) as well as biochemical markers.

2.10 Quantification of cell death

To quantify for the cell death induced under different conditions, cells were harvested by trypsinization, washed in PBS pH 7.2 then stained with propidium iodide (10 μg/ml) that was added just prior to analysis. Cell fluorescence was then measured on a flow cytometer (FACSCalibur; Becton Dickinson, CA) and analyzed using CellQuest software.

2.11 Nuclear staining with Hoechst dye

Nuclei of cells treated with different treatments were visualized by staining with Hoechst dye. Hoechst dye was added directly to the cell culture at a final concentration of 20nM and nuclei were examined under an Olympus fluorescence microscope.

2.12 Quantification of autophagy

Autophagy induced under various conditions was quantified by transfecting the cells with GFP-tagged LC3 and counting the percentage of cells exhibiting punctate versus diffuse GFP-LC3 under laser confocal microscope (Olympus FV1000).

2.13 Clonogenic Assay

To test the effect of different conditions on the clonogenic survival capabilities of the cells, cells were subjected to clonogenic assay illustrated in Figure (2.1). Briefly, HOSE-Ras^{V12} cells were nucleofected with the indicated plasmids. After nucleofection with the indicated plasmids, cells were left for 48 to un-stress and to fully express the nucleofected plasmids. In the case of nucleofection with shRNA against different BH3-only proteins, nucleofected cells were selected by treatment with 300 ng/ml of puromycin as all the shRNA constructs included puromycin-resistance gene. After 7 days of selection with puromycin, cells were allowed to grow for another 7 days in a medium containing 150 ng/ml puromycin to maintain the initial selection. Protein expression or knockdown efficiency was checked at this stage by on samples from different nucleofection conditions.

Another selection round with was also run on a subset of the cells to ensure that the maintenance period did not compromise the initial selection. Cells were then treated with or without 100ng/ml Dox for 3 or 5 days. 1000 or 2000 were then plated in a 6-well plate and treatment with or without Dox was continued for 3 more weeks. Colonies were stained with 0.5% crystal violet in 20% methanol and washed three times with PBS pH 7.2. Colonies bigger than 1mm were counted for colonies number assay or images of the plates taken by Canon digital camera were analyzed by ImageJ software for surface coverage assay.

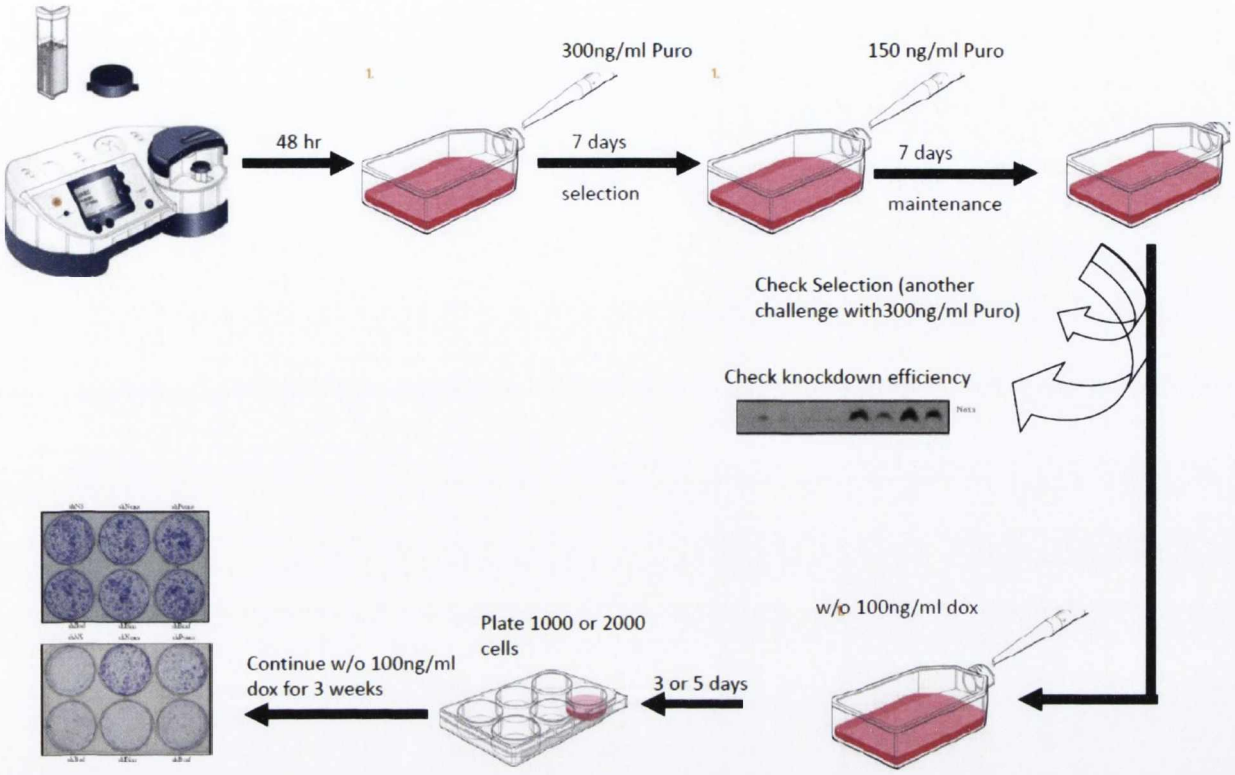


Figure 2.1 Clonogenic survival assay

Schematic presentation showing the steps of clonogenic survival assay .

Chapter III

Characterization of Oncogenic Ras- Induced Autophagy and Cell Death

3.1 Introduction

RAS is a proto-oncogene that is found mutated in 20-25% of all human cancers, making it an important subject for intensive study. Expression of oncogenic Ras has been described to trigger a wide spectrum of responses (reviewed in chapter one). A few sporadic studies have suggested that expression of oncogenic Ras induces cell death in cultured cells *in vitro* (Chi *et al.,* 1999; Kitanaka *et al.,* 2002). It has also been suggested that Ras-induced cell death contributes to the spontaneous regression of neuroblastoma *in vivo* (Kitanaka *et al.,* 2002). This supports the notion that Ras-induced cell death may constitute one of anti-proliferative responses evoked by mutational activation of Ras to restrain transformation. However, very little is known to date about the characteristics of Ras-induced cell death and the molecular mechanisms that execute it. We aimed to study in more detail the cellular and molecular consequences of gain of function mutations in the *RAS* gene with special focus on the anti-transformative responses triggered by mutational activation of this oncogene. To this end, we made use of a previously described system "HOSE-Ras^{V12} cells" (Nicke *et al.*, 2005). HOSE-Ras^{V12} cells are stably transfected with pcDNA4/TO-^{V12} H-Ras and thereby have the constitutively active H-Ras^{V12} under the control of a tetracycline-inducible promoter. HOSE-Ras^{V12} cells are immortalized by the human papilloma virus oncogenes E6 and E7 in combination with hTERT and have a lifespan of over 100 population doublings (Nicke *et al.*, 2005). Although many of the observations in this study were initially made in HOSE-RAS^{V12} system, central observations were reproduced in other cell lines as well to exclude cell linage specificity.

3.2 Results

3.2.1 Expression of oncogenic Ras cripples cell growth and induces cell death

The induction of oncogenic H-Ras^{V12} expression by doxycycline (Dox)-treatment of HOSE-Ras^{V12} cells resulted in drastic changes in cellular morphology. While untreated cells looked healthy and proliferated normally, Dox-treated cells became flatter and exhibited massive cytoplasmic vacuolation, cell rounding and detachment from the surface (Figure 3.1 A).

Assessment of the proliferation rate of untreated versus Dox-treated cells revealed that oncogenic H-Ras induction resulted in initial mitotic burst that lasted two to three days but was surprisingly followed by a drop in proliferation rate and decrease in cell numbers (Figure 3.1 B). The drop in cell number that becomes obvious five to seven days post induction of H-Ras^{V12} expression suggested that cell death was taking place. Staining with propodium iodide (PI) revealed that a considerable number of Dox-treated HOSE-Ras^{V12} cells were indeed dying 5-7 days post Dox-treatment. These dead cells become permeable to PI vital dye and thus fluoresce bright red while untreated cells excluded the dye and looked healthy (Figure 3.2 A and B).

Although the concentration of doxycycline (Dox) used throughout the study was within nanograms per ml, we wished to ensure that Dox-treated cells were not dying as a consequence of Dox treatment *per se* rather than the induction of H-Ras^{V12}. To this end, control HOSE cells, not transfected with the Dox-inducible H-Ras^{V12} construct, and HOSE-Ras^{V12} cells were treated with Dox and cell death was assessed. As Figure 3.2 B shows, only HOSE cells expressing Dox-inducible H-Ras^{V12} died upon Dox treatment while neither untreated HOSE-Ras^{V12} nor Dox-treated control HOSE were affected which confirms that the cell death is a direct consequence of H-Ras^{V12} induction.

3.2.2 Expression of oncogenic Ras limits clonogenic capabilities

Next, we sought to examine the effect of H-Ras^{V12} expression on long-term clonogenic survival. Consistent with the effect of H-Ras^{V12} expression on short term growth rate, cells expressing H-Ras^{V12} (Dox-treated) did not survive the clonogenic challenge outlined in the previous chapter. While untreated cells readily formed many colonies that covered most of the well surface, Dox-treated cells formed markedly less colonies (Figure 3.2 C). Ras-induced cell death that may contribute to the diminished clonogenic survival observed in cells expressing Ras.

3.2.3 Ras induces non-apoptotic cell death

From the previous experiments it was obvious that induction of H-Ras^{V12} induces cell death that impacted the long term clonogenic survival. We sought to get insight into the characteristics of this form of cell death triggered by oncogenic H-Ras. Expression of other oncogenes such as c-myc and E2F has been shown to induce apoptosis (Prendergast, 1999, Thompson, 1998, Lowe *et al.*, 2004). We, therefore, asked whether oncogenic H-Ras expression similarly promoted apoptosis. To address this question we initially examined the morphology of cells dying in response to oncogenic H-Ras expression. Cells dying by apoptosis typically exhibit characteristic morphological features which distinguishably characterize this form of cell death such as cell retraction, membrane blebbing and chromatin condensation. Close inspection of HOSE-Ras^{V12} cells dying upon H-Ras.V12 induction did not suggest that these cells were dying by classical apoptosis as they did not show any of the gross morphological features of apoptosis. Such features, however, were clearly detectable when HOSE-Ras^{V12} cells were treated with Actinomycin D (Act D), a widely used apoptosis-inducing agent (Figure 3.3 A). These initial morphological observations were further confirmed by staining with the nuclear dye Hoechst dye. As figure 3.3 A illustrates, cells dying upon H- Ras^{V12} expression did not show chromatin condensation which is characteristic for apoptotic cell death and was readily obvious in the apoptotic HOSE-Ras^{V12} cells dying upon treatment with Act D.

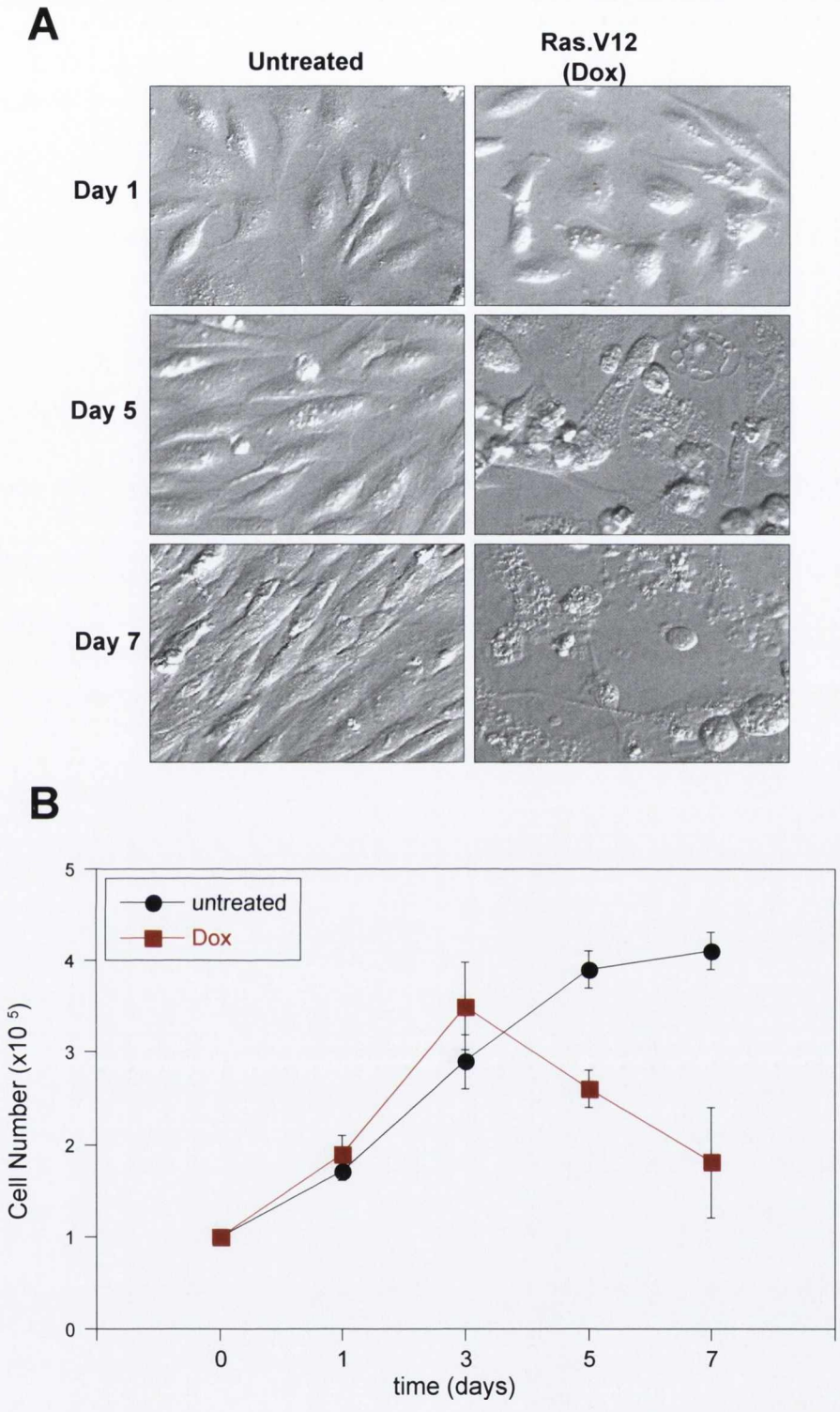


Figure 3.1 Expression of oncogenic H- Ras impedes cell growth

HOSE.RasV12 cells were plated at 10^5/10cm dish followed 24 h later by treatment with or without 100ng/ml Dox. . Pictures of the cultures at different time points were taken on a light microscope with 40X magnification (A). Cells were counted at the indicated time points (B)

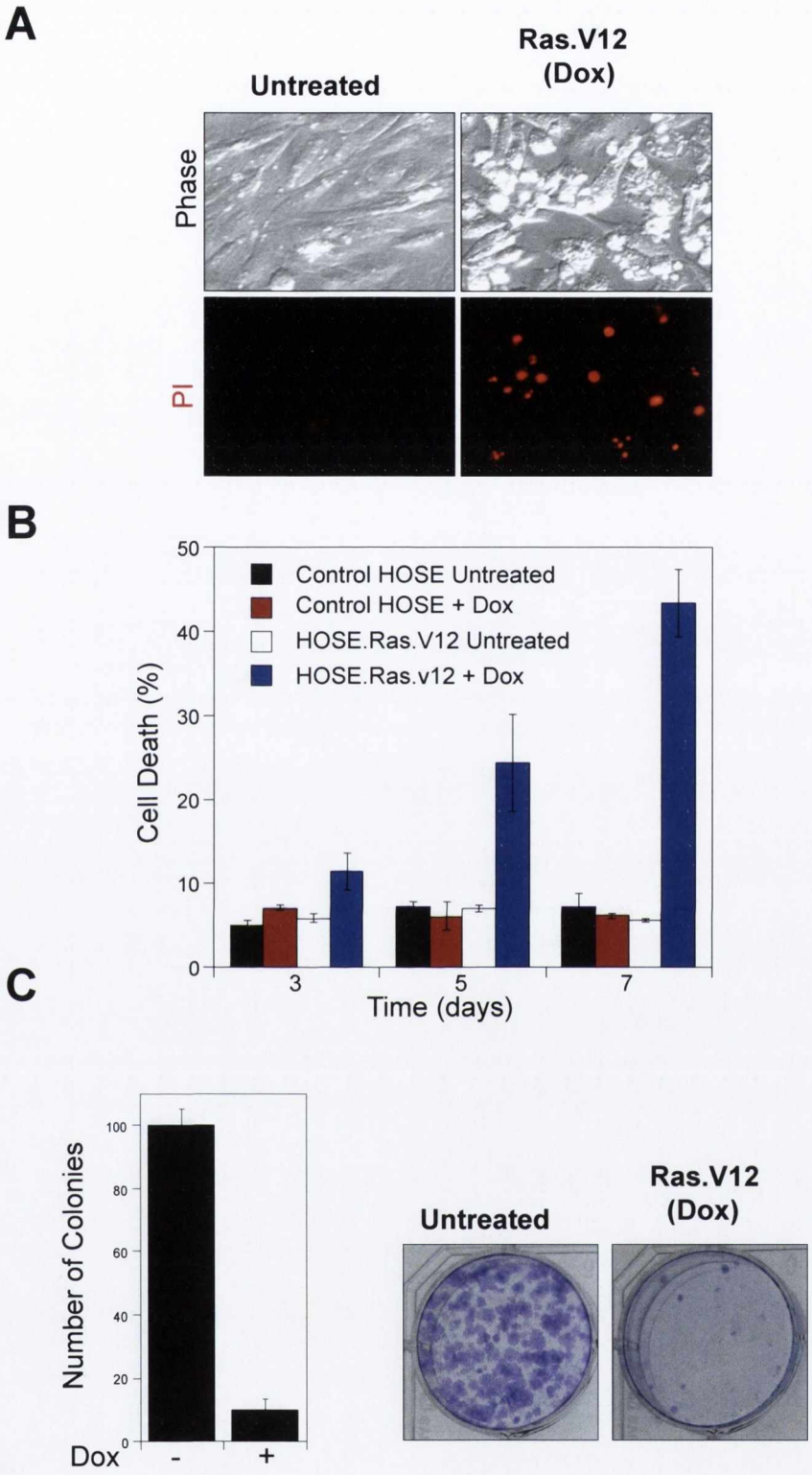


Figure 3.2 Expression of oncogenic H-Ras induces cell death and limits clonogenic survival

Control HOSE or HOSE.RasV12 cells were plated at 10^5/10cm dish followed 24 h later by treatment with or without 100ng/ml Dox. Dead cells were stained using propodium iodide (PI) added directly to the culture at final concentration of 10 μg/ml (A). The percentage of dead cells was assessed at the indicated time points (B). HOSE.RasV12 cells treated with or without 100ng/ml Dox were subjected to clonogenic survival assay as described in chapter two. Colonies were stained with 0.5% crystal violet in 20% methanol. Pictures were taken with Canon G9 digital camera and colonies bigger than 1mm were counted (C).

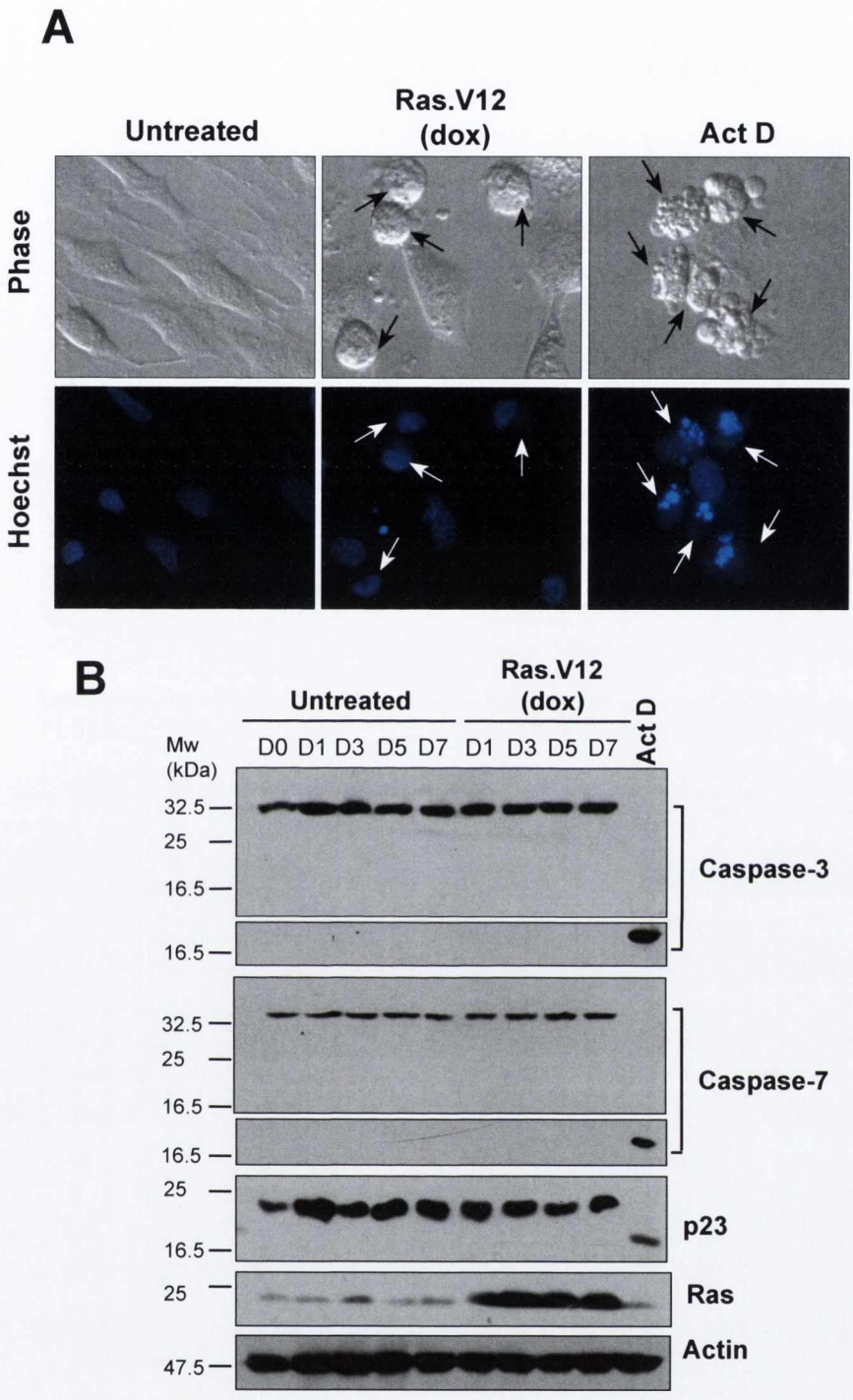


Figure 3.3 Ras induces caspase-independent cell death

HOSE.RasV12 cells were either treated with 100ng/ml Dox, 2 μM Actinomycin D (Act D) or left untreated. Cells were stained with Hoechst dye (10 μM). Pictures were taken on a fluorescence microscope with 40X magnification (A). Arrows point at dying cells in each condition. Note chromatin condensation in the case of Act D-induced apoptosis. HOSE.RasV12 cells treated with Act D, Dox or left untreated over a time course of 7 days were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (B).

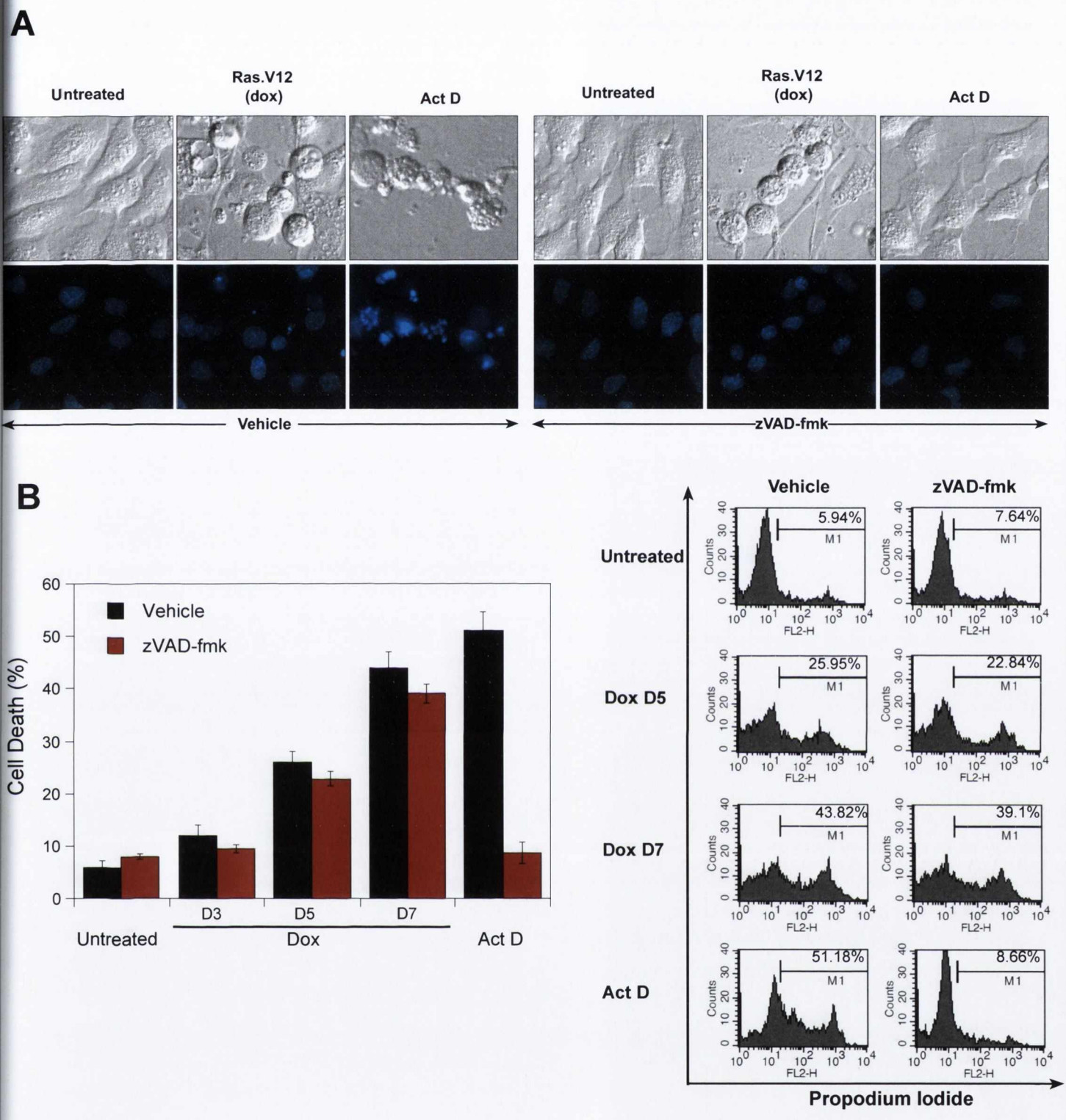


Figure 3.4 Caspase inhibition does not block Ras-induced cell death

HOSE.RasV12 cells were either treated with 100 ng/ml Dox or 2 μM Actinomycin D (Act D) in the presence or absence of zVAD-fmk (50 μM) . Cells were stained with Hoechst dye (20 nM). Pictures were taken on a fluorescence microscope with 40X magnification (A) .

At the indicated time points, cells were harvested by trypsinization. Percentage of cell death in each condition was assessed by flow cytometer analysis of PI uptake (B). PI was added immediately prior to analysis at final concentration of 10 μg/ml. The percentage of PI-positive cells in each treatment is indicated in the top right-hand corner of each panel.

Furthermore, immunoblotting of cell lysates prepared from HOSE-Ras^{V12} cells treated with or without Dox over the time course of seven days revealed that Ras-induced cell death was not associated with caspase activation as indicated by the absence of caspase 3 and caspase 7 processing. In contrast, cleavage of these caspases was evident in Act D-treated cells (Figure 3.3 B). Moreover, the caspase substrate p23 was not processed during Ras-induced cell death while it was clearly processed during Act D-induced apoptosis (Figure 3.3 B).

Consistent with the previous results, the broad caspase inhibitor zVAD-fmk did not seem to modulate the morphology (Figure 3.4 A) or the magnitude of Ras-induced cell death to any significant extent (Figure 3.4 B) It, however, profoundly inhibited Act D-induced apoptosis as assessed by morphological analysis (Figure 3.4 A) and quantified by flow cytometer analysis of PI uptake (Figure 3.4 B). Absence of any sign of caspase activation and the inability of zVAD-fmk to modulate Ras-induced cell death suggested that cell death was occurring in a caspase-independent manner.

Taken all together, these results suggest that cell death induced by H-Ras^{V12} is a form of cell death that is morphologically and mechanistically distinct from apoptosis.

3.2.4 Ras promotes autophagy that precedes cell death

Autophagy is the process of sequestrating cytoplasmic proteins into the lytic component and is characterized by the formation and promotion of acidic vesicular organelles (AVOs). As mentioned earlier, expression of oncogenic Ras resulted in massive cytoplasmic vacuolation suggestive of enhanced autophagic activities (Figure 3.5 A). To investigate the nature of these vacuoles and to test whether or not they represent a sign of undergoing autophagy, we first sought to test if these vacuoles were acidic, a characteristic feature of autophagic vacuoles. HOSE-Ras^{V12} cells treated with or without Dox were stained with the lysosomo-tropic agent acridine orange (AO). In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green and dim red, whereas acidic compartments fluoresce bright red. That is because acridine orange moves freely

across biological membranes when uncharged. The protonated form accumulates in acidic compartments where it forms aggregates that fluoresce bright red. Using this staining we were able to demonstrate that Ras-induced vacuoles were acidic as they specifically fluoresced bright red (Figure 3.5 B). To further confirm this result, we used another acidotropic dye, monodansyl cadaverine (MDC). Staining of Dox-treated HOSE-Ras^{V12} cells with MDC exhibited punctuate bright blue fluorescence in contrast to dimmer diffuse blue fluorescence exhibited by the control untreated cells stained with the same dye (Figure 3.5 C).

The previous results initially suggested that the vacuoles that become readily detectable 2-3 days after turning on the expression of oncogenic H-Ras are acidic and might indeed be acidic vesicular organelles (AVOs), a characteristic feature of underlying autophagy. To further explore this possibility, we examined the expression pattern of microtubule-associated protein 1 light chain 3 (LC3), a widely used specific marker for autophagy. LC3 is the mammalian ortholog of yeast Atg8 and is involved in autophagosome formation during autophagy. Immediately after synthesis, LC3 is processed by Atg4 and becomes LC3I. Upon induction of autophagy, the C-terminal glycine of LC3I gets conjugated to phosphatidylethanolamine, resulting in the formation of membrane-bound LC3II that localizes to the outer and inner membranes of the autophagosomes. Conversion of LC3 from cytoplasmic LC3I to autophagosome-bound LC3II results in change in distribution from diffuse to punctuate LC3. When an autophagosome subsequently fuses with a lysosome, inside materials including membrane-bound LC3II, are degraded.

The expression level of LC3II generally correlates with the number of autophagosomes and gives a good estimate of the extent of autophagic activities. Morphological analysis of puncta formation of a tagged LC3 (typically GFP-LC3) and/or detection of the conversion of LC3I to LC3II by western blotting are widely used and generally accepted specific markers for autophagy.

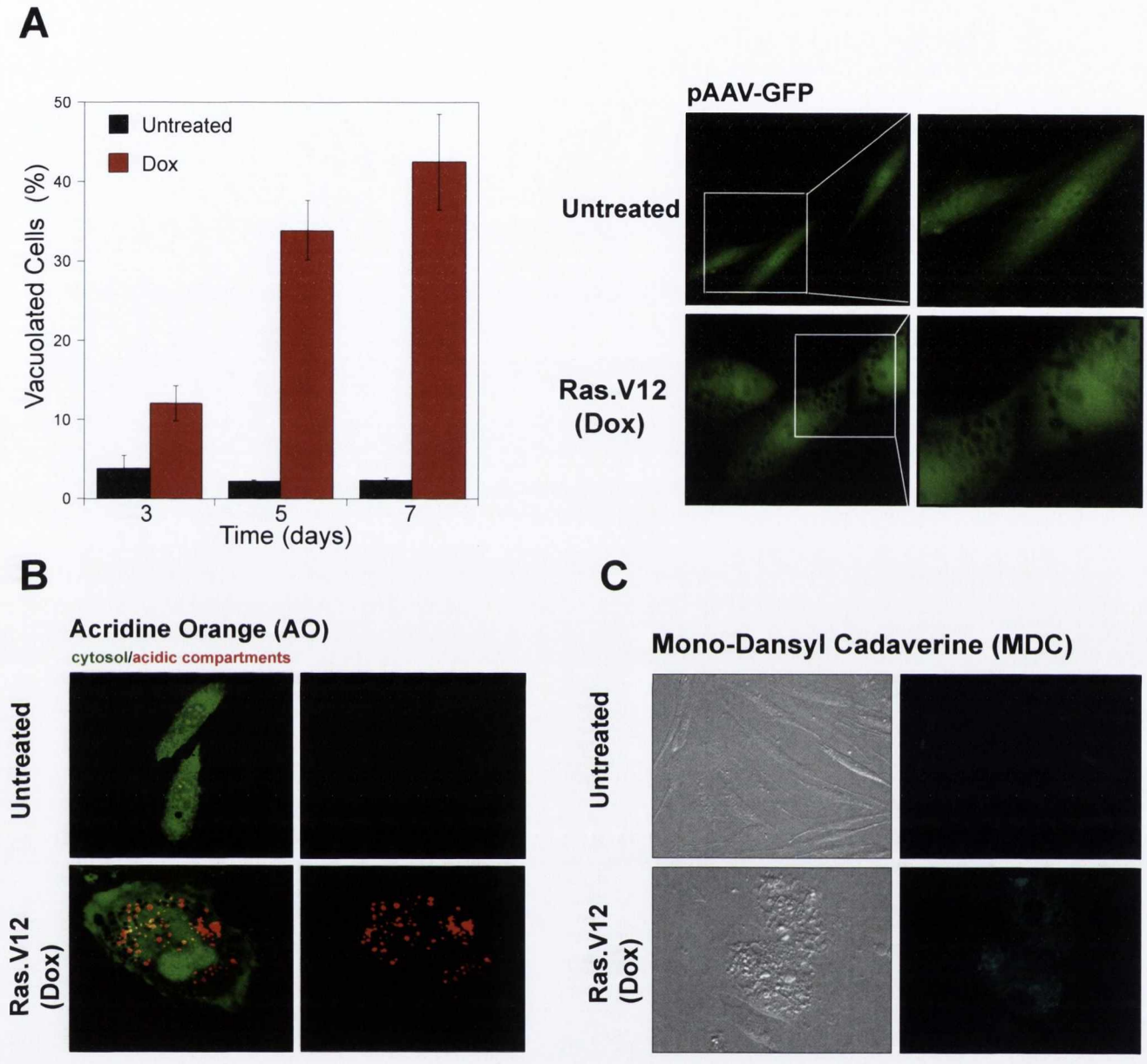


Figure 3.5 Expression of oncogenic H-Ras induces the formation of acidic vesicular organelles (AVO's)

HOSE.RasV12 cells nucleofected with pAAV-GFP were treated with or without 100 ng/ml Dox for the indicated time points. The percentage of cells exhibiting visible vacuolation under fluorescence microscope (20X magnification) was scored (A). To test if these vacuoles were acidic, cells treated with or without Dox were incubated with Acridine Orange (1μg/ml) (B) or Mono-Dansyl Cadaverine (0.05 mM) (C) at 37°C for 15 min. Pictures were taken on a confocal microscope with 40X magnification.

To test whether the expression of H-Ras^{V12} leads to autophagy manifested by changes in LC3 distribution, HOSE-Ras^{V12} cells were nucleofected with a GFP-tagged LC3 construct and then treated with or without Dox. As figure 3.6 A illustrates, induction of H-Ras^{V12} expression was accompanied by change in LC3-GFP distribution from diffuse to punctate. Additionally, high magnification images generated by confocal microscopy showed that LC3-GFP puncta localized to the membranes of the vacuoles (Figure 3.6 B). Furthermore, immunoblotting of cell lysates prepared from HOSE-Ras^{V12} cells treated with or without Dox over 7 days showed time-dependent conversion of LC3I to LC3II upon Dox treatment of HOSE-Ras^{V12} cells (Figure 3.6 C).

Additionally, to further confirm these results, we checked the levels of another marker of autophagy, p62/SQSTM1. The p62 protein serves as a link between LC3 and ubiquitinated substrates. p62 becomes incorporated into the autophagosomes and is degraded in the autolysosomes. Reduction in p62 levels therefore marks enhanced autophagy. Immnuoblotting showed a gradual decrease in p62 level over time after the induction of H-Ras^{V12} expression in HOSE-Ras^{V12} cells (Figure 3.6 C).

The decrease in p62 and the elevation of LC3II levels suggested that H-Ras^{V12} induced autophagy. However, these results need to be interpreted with caution as elevated LC3II levels may mark enhanced autophagic activity but paradoxically it may also indicate a decrease in the autophagic flux i.e. maturation of autophagosomes to autolysosomes. Both events, either increase in the number of autophagosomes due to enhanced autophagy or blockage of the autophagic flux and hence inhibition of LC3II delivery and subsequent degradation in the autolysosomes, can result in elevation in LC3II level. To determine which of the two scenarios contributes to Ras-induced LC3II upregulation, HOSE-Ras^{V12} cells were treated with Dox in the presence or absence of lysosomal enzyme inhibitors pepstatin and E64d and the protein levels of LC3II and p62 in different treatments were analyzed. LC3II and p62 are both degraded inside the autolysosomes as a result of autophagic flux and can

thus be used to distinguish complete autophagy from inhibited flux. If H-Ras^{V12} induces complete autophagy that drives the degradation of these proteins inside the autolysosomes then inhibiting the lysosomal enzymes should result in their accumulation. If, however, H-Ras^{V12} was only inhibiting the flux then the use of lysosomal enzyme should not make a difference as the process is interrupted in an earlier step. As figure 3.6 C illustrates, Dox treatment of HOSE-Ras^{V12}cells elevated the level of LC3II which further accumulated when Dox was combined with the lysosomal enzymes inhibitors E64d and pepstatin. Similarly, p62 accumulated in the presence of E64d and pepstatin (Figure 3.7 A). These results give evidence that oncogenic H-Ras expression leads to a complete autophagic response not inhibition of the autophagic flux.

Furthermore, we sought to check another marker that is typically associated with the induction of autophagy. As outlined in the introduction, mTOR is an important regulator of autophagy at least in response to starvation. mTOR is a serine/threonine protein kinase which negatively regulate autophagy when nutrients are in supply. Starvation leads to the inhibition of mTOR and thus the induction of autophagy. To examine the activity of mTOR upon H-Ras^{V12} expression, we assessed the phosphorylation status of p70S6 Kinase, an mTOR substrate. Immunoblotting showed that oncogenic H-Ras expression correlated with reduced phosphorylation of p70S6K indicating inhibited activity of mTOR (Figure 3.7 B).

3.2.5 Ras-induced autophagy is not restricted to HOSE-Ras^{V12} cells

As shown above, induction of Ras^{v12} expression in HOSE-Ras^{V12} cells resulted in the induction of autophagy. To test whether Ras-induced autophagy is a general consequence of Ras activation or a confined phenomenon to HOSE-Ras^{V12} cells, primary Human Umbilical Vein Epithelial Cells (HUVECs), MCF-7 cells and HeLa cells were transfected with either oncogenic H-Ras^{V12} or dominant negative Ras^{N17} construct along with a GFP-tagged LC3 construct. Induction of autophagy was assessed by morphological analysis of LC3-GFP puncta formation. Consistent with the observations made in HOSE-Ras^{V12} system, overexpression

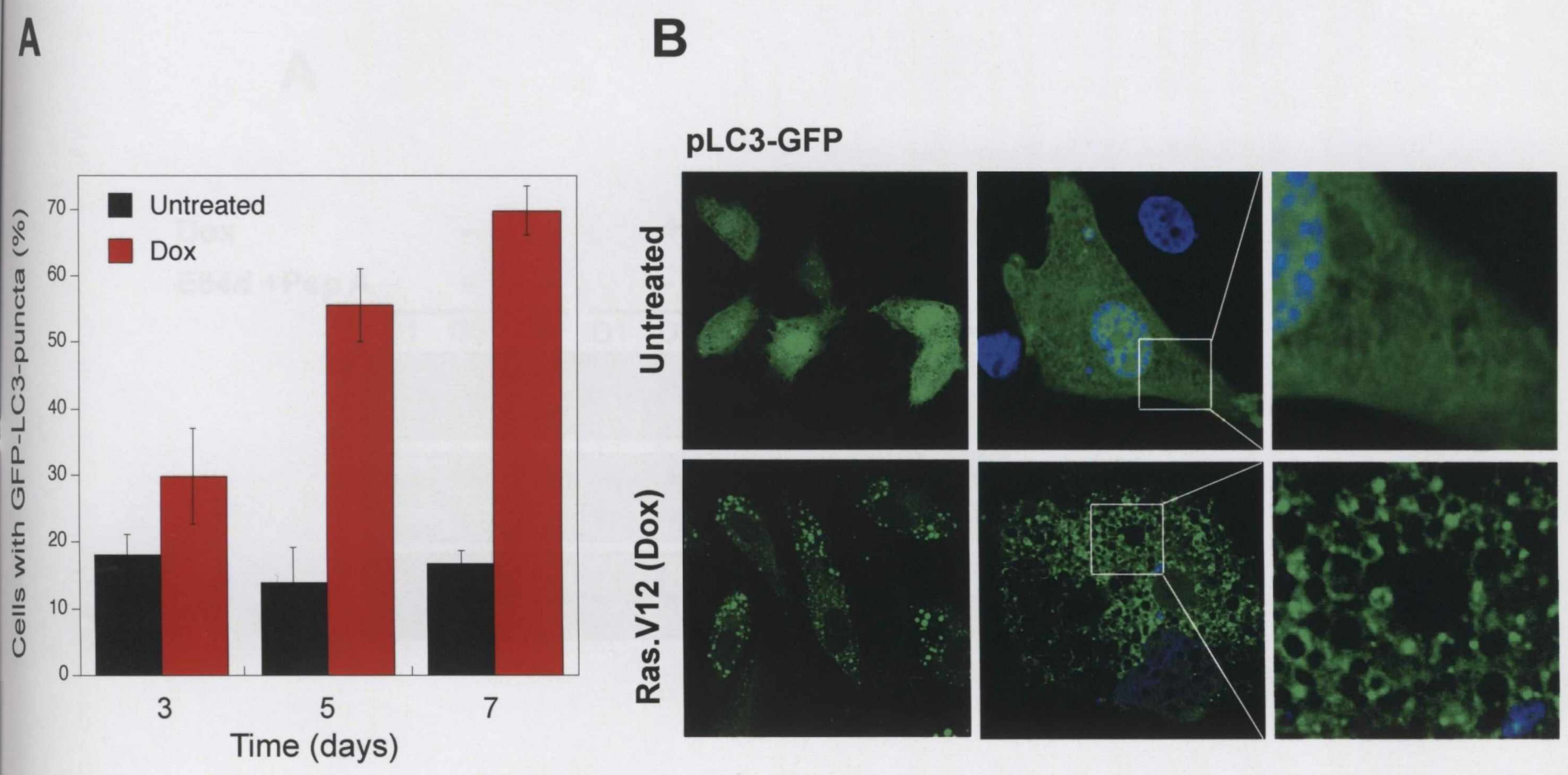


Figure 3.6 Expression of oncogenic H-Ras induces autophagy markers

HOSE.RasV12 cells nucleofected with pLC3-GFP (250 ng) were treated with or without 100 ng/ml Dox for the indicated time points. The percentage of cells exhibiting LC3-GFP punctate morphology was scored under fluorescence microscope (20X magnification) (A). Pictures were taken on a confocal microscope with 60X magnification (B). HOSE.RasV12 cells treated with or wthout Dox over a time course of 7 days were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (C).

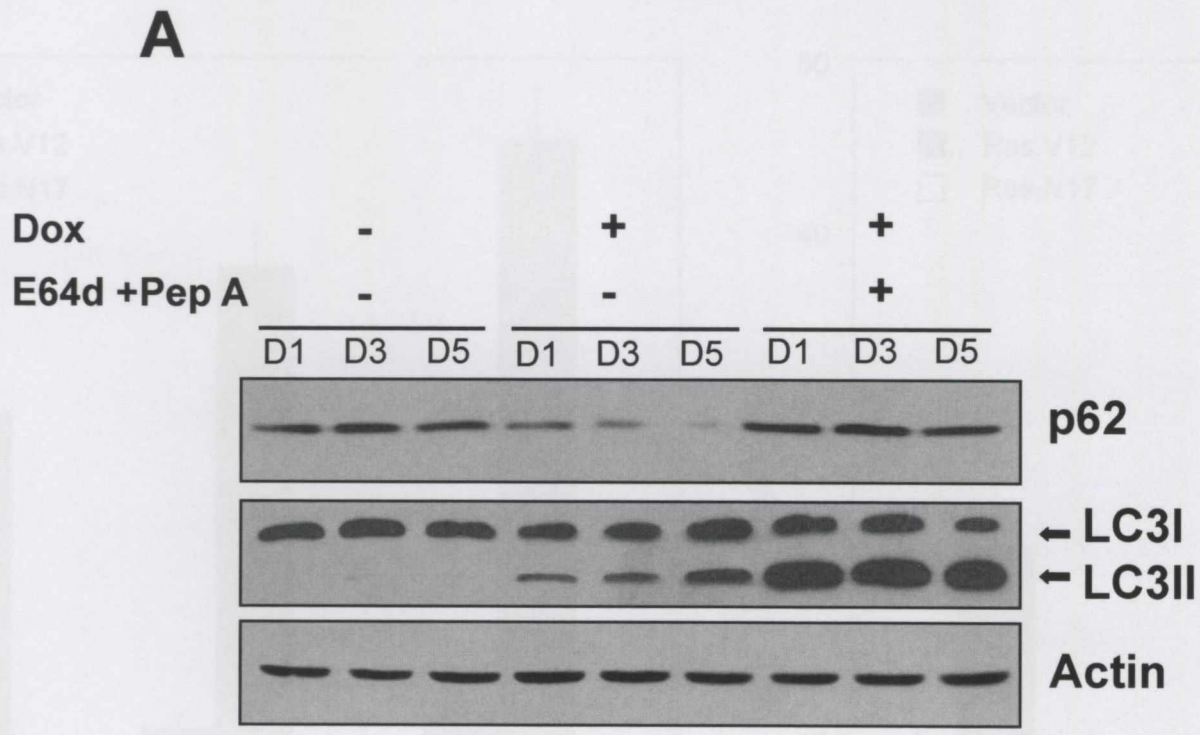


Figure 3.7 Expression of oncogenic H-Ras induces complete autophagic response

HOSE.RasV12 cells treated with or wthout 100 ng/ml Dox in the presence or absence of the lysosomal enzymes inhibitors, E64d (10 μg/ml) and pepstatin A (10 μg/ml) for the indicated time points (A) or HOSE.RasV12 cells treated with or wthout 100ng/ml Dox over a time course of 7 days (B), were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies .

HUVECs

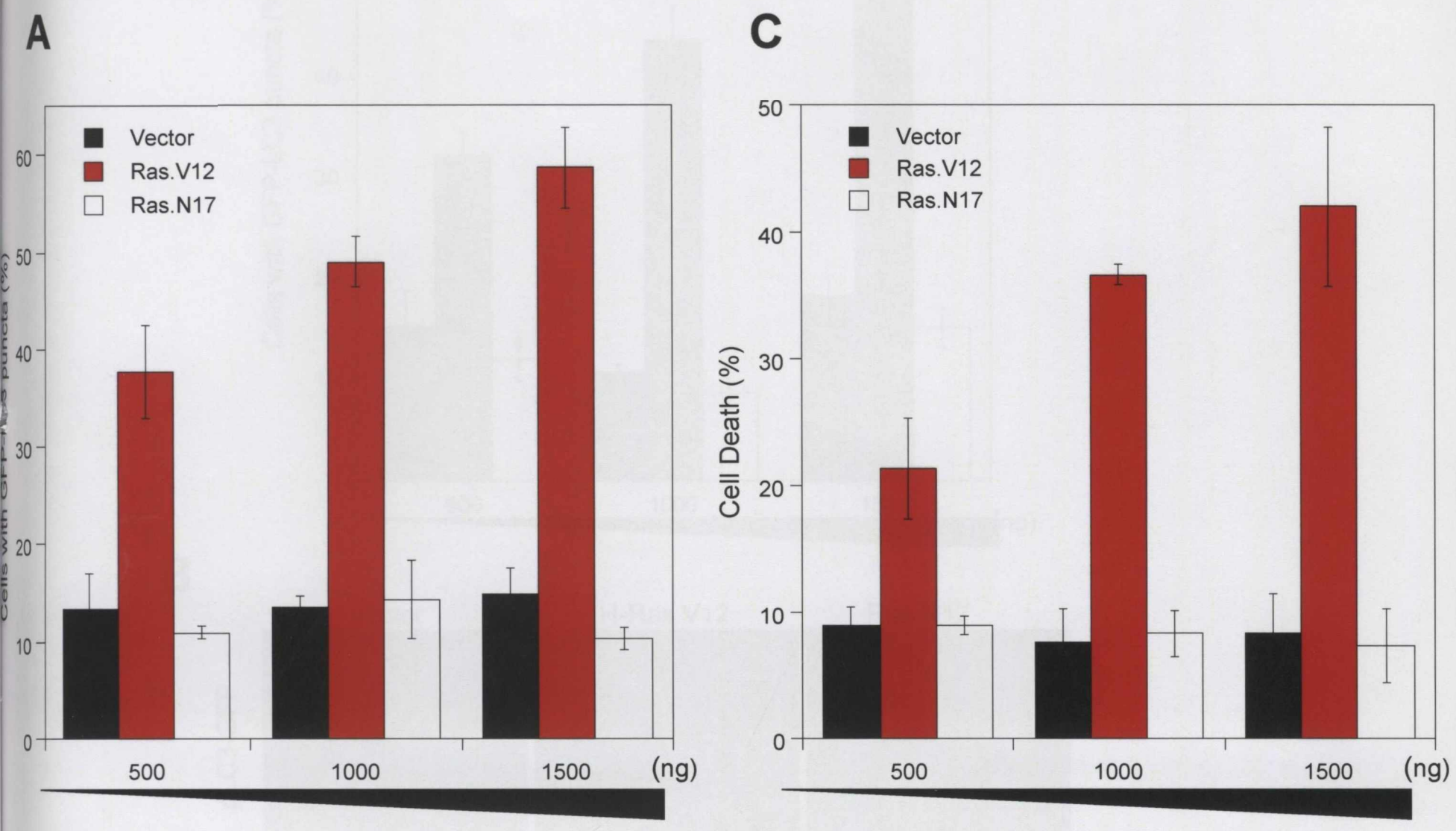
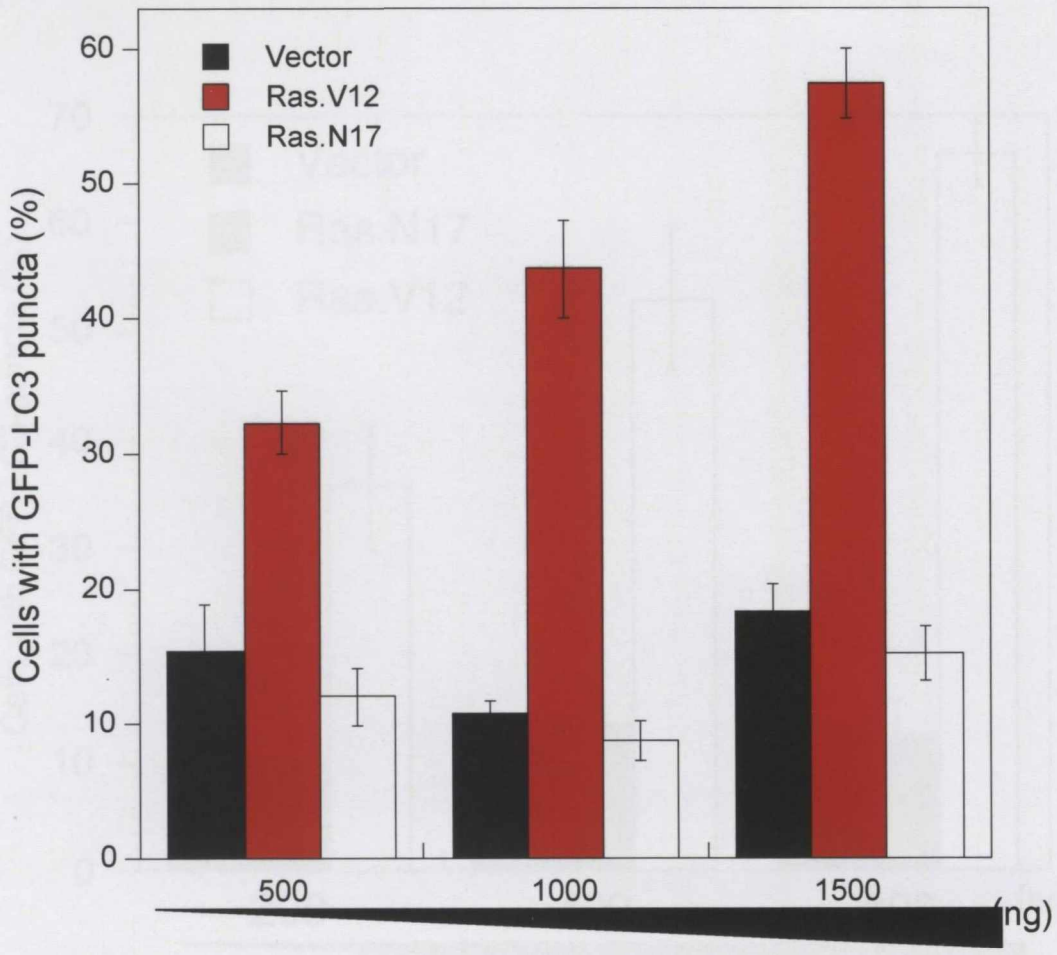


Figure 3.8 Ras-induced autophagy and cell death in HUVECs

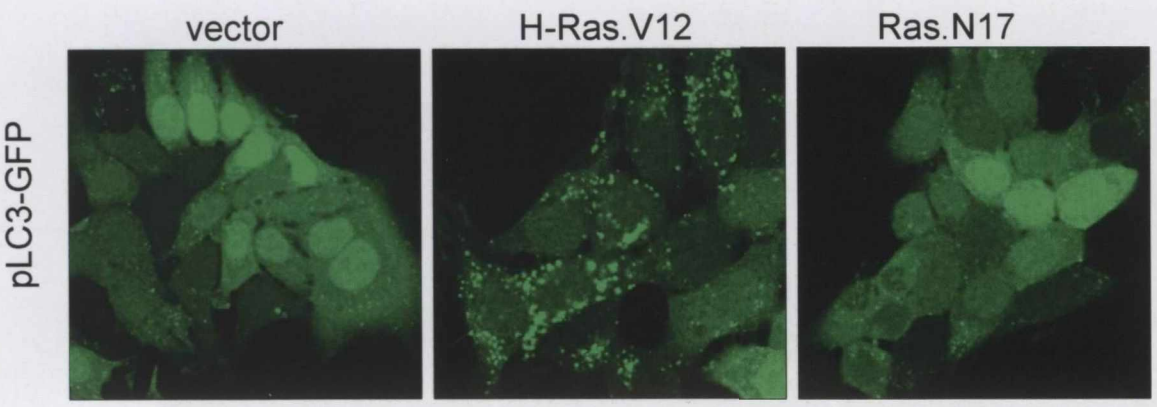
HUVECs were nucleofected with the indicated plasmids using Amaxa nucelofection system (program T20). Follwoing 24h of expression, the percentage of cells exhibiting LC3-GFP punctate morphology was scored (A) and pictures were taken on a confocal microscope with 60X magnification (B). At day 3, dead cells were stained with propodium iodide (10 μg/ml) and the percentage of cell death in each treatment was scored (C). Pictures were taken on a fluorescence microscope with 20X magnification (D)

MCF-7 cells

A



B



C

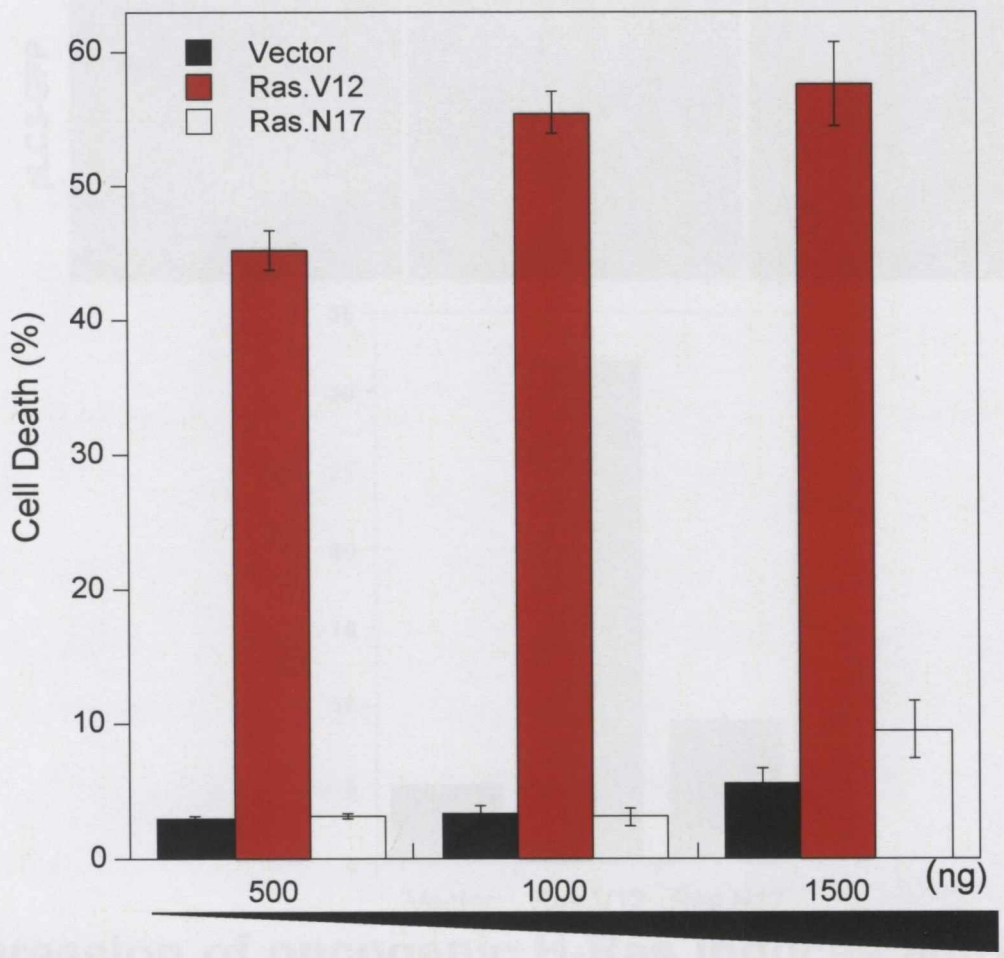
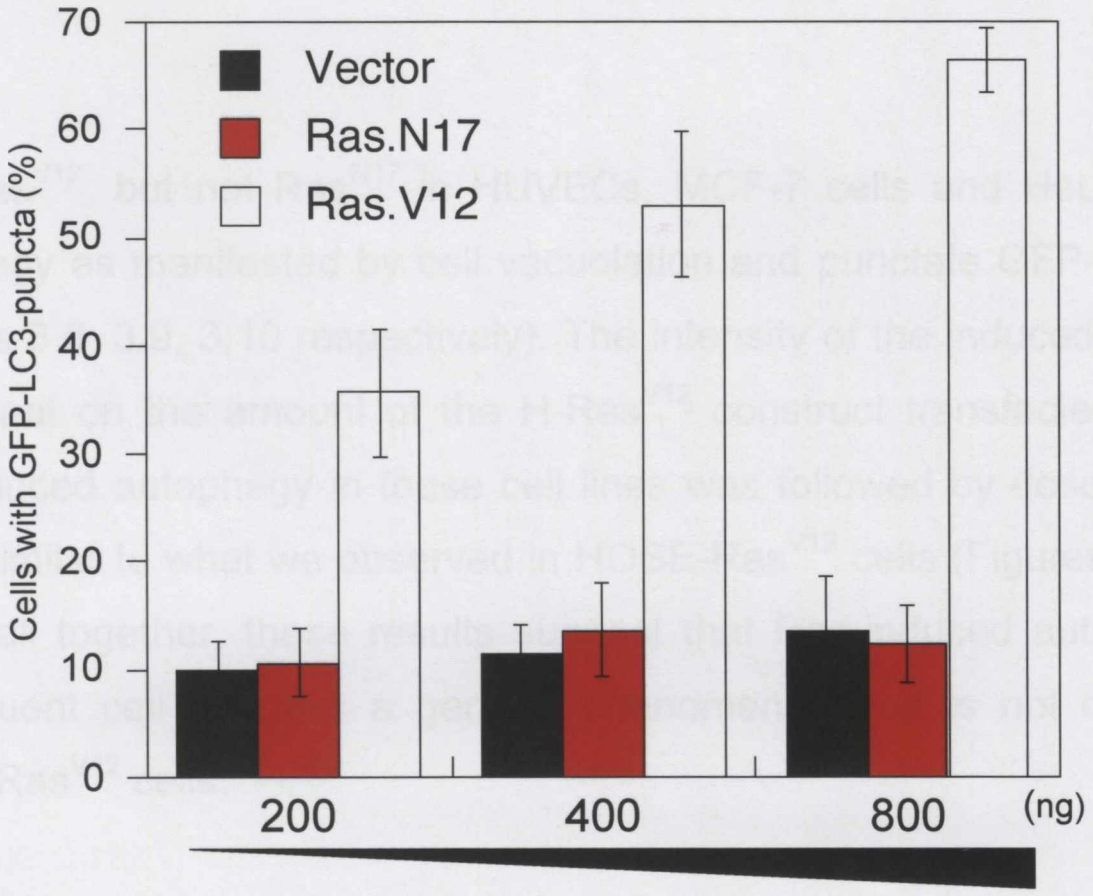


Figure 3.9 Ras-induced autophagy and cell death in MCF-7 cells

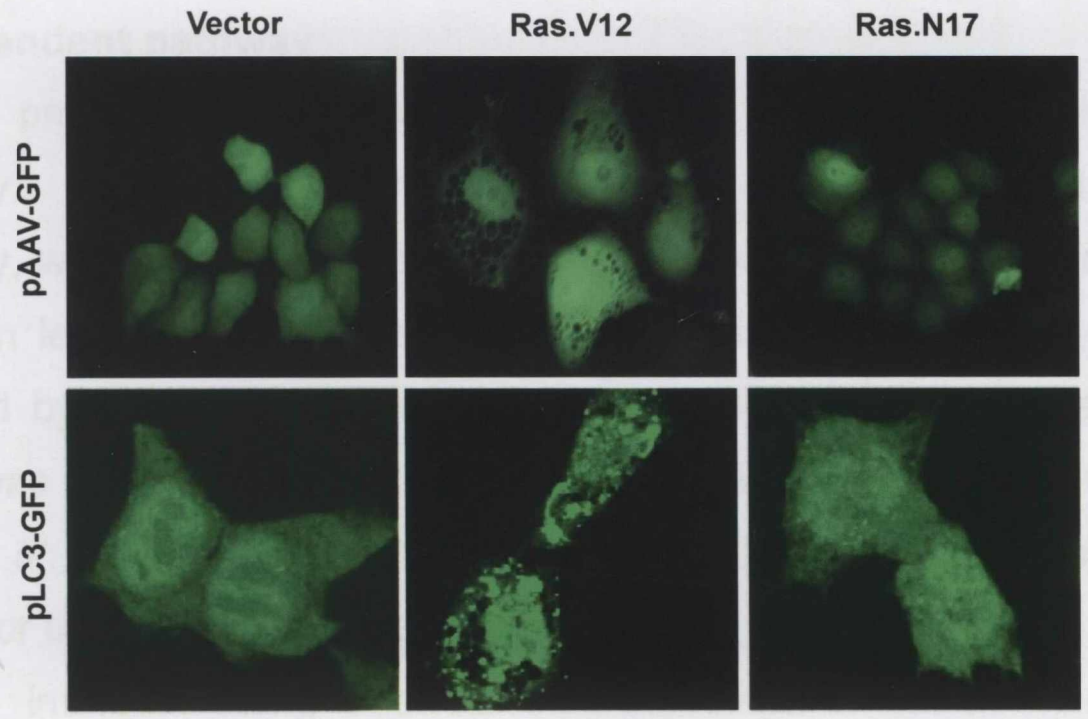
MCF-7 cells were nucleofected with the indicated plasmids using Amaxa nucelofection system (program T20). Follwoing 24h of expression, the percentage of cells exhibiting LC3-GFP punctate morphology was scored (A) and pictures were taken on a confocal microscope with 60X magnification (B). At day 3, dead cells were stained with propodium iodide (10 μg/ml) and the percentage of cell death in each treatment was scored (C).

HeLa cells

A



B



C

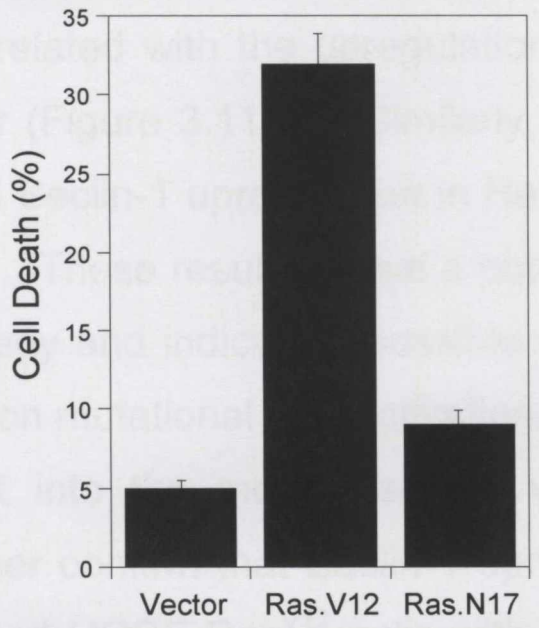


Figure 3.10 Expression of oncogenic H-Ras induces autophagy and cell death in HeLa cells

HeLa cells were transfected with the indicated plasmid combinations using Genejuice transfection reagent. Follwoing 48h of expression, the percentage of cells exhibiting autophagic vacuolation or LC3-GFP punctate morphology was scored (A) and pictures were taken on a confocal microscope with 60X magnification (B). Four days after transfection, cells were enumerated for cell death based on morphological examination (C).

of H-Ras^{V12}, but not Ras^{N17} in HUVECs, MCF-7 cells and HeLa cells induced autophagy as manifested by cell vacuolation and punctate GFP-LC3 distribution (Figures 3.8, 3.9, 3.10 respectively). The intensity of the induced autophagy was dependent on the amount of the H-Ras^{V12} construct transfected into the cells. Ras-induced autophagy in these cell lines was followed by dose-dependent cell death similar to what we observed in HOSE-Ras^{V12} cells (Figures 3.8, 3.9, 3.10). Taken all together, these results suggest that Ras-induced autophagy and the subsequent cell death is a general phenomenon and is not confined to only HOSE-Ras^{V12} cells.

3.2.6 Expression of oncogenic H-Ras induces Beclin-1 upregulation via an ERK-dependent pathway

From the previous experiments, it was clear that oncogenic H-Ras induces autophagy. In order to gain insight into the mechanism of H-Ras induced autophagy, we first sought to examine whether oncogenic H-Ras modulates the expression level of Beclin-1, an essential mediator of autophagy that is often modulated by inducers of autophagy. Beclin-1 is the mammalian ortholog of Atg6. It was the first human protein shown to be indispensable for autophagy (Liang *et al.*, 1999). Beclin-1 forms a complex with PI3K class 3, which is required for the initiation of autophagy.

Strikingly, immunoblotting showed that induction of H-Ras^{V12} expression in HOSE-Ras^{V12} cells correlated with the upregulation of Beclin-1 expression in a time-dependent manner (Figure 3.11 A). Similarly, overexpression of H-Ras^{V12}, but not Ras^{N17}, induced Beclin-1 upregulation in HeLa cells in a dose-dependent manner (Figure 3.11 B). These results reveal a potentially important mechanism of Ras-induced autophagy and indicate a possible role for Beclin-1 in mediating autophagy induction upon mutational Ras activation.

To gain further insight into the mechanism by which Ras induces Beclin-1 expression and to further confirm that Beclin-1 upregulation is a direct effect of Ras signaling, we treated HOSE-Ras^{V12} cells with Dox alone or in combination with inhibitors of different signaling pathways activated downstream of Ras,

MEK/ERK, JNK and PI3K. Inhibition of JNK or PI3K by treatment with SP600125 or LY294002 respectively did not modulate induction of Beclin-1 expression by H-Ras^{V12} to any extent. Conversely, inhibition of MEK/ERK pathway by treatment with the widely used MEK inhibitor UO126 completely abolished Ras-induced Beclin-1 upregulation (Figure 3.11 C). These results suggest that Ras induces Beclin-1 expression via an ERK regulated pathway.

3.2.7 Ras-induced cell death is a consequence of excessive autophagy

As discussed in chapter one, autophagy is a protein recycling process that has been linked to both survival and death in different contexts. On the one hand, autophagy plays an important role in cell survival under the conditions of nutrient deprivation and helps maintain cell viability until nutrients are supplied (Levine *et al.*, 2008). On the other hand, excessive or prolonged autophagy has been suggested to ultimately lead to cell demolition and cell death (Yu *et al.*, 2004, Shimizu *et al.*, 2004).

In multiple experimental paradigms, autophagy has been associated with a particular mode of cell death that is characterized by the massive accumulation of cytoplasmic acidic vacuoles. To distinguish it from apoptosis (type I cell death), this has been termed autophagic or type II cell death. Although these pathways exhibit rather distinctive morphological features, it is still a matter of debate whether autophagy truly accounts for an independent cell death mode, or whether autophagic vacuolization represents a fruitless attempt of cells to adapt before succumbing to overwhelming stress.

As shown earlier, induction of H-RasV12 expression in HOSE-RasV12 cells induced a strong autophagic response that correlated with massive accumulation of acidic vacuoles and Beclin-1 induction and was followed by cell death. We, therefore, sought to examine whether Ras-induced autophagy serves a pro-survival function or contributes to cell death in this system. To this end, the two essential mediators of autophagy, Beclin-1 and Atg5 were efficiently knocked

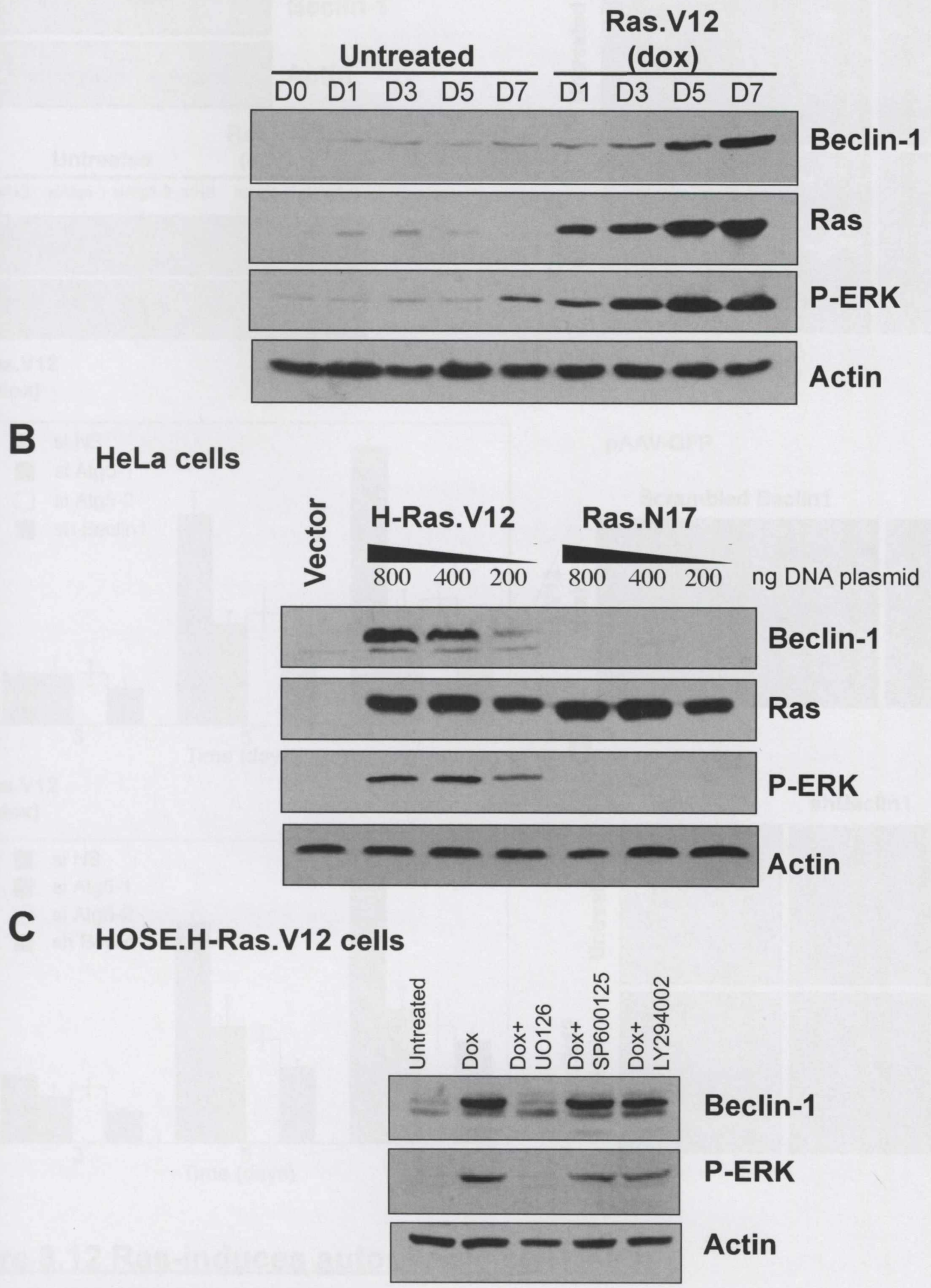


Figure 3.11 Ras-induced Beclin-1 upregulation

HOSE.RasV12 cells treated with or wthout 100 ng/ml Dox over a time course of 7 days (A) or Hela cells transfected with the indicated plasmids and left 48 hours (B), were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies. HOSE.RasV12 cells were treated with 100 ng/ml Dox either alone or in combination with either MEK inhibitor UO126 (10μM), JNK inhibitor SP600125 (20μM) or PI3K inhibitor LY294002 (20μM). Three days later, cells were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (C).

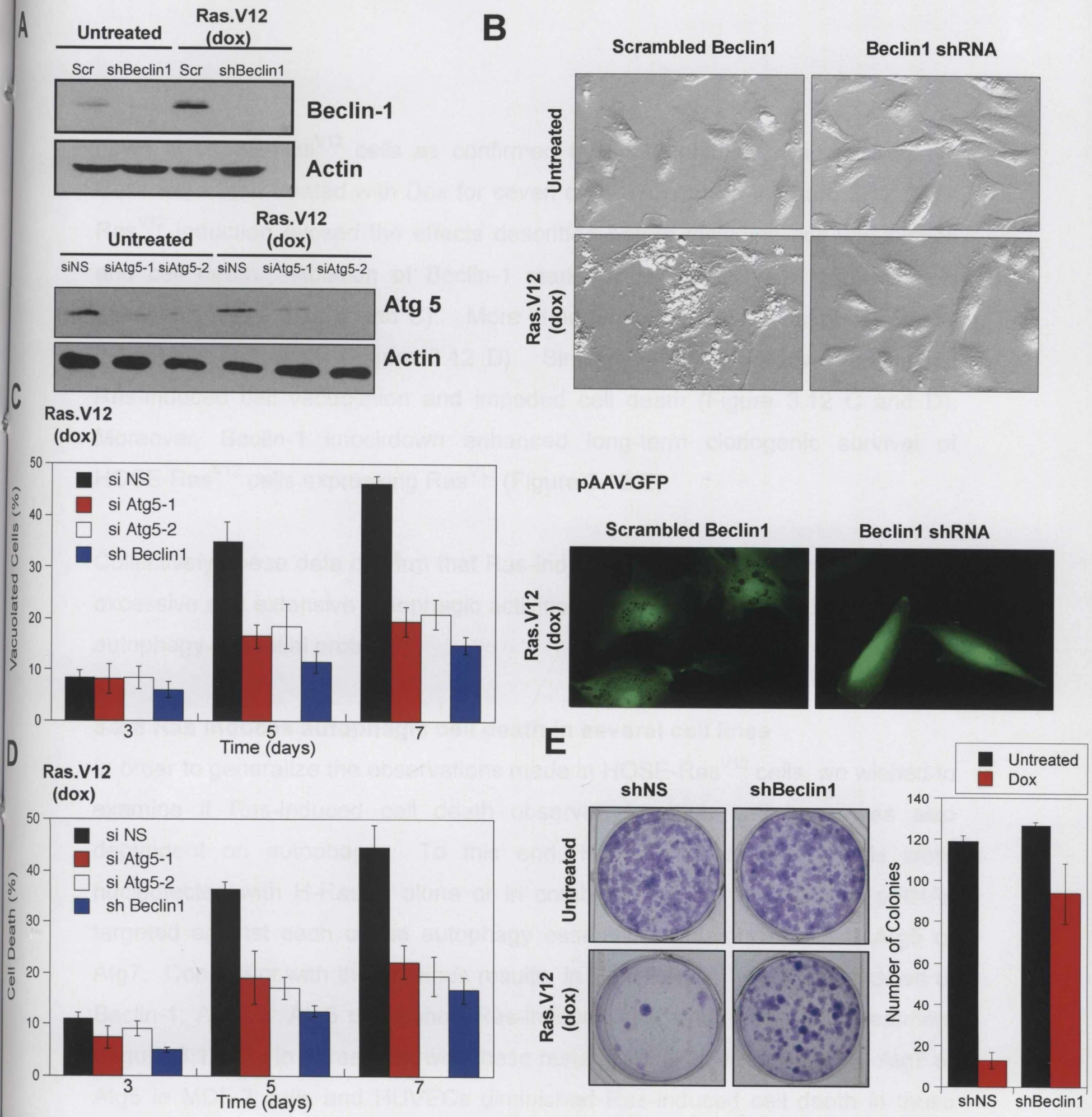


Figure 3.12 Ras-induces autophagic cell death

To ablate Beclin-1 or Atg5, HOSE.RasV12 cells were nucleofected with shRNA construct targeting Beclin-1 (2.5μg) or siRNA against Atg5 (200nM) using Amaxa nucleofection system (program T 20). Cells were allowed 72 h for full expression followed by treatment with or without 100 ng/ml Dox. Efficiency of knockdown was verified by Western Immunoblotting (A). Cells were followed up for morphological changes and pictures were taken on a light micrscope (40X magnification) at day 7 post Dox treatment (B). The percentage of cells exhbiting cytoplasmic vacuolation (C) or the percentage of cell death (D) were assessed at the indicated time points. Cells ablated for Beclin-1 were subjected to clonogenic survival assay for 3 weeks as described in chapter two. Colonies were stained with 0.5% crystal violet in 20% methanol. Pictures were taken with Canon G9 digital camera and colonies bigger than 1mm were counted (E).

down in HOSE-Ras^{V12} cells as confirmed by immunoblotting (Figure 3.12 A). Cells were then treated with Dox for seven days. As shown in figure 3.12 B, H-Ras^{V12} induction evoked the effects described before including cell vacuolation and cell death. Ablation of Beclin-1 markedly reduced these morphological changes (Figure 3.12 B and C). More importantly, Beclin-1 knockdown greatly diminished cell death (Figure 3.12 D). Similarly, Atg5 knockdown diminished Ras-induced cell vacuolation and impeded cell death (Figure 3.12 C and D). Moreover, Beclin-1 knockdown enhanced long-term clonogenic survival of HOSE-Ras^{V12} cells expressing Ras^{V12} (Figure 3.12 E).

Collectively, these data confirm that Ras-induced cell death is a consequence of excessive and extensive autophagic activities as it can be blocked by ablation of autophagy-essential proteins.

3.2.8 Ras induces autophagic cell death in several cell lines

In order to generalize the observations made in HOSE-Ras^{V12} cells, we wished to examine if Ras-induced cell death observed in other cell lines was also dependent on autophagy. To this end, HUVECs and MCF-7 cells were nucleofected with H-Ras^{V12} alone or in combination with two different siRNAs targeted against each of the autophagy essential regulators Beclin-1, Atg5 or Atg7. Consistent with the previous results, in HOSE-Ras^{V12} cells, knockdown of Beclin-1, Atg7 or Atg5 diminished Ras-induced cell death upon Dox treatment (Figure 3.13 A). In agreement with these results, knockdown of Beclin-1, Atg7 or Atg5 in MCF-7 cells and HUVECs diminished Ras-induced cell death in these cells (Figure 3.13 B and C respectively). These results strongly suggest that autophagy triggered by oncogenic H-Ras mediates the subsequent cell death and does not serve a pro-survival function in this context.

3.2.9 Differential induction of autophagy by Ras isoforms

In human, three Ras proto-oncogenes encode four isoforms (H-Ras, K-Ras4A, K-Ras4B and N-Ras). Ras isoforms were initially assumed to be functionally

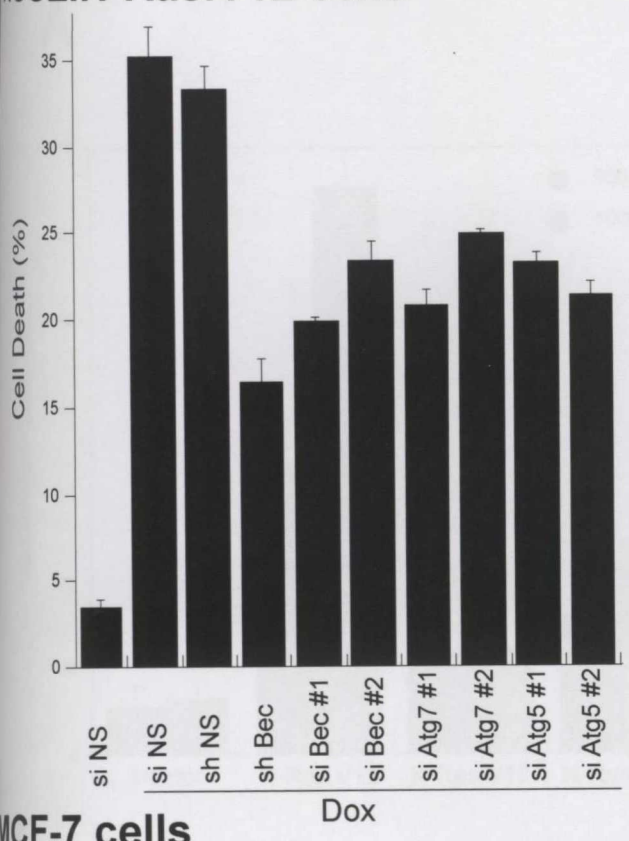
redundant due to their high degree of sequence homology however, many studies point to specific roles for each isoform.

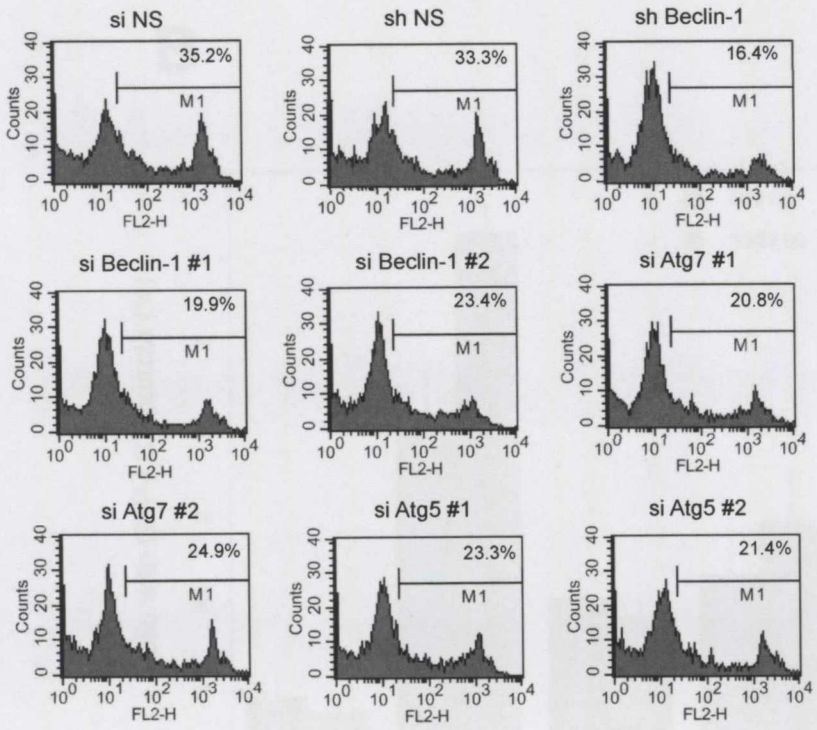
As mentioned earlier, HOSE-Ras^{V12} cells are stably transfected with oncogenic H-Ras^{V12}. Observations made in HOSE-Ras^{V12} cells were replicated in other cell lines by transfecting H-Ras^{V12} construct. Therefore the observed induction of autophagy was mainly a consequence of mutational activation of H-Ras gene. This implicated an important question whether activation of other isoforms of Ras triggers the same phenotypic changes. To answer this question we transiently transfected HeLa cells with oncogenic H-Ras^{V12}, K-Ras^{V12} or N-Ras^{61K}. As observed before, transfection of HeLa cells with H-Ras^{V12} triggered autophagy as indicated by cytoplasmic vacuolation and GFP-LC3 puncta formation (Figure 3.14 A, B and C). The magnitude of autophagy induced by K-Ras.V12 and N-Ras.61K was however markedly less than that triggered by H.Ras.V12. N-Ras.61K induced slightly higher level of autophagy than K-Ras.V12 (Figure 3.14 A, B and C). Strikingly, immunoblotting analysis of lysates prepared from HeLa cells transfected with titrated doses of each of the three oncogenic Ras isoforms revealed that only oncogenic H-Ras exclusively induced the expression of Beclin-1 while oncogenic K-Ras and N-Ras did not seem to do so (Figure 3.14 D). This result might help explain the ability of H-Ras^{V12} to induce autophagy and the failure of other isoforms to exert a similar effect. Interestingly, the differential induction of autophagy and Beclin-1 expression by oncogenic H-Ras correlated with stronger induction of the MEK/ERK pathway compared to other isoforms as indicated by the level of ERK phosphorylation (Figure 3.14 D). These results are consistent with the observation shown earlier that Beclin-1 induction by H-Ras^{V12} in HOSE-Ras^{V12} cells was regulated via an ERK-dependent pathway and strongly suggest a crucial role for MEK/ERK pathway in mediating autophagy downstream of Ras.

3.2.10 Duration and intensity of Ras activation determine cell's fate

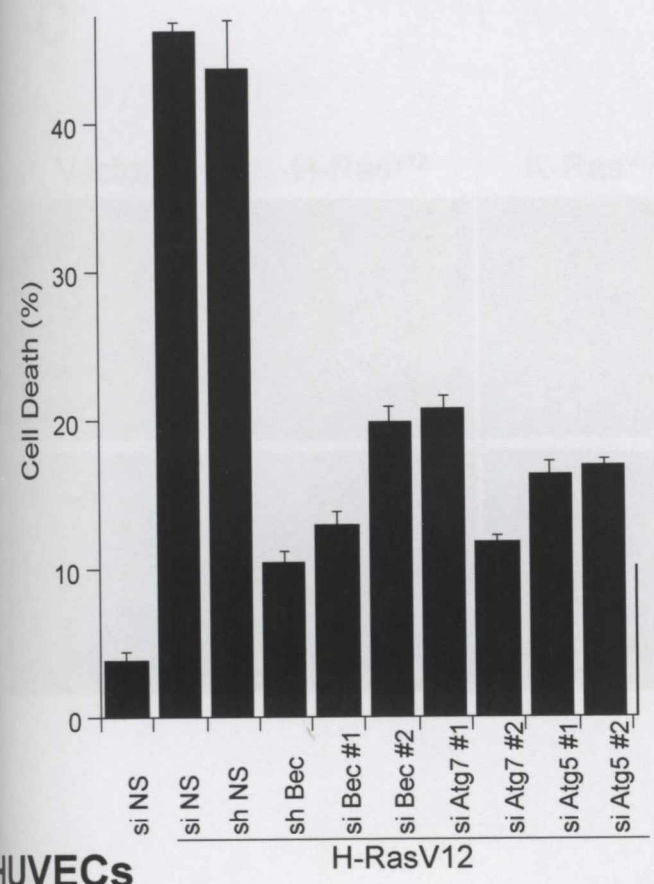
Expression of oncogenic Ras has been described to trigger cellular senescence especially in primary immortalized human and rodent cells (Serrano *et al.*, 1997).

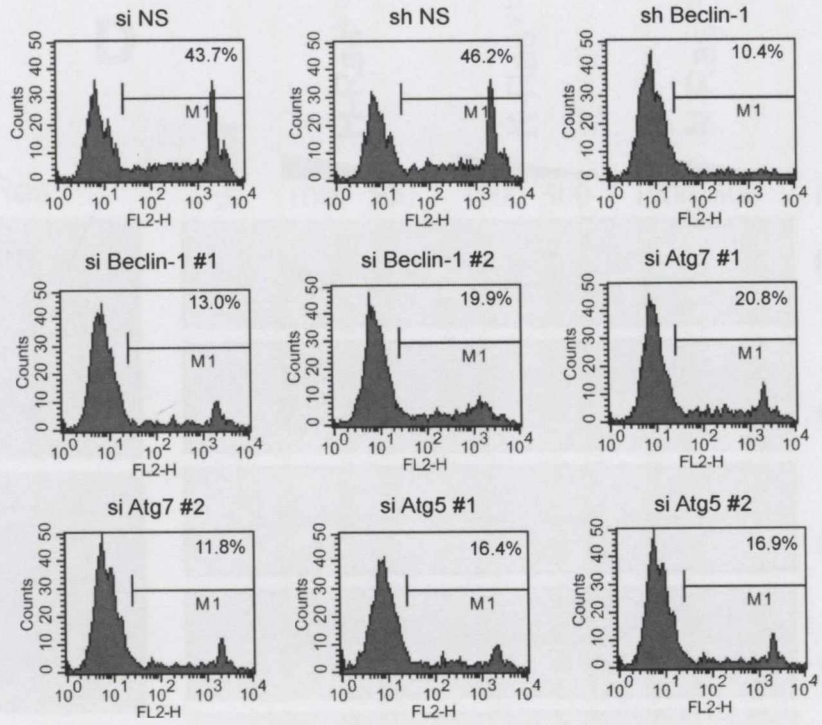
HOSE.H-Ras.V12 cells



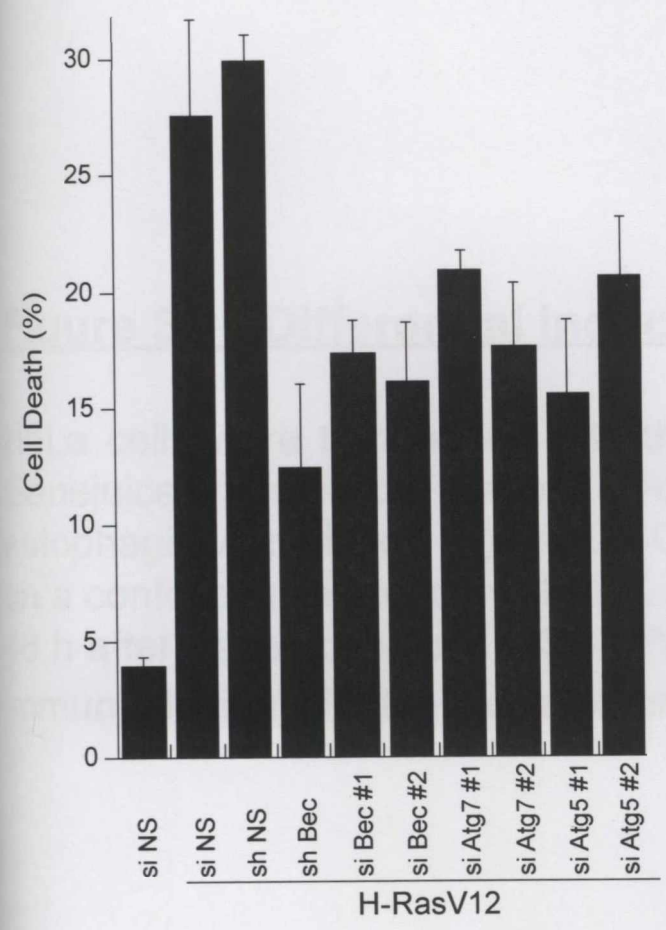


MCF-7 cells





HUVECs



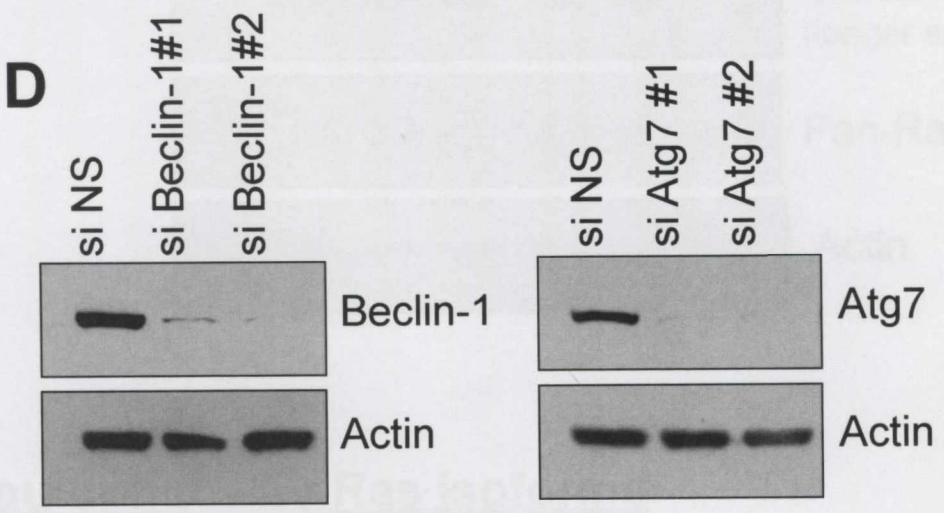


Figure 3.13 Ras-induces autophagic cell death in different cell lines

HOSE.RasV12 cells (A), MCF-7 cells (B) or HUVECs (C) were nucleofected with H-RasV12 (or treated with Dox in case of HOSE. Rasv12 cells) either alone or in combination with siRNA's against Beclin-1, Atg7 or Atg5 (200nM). Percentage of cell death in each condition was assessed by flow cytometer analysis of PI uptake. PI was added immediately prior to analysis at final concentration of 10 μg/ml. Efficiency of silencing Beclin-1 and Atg7 by siRNA was confirmed by immunoblotting of lysates prepared from HOSE. RasV12 nucleofected with the indicated siRNA's.

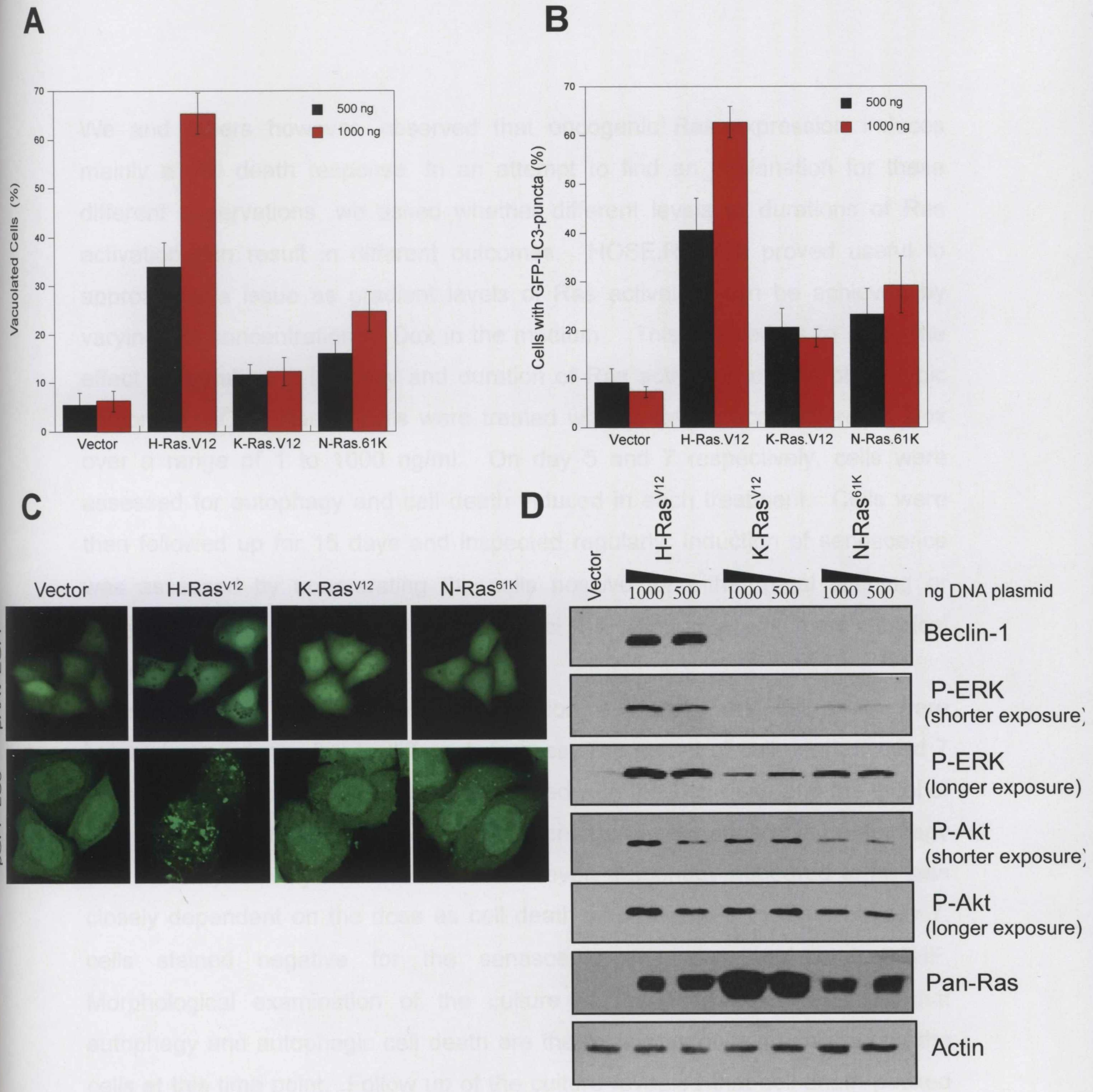


Figure 3.14 Differential induction of autophagy by Ras isoforms

HeLa cells were transfected with the indicated amounts of each of different Ras isoforms using Genejuice transfection reagent. Follwoing 48h of expression, the percentage of cells exhibiting autophagic vacuolation (A) or LC3-GFP punctate morphology (B) was scored. Pictures were taken on a confocal microscope with 60X magnification (C). HeLa cells transfected as above were lysed 48 h after transfection in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (D).

We and others however, observed that oncogenic Ras expression induces mainly a cell death response. In an attempt to find an explanation for these different observations, we asked whether different levels or durations of Ras activation can result in different outcomes. HOSE.RasV12 proved useful to approach this issue as gradient levels of Ras activation can be achieved by varying the concentration of Dox in the medium. This enabled us to study the effect of variation in intensity and duration of Ras activation on the phenotypic outcome. HOSE-Ras^{V12} cells were treated with titrated concentrations of Dox over a range of 1 to 1000 ng/ml. On day 5 and 7 respectively, cells were assessed for autophagy and cell death induced in each treatment. Cells were then followed up for 15 days and inspected regularly. Induction of senescence was assessed by enumerating the cells positive for either β-gal staining or senescence-associated heterochromatin foci (SAHF), both of which are classical markers of senescence.

Over the tested range of Dox concentration, autophagy and cell death were induced at all doses but to different degrees. The extent of cell death scored 7 days post Dox-treatment positively correlated with the Dox dose and the level of Ras expression (Figure 3.15 A). The percentage of cells undergoing autophagy also varied positively with the dose. However, autophagy appeared to be less closely dependent on the dose as cell death was (Figure 3.15 B). On day 7, cells stained negative for the senescence markers β-gal and SAHF. Morphological examination of the culture at this time point revealed that autophagy and autophagic cell death are the main phenotypes exhibited by the cells at this time point. Follow up of the culture revealed that cell death peaked at day 7 with no or little further death observed after this time point. Surviving cells, however, started to acquire a senescence-like phenotype, which was confirmed by positively staining for β-gal and SAHF on Day 15. Interestingly in contrast to cell death, the percentage of β-gal- and SAHF-positive cells varied in an inverse correlation with the Dox dose applied (Figure 3.15 C and D). Immunoblotting of cell lysates prepared from the cells in this experiment confirmed the data obtained from morphological analysis and gave further insight

into the molecular mechanisms governing the ultimate outcome. The senescence-associated markers p16 and p21 were highly upregulated in the senescence phase (Day 15) but not in the death phase (Day 7) (Figure 3.16 A). However, p53, another marker that is sometimes associated with Ras-induced senescence was not upregulated in these cells.

As shown earlier, cell death observed within 7 days seems to be a direct consequence of Ras-induced excessive autophagy. To test whether or not autophagy is also required for the establishment of the senescence phenotype, we knocked down Beclin-1 in HOSE-Ras^{V12} cells followed by treatment with or without Dox for 21 days. Interestingly, Beclin-1 knockdown, which was shown earlier to diminish Ras-induced cell death, also diminished Ras-induced senescence which suggests a role for autophagy in the establishment of both cell death and senescence (Figure 3.16 B).

In conclusion, our results suggest that variation of the intensity of Ras activation and the time elapsed between the point of Ras activation and the point of outcome assessment seem to dictate the phenotype observed and may account for the conflicting observations made by different groups. Autophagy is induced as an initial response to Ras activation and depending on the intensity of the induced autophagic program, the cell's ultimate fate is determined. Higher intensities of Ras activation trigger stronger autophagic response which tends to induce more cell death while weaker autophagic response that is not intense enough to kill the cell can result in cell cycle exit and senescence.

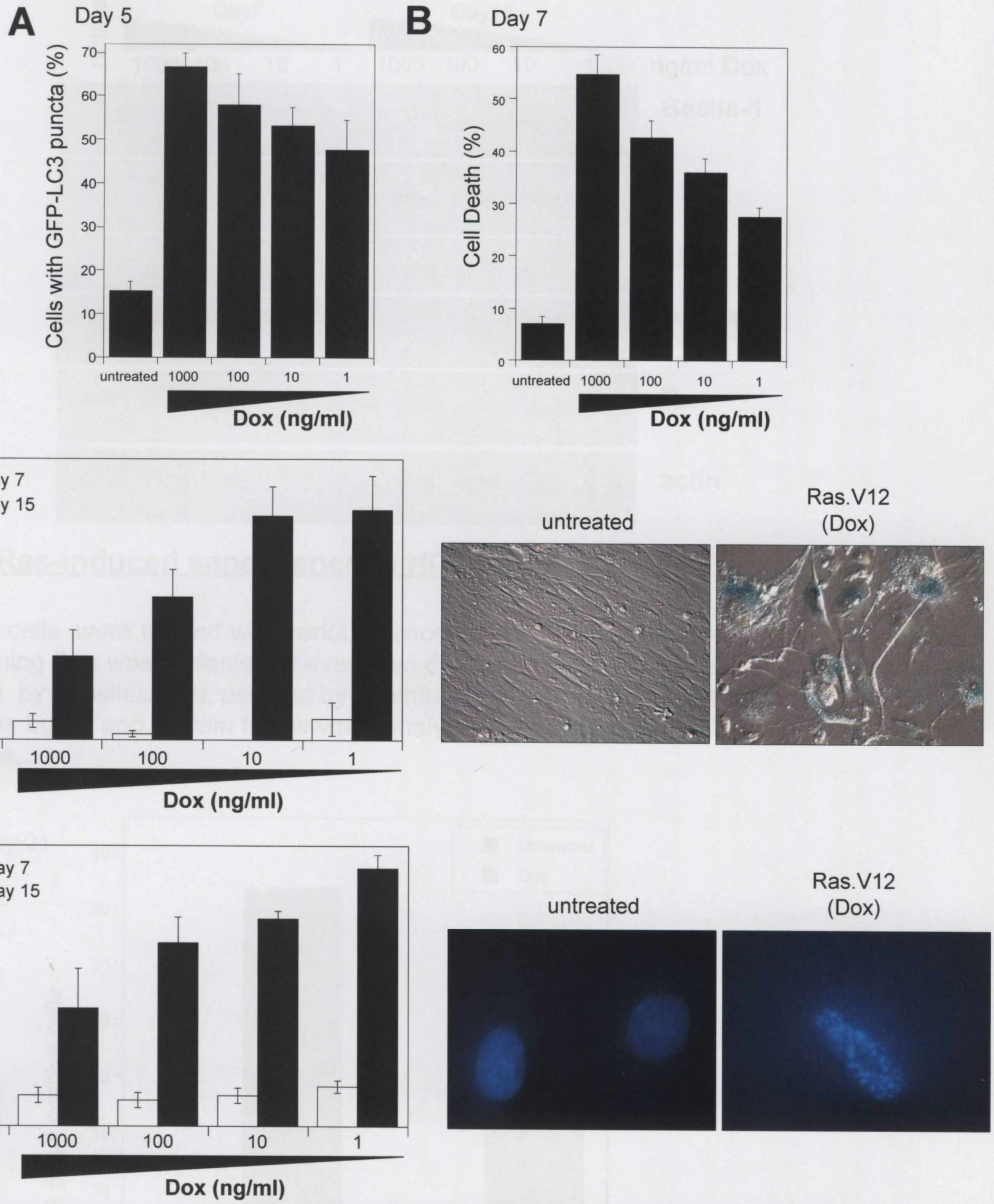


Figure 3.15 Intensity and duration of Ras activation determines outcome

HOSE.RasV12 cells or HOSE.RasV12 cells nucleofected with pLC3-GFP were treated with various concentrations of Dox for the indicated time points. Medium containing Dox was replenished every two days. At day 5 post Dox treatment , the percentage of cells showing LC3-GFP puncta was scored (A). At day 7 , the percentage of cell death was assessed using PI staining (B). HOSE.RasV12 cells treated for 7 or 15 days were washed twice with PBS pH7.2, then fixed with 0.5% glutaraldehyde in PBS, then washed three times with PBS pH7.2 supplemented with 1 mM MgCl2. Cells were assessed for senescence by staining for 4–6 h with X-Gal solution. β-gal positive cells (blue-stained) were counted using Olympus microscope under bright phase (C). Cells treated as above were stained with Hoechst (10 μM) and cells with nuclei exhbiting SAHF were counted under fluoresence microscope (20X) (D).

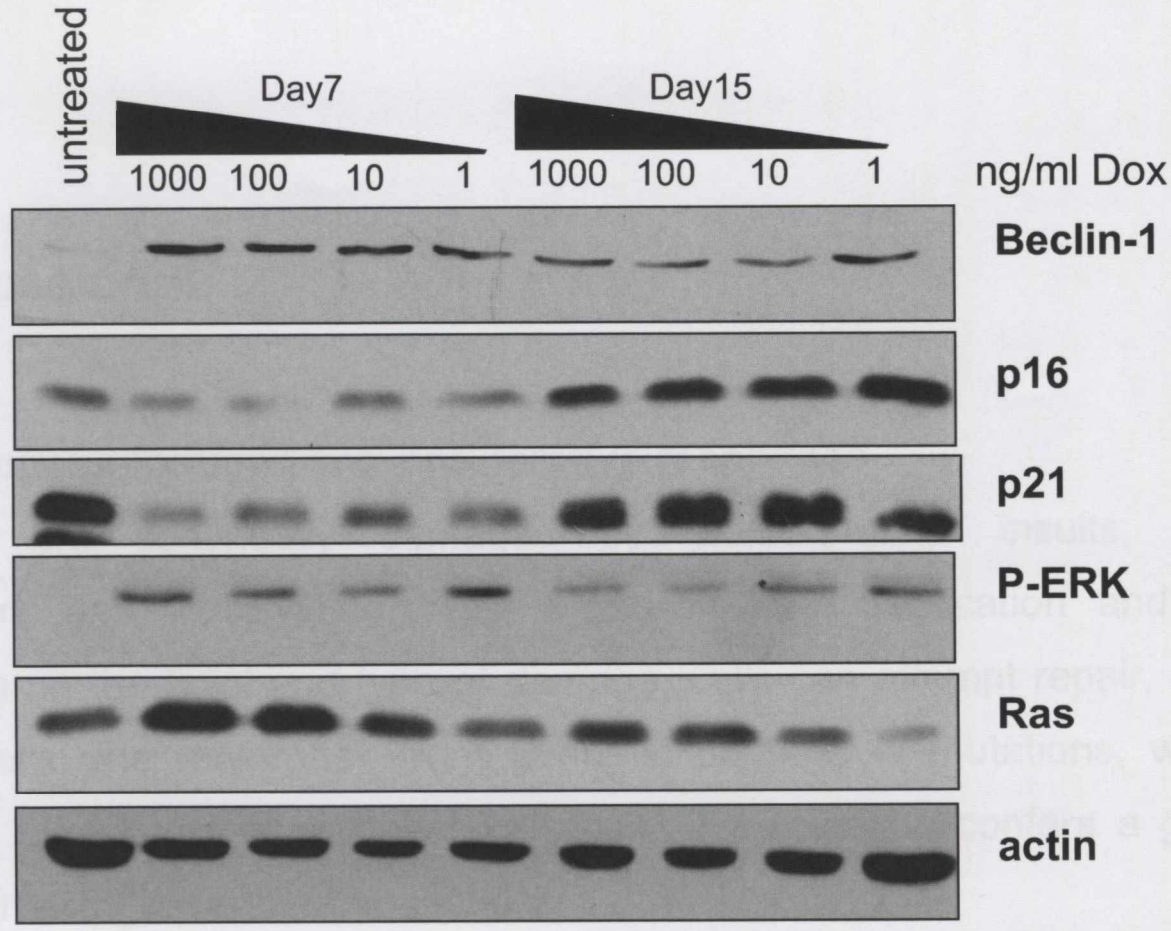


Figure 3.16 Ras-induced senescence in HOSE.RasV12 cells

HOSE.RasV12 cells were treated with various concentrations of Dox for the indiacted time points. Medium containing Dox was replenished every two days. At day 7 or 15 post Dox treatment , cells were harvested by trypsinization, pelleted by centrifugation at 250 g. Cell pelletes were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies.

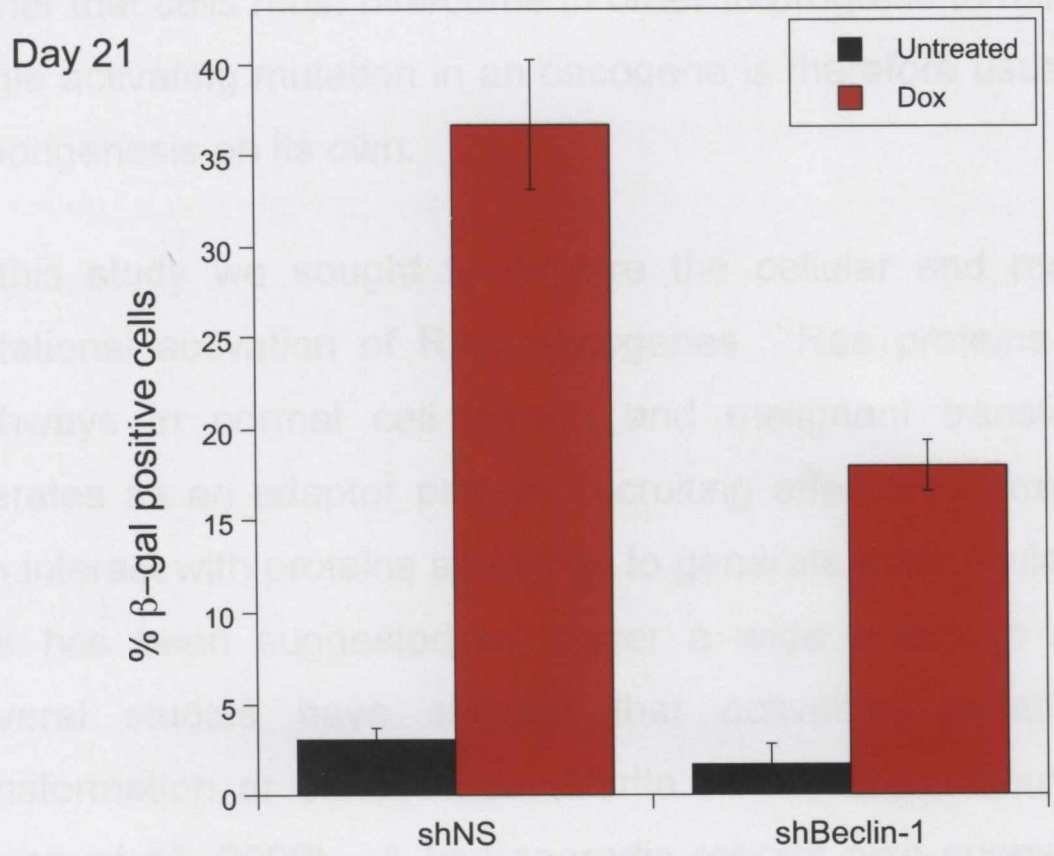


Figure 3.17 Autophagy mediates Ras-induced senescence

Beclin-1 or Noxa were ablated in HOSE.RasV12 cells by nucleofection with shRNA constructs targeting Beclin-1 using Amaxa nucleofection system (program T 20). Cells were allowed 72 h for full expression followed by treatment with or without 100 ng/ml Dox for 21 days. Medium containing Dox was replenished every two days. At day 21, cells were washed twice with PBS pH7.2 then fixed with 0.5% glutaraldehyde in PBS then washed three times with PBS pH7.2 supplemented with 1 mM MgCl2. Cells were assessed for senescence by staining for 4–6 h with X-Gal solution. β-gal positive cells (blue-stained) were counted using Olympus microscope under bright phase (A).

3.3 Discussion

3.3.1 Oncogene-induced anti-proliferative responses

Genomes are continually damaged by environmental insults, oxidative metabolism, and, in dividing cells, errors in DNA replication and mitosis. Depending on the level and type of damage, cells can attempt repair, or die. In dividing cells, the major risk from genomic damage is mutations, which are generated by failures or mistakes in repair. If a mutation confers a growth or survival advantage, cancer can develop.

Complex organisms have evolved at least two cellular mechanisms to suppress the proliferation should an activating mutation occur in an oncogene: programmed cell death and cellular senescence. Programmed cell death eliminates the cells that represent a tumorigenic thread while cellular senescence irreversibly locks them in permanent growth arrest. Both responses form a major barrier that cells must overcome in order to progress to full-blown malignancy. A single activating mutation in an oncogene is therefore usually insufficient to drive tumorigenesis on its own.

In this study we sought to explore the cellular and molecular responses to mutational activation of RAS oncogenes. Ras proteins control key signaling pathways in normal cell growth and malignant transformation. Active Ras operates as an adaptor protein, recruiting effectors to membranes, where they can interact with proteins and lipids to generate intracellular signals. Activation of Ras has been suggested to trigger a wide spectrum of cellular responses. Several studies have showed that activating mutations in *RAS* induce transformation or senescence (Narita *et al.*, 2003; Courtois-Cox *et al.*, 2006; Young *et al.*, 2009). A few sporadic reports also suggested that activation of *RAS* can trigger cell death (Chi *et al.*, 1999; Kitanaka *et al.*, 2007). The characteristics and molecular mechanism of Ras-induced cell death however remained elusive.

3.3.2 Oncogenic H-Ras induces non-apoptotic cell death

We have observed that the expression of H-Ras^{V12} in several cell lines induced cell death. As the expression of some oncogenes such as c-myc and E2F was shown to induce apoptosis (Prendergast, 1999, Thompson, 1998, Lowe *et al.*, 2004), we explored the possibility that expression of oncogenic Ras could similarly trigger apoptosis. However, we found that cells dying as a consequence of oncogenic Ras expression did not exhibit any of the characteristic morphological features of apoptosis such as membrane blebbing, chromatin condensation and fragmentation. Furthermore, we did not detect any sign of caspase activation, another hallmark of apoptosis. In addition, the broad caspase inhibitor zVAD-fmk did not have any significant inhibitory effect on Ras-induced cell death. Taken all together, these results suggested that oncogenic Ras expression evokes a form of cell death that is distinct from apoptosis. Our observations are in agreement with previous studies that reported Ras-induced caspase-independent cell death in other systems (Chi *et al.,* 1999; Kitanaka *et al.*, 2007).

3.3.3 Ras-induced autophagy precedes cell death

In the HOSE-Ras^{V12} system, Ras-induced non-apoptotic cell death was preceded by massive cell vacuolation that suggested that autophagy may be induced by Ras. We investigated this possibility by checking a panel of autophagy markers including: detection of LC3I-to-LC3II conversion, p62 degradation by immunoblotting, GFP-LC3 punctate formation, LC3 localization to the vacuoles membranes and positive staining for acidotropic agents, monodansyl cadaverine and acridine orange. We also assessed the status of the autophagic flux and we did not find that it was inhibited when oncogenic Ras was expressed. All these markers clearly demonstrated that Ras indeed induces complete autophagy. The observation that induction of autophagy preceded Ras-induced cell death raised two important questions: first how Ras mechanistically induces autophagy and second whether autophagy contributes to the cell death or serves as an attempt to maintain viability.

To get an insight into the mechanisms by which Ras induces autophagy, we examined whether oncogenic Ras modulates the level of the autophagy-regulating protein Beclin-1. Beclin-1 is an essential regulator of autophagy that was identified in 1998 as a Bcl-2 binding protein and was described later as the mammalian ortholog of Atg6. The BECN1 gene is found mono-allelically deleted in 40-75% of sporadic breast and ovarian cancers (Yue *et al.*, 2003), which suggests it serves as tumor suppressor. This was the first direct link between tumorigenesis and disruption of autophagy. We found that oncogenic H-Ras^{V12} but not the dominant negative Ras^{N17} induces Beclin-1 upregulation in a dose and time-dependent manner which suggests that Beclin-1 upregulation is an important mechanism of Ras-induced autophagy. Given the diversity of Ras effectors, it is plausible to think that other mechanisms may also contribute to Ras-induced autophagy. Therefore we also checked the activation status of mTOR, another important autophagy regulator. We observed that H-RasV12 expression correlated with the inhibition of mTOR activity. The activation of mTOR downstream of Ras/PI3k/Akt pathway and the well characterized role of TOR in the inhibition of autophagy in yeast led to the hypothesis that Ras may be involved in the negative regulation of autophagy (Furuta *et al.,* 2004). This hypothesis however was postulated before the introduction of LC3, the specific marker of autophagy, and also prior to the discovery of a negative feedback loop between mTOR and PI3K (Park *et al* , 2009, Young *et al* , 2009, Courtois-Cox *et al* , 2006, Tamburini *et al* , 2005). Activated PI3K/Akt pathway downstream of Ras can lead to increased activation of mTOR which in turn results in the activation of p70S6K, which then phosphorylates IRS1 resulting in inhibition of IRS1. The IGF/insulin and IRS1 signalling pathways are major activators of the PI3K-Akt pathway. Thus, mTOR-, p70S6K-mediated inhibition of IRS1 results in decreased activation of PI3K-Akt in a negative signalling feedback loop. Moreover, while TOR is an essential regulator of autophagy in yeast in response to nutrient-rich or starvation condition, it is not clear whether mTOR plays the same crucial role in the regulation of autophagy in mammalian cells especially

when autophagy is induced in response to stimuli other than starvation. Much of the cumulative knowledge on autophagy regulation comes from studies that used starvation to induce autophagy. It is not clear however, whether autophagy regulators in response to starvation play the same roles when autophagy is induced by other stimuli such as oncogenic activation or treatment with autophagy-inducing agents.

3.3.4 Autophagy mediates Ras-induced cell death

The answer to the other question as whether or not Ras-induced autophagy contributes to Ras-induced cell death is even more complicated. The role of autophagy in cell death or survival is controversial with some evidence pointing to a mainly pro-survival function for autophagy and a growing body of evidence supporting the notion that autophagy could contribute to cell death in certain contexts. The presence of autophagic vesicles in dying cells may reflect a causative role of excessive autophagy in that form of cell death but may also represent an adaptive response or a desperate attempt to maintain cell survival under stress condition and thus represents a rescue mechanism activated during cellular distress.

While autophagy is mainly initiated under starvation conditions as a selective mechanism that aims to provide the cell with the nutrients it needs to survive for a short period of time until nutrients are re-supplied, prolonged and probably overwhelming autophagy induction in a case like Ras-induced autophagy may evoke a non-selective autophagic response that can lead to cell demolition. Thus, it is plausible to think that while physiological levels of selective and specific autophagic activities may serve pro-survival functions under normal circumstances, extensive and excessive levels of non-selective autophagy may demolish the cell and ultimately lead to cell death. This concept fits with the observation that autophagy contributes to tumor suppression, as several positive regulators of autophagy (e.g. Beclin-1, Bif-1, UVRAG) were shown to act as

tumor suppressors and many negative regulator (e.g. PI3K, Akt) are known to be proto-oncogenes (Maiuri *et al.*, 2007a).

Another possibility in the light of the very recent discovery of Atg5/Atg7-indpenedent, but Beclin-1-dependent, alternative autophagy (Nishida *et al.*, 2009) is that mechanistically or functionally distinct forms of autophagic responses exist and are preferentially induced in different cellular contexts and may ultimately lead to different outcomes. Elevated levels of Beclin-1 may thus be playing a more important role in destructive rather than the housekeeping form of autophagy as has been suggested before (Wang *et al.*, 2009a).

In the context of Ras the dilemma of whether autophagy serves a pro- or anti-death function is even more complicated by the diversity of downstream targets of Ras that regulate both survival and death.

Previous reports however, hinted to that Ras-induced autophagy serves mainly a pro-death function and that autophagic cell death may act as a safeguard mechanism to limit Ras-driven tumorigenesis. For instance, in the light of the observation that mutations in the *RAS* gene are common in many human cancers but quite rare in glioblastomas and gastric cancer (Bos, 1989), Chi and co-workers investigated the reason behind this and demonstrated that the expression of oncogenic H-Ras in human glioma cell U251 and U343 and gastric cell lines MKN-1 and TMK-1 triggers a caspase-independent cell death that was distinct from apoptosis. Cell death in that case was preceded by a massive cell vacuolation. Those vacuoles were found to be derived from lysosomes as staining for lysosomal markers LAMP -1, 2 and LGP-85 colocalized with vacuolar membrane (Chi *et al.,* 1999). Those results suggested for the first time that expression of H-Ras^{V12} can trigger a non-apoptotic cell death with autophagic features which constrains the clonogenic capability of the cells. Notably, expression of oncogenic H-Ras in T24 human bladder cancer cells which often harbour a mutation in the *RAS* gene did not provoke a similar autophagic cell death response. This may reflect an adaptive response whereas those cells have acquired other oncogenic lesions during the multistep carcinogenesis process that enabled them to bypass autophagy. Ras-induced cell death was later

described to occur *in vivo* and to play an important role in the spontaneous regression of neuroblastoma (Kitanaka *et al.*, 2002). H-Ras expression is a biological marker that correlates with positive prognosis in neuroblastoma. Kitanaka and co-workers conducted immunohistochemical analysis of neuroblastoma tissues and demonstrated that neuroblastoma cells overexpressing Ras show morphological changes indicative of cell death. Degenerating neuroblastoma cells positive for Ras did not have morphological features of apoptosis and were negative for TUNEL and active caspase-3 staining but generally showed autophagic features and stained positive for PAS, a marker of autolysosome.

To test whether or not autophagy contributes to cell death in HOSE-RasV12 system, we ablated two essential autophagy-regulating proteins Beclin-1 and Atg5. Inhibition of autophagy via pharmacological modulators and/or RNAi (RNA interference) would enhance long-term cell survival only when autophagy is the cause of cell death, rather than an accompanying stress response or a mere withstander phenomenon. Knockdown of either Atg5 or Beclin-1 diminished H-Ras^{V12} induced vacuolation which indicates that those vacuoles are autophagosomes and furthermore greatly reduced cell death. Moreover, Beclin-1 knockdown enhanced long-term clonogenic survival upon H-Ras^{V12} induction. Collectively, these data confirm that Ras-induced cell death is a consequence of excessive and extensive autophagy as it can be rescued by knocking down autophagy-essential proteins.

3.3.5 Ras isoforms vary in their ability to induce autophagy

H-, N-, and K-Ras are ubiquitously expressed in mammalian cells and can all interact with the same set of effector proteins (Bos, 1988). An interesting yet unresolved issue is whether the Ras isoforms that are ubiquitously expressed in mammalian cells serve distinct functions. Several lines of evidence suggest that they may. First, specific Ras isoforms are mutated in different tumors: K-*Ras* mutations occur in 50% of colon cancers and 90% of pancreatic cancers,

whereas N- and H-*Ras* mutations are extremely uncommon. Conversely, N-*Ras* mutations occur in 25% of acute leukemias, whereas H-*Ras* and K-*Ras* mutations are much less common in this type of cancer (Omerovic *et al.*, 2007). Secondly, recent knockout studies have shown that genetic deletion of different Ras isoforms has differential outcomes. K(A)-,H-, N- and double N-, H-homozygous Ras knockouts apparently develop normally, which suggests that these isoforms are dispensable for mouse development, K(B)-Ras is required during embryogenesis. K(B)-Ras knockout mice die during embryogenesis between days 12 and 14 (Koera *et al.*, 1997). Thirdly, the four isoforms differ in their ability to drive tumourigenesis. K-Ras is the most potent inducer of transformation among the four isoforms (Omerovic *et al.*, 2007).

These observations suggest that the activation of different Ras proteins has distinct biological consequences. The H-, K-, and N-Ras are almost identical and the only area of significant sequence divergence between isoforms lies in the C-terminal hypervariable region (HVR). This short (23–24 aa) stretch terminates with a CAAX motif that undergoes a series of post-translational modifications that promote membrane binding (Choy *et al.*, 1999). An important feature of Ras isoforms that may contribute to their differential effects is their differential localization. H-Ras and K(B)-Ras represent the two most cell surface-localised isoforms, whereas in many cell types, N-Ras is a prominent endomembranous component (Omerovic *et al.*, 2007). Individual Ras isoforms preferentially associate with different organelles and microdomains depending upon the type of membrane anchor, post-translational modification and activation state. This differential localization may contribute to differential signaling by Ras isoforms simply by interaction with different sets of effectors that are present in the proximity of each isoform differential location. This compartmentalised Ras isoform signalling may well contribute to distinct signalling.

Several studies were dedicated to comparing the differential signalling triggered by different Ras isoforms. These studies led to conflicting conclusions. For

instance, Li and co-workers suggested that N-Ras and K-Ras were more important than H-Ras in the activation of the Raf-1/MEK/ERK effector pathway among the three isoforms and that H-Ras induces PI3K/Akt pathway more efficiently that than the other isoforms (Li *et al.*, 2004). Conversely, Rosseland and co-workers reported a more important role for H-Ras than K-Ras in signalling through the ERK pathway (Rosseland *et al.*, 2007). These conflicting results are not surprising given the complexity of Ras signalling which is likely to be cell line- and context- dependent.

We have shown here that there are marked quantitative differences in the ability of Ras isoforms to induce autophagy that correlated to their differential ability to activate ERK pathway and thus to induce the expression of Beclin-1. In the experimental settings we used, we observed that oncogenic H-Ras correlated with the most potent activation of the ERK pathway followed by N.Ras then K-Ras. Correspondent with its ability to activate the ERK pathway, H-Ras was the most potent in the induction of autophagy. N-Ras triggered only a very mild level of autophagy while K-Ras did not seem to induce autophagic response above the level of control.

In the light of the observation that H-Ras mutations are the least common in cancer among the three isoforms, it is tempting to speculate that autophagy and autophagic cell death selectively triggered by that isoform may serve anti-proliferative function to eliminate cells that acquire mutations in that gene. K- and N-Ras mutations which do not trigger a similar fail safe response are therefore more likely to drive tumorigenesis. Some observations further support this speculation: H-Ras driven tumors in humans and in animal models are often self-regressing (Leon *et al.*, 1998), H-Ras expression is generally considered as a positive prognostic marker in neuroblastoma. Overexpression of H-Ras in neuroblastoma cells induced cell death that was accompanied with some markers that hinted to a role for autophagy in that form of cell death (Kitanaka *et al.*, 2002).

3.3.6 Duration and intensity of Ras activation dictate the outcome

Several studies have demonstrated that the expression of oncogenic Ras induces cellular senescence in non-transformed cells (Serrano *et al.*, 1997, Lin *et al.*, 1998, Narita *et al.*, 2004). We and others observed that oncogenic Ras expression can provoke a cell death response (Chi *et al.,* 1999; Kitanka *et al.*, 2002). In attempt to understand the reason behind those different observations, we studied the effect of variations in duration and intensity of Ras activation to test if different levels or durations of Ras activation may lead to different outcomes. Our observations suggest that autophagy is initially induced by all levels of Ras activation albeit to varying degrees. Autophagic response induced by higher levels of Ras activation is overwhelming and lead mainly to cell death with few surviving cells that may later senesce or if they have acquired some selective advantages during the elimination phase may proceed to full transformation. Milder levels of Ras activation however trigger subsequently milder autophagic response that tends to result in less cell death but instead drives more cells over time to cell cycle exit and senescence. Ras-induced autophagic cell death might thus represent an acute response triggered by rather stronger Ras signals shortly after the activation of Ras, while senescence might be a chronic, long-term response triggered by milder Ras signals. Additionally, as has been suggested by others, autophagic cell death might still represent an end point for long-lived senescent cells (Gosselin *et al.*, 2009).

Another possibility that may account for the different results is cell linage specificity. Although we observed that Ras triggered autophagy and cell death in several cell lines that we tested, Ras-induced senescence was differentially induced in different cell lines. For instance, while senescent cells were readily detectable in HUVECs transfected with Ras^{V12} five days post transfection, HOSE-Ras^{V12} only showed positive markers of senescence 15-21 days post induction of Ras^{V12} expression. One reason that may account for that difference in senescence kinetics is that HOSE-Ras^{V12} cells express low levels of p53 possibly due to immortalization of those cells with viral E6. As reviewed in chapter one, senescence is executed through two axes that involve cell cycle

regulators: the ARF-p53 and p16-Rb axes. p53 levels in HOSE-Ras^{V12} cells is further decreased after the induction of H-Ras^{V12} expression, which suggests that the p53-driven axis to senescence is disabled and that those cells have to rely solely on the p16 axis for the establishment of senescence. The p16 axis in HOSE-Ras^{V12} cells appears to be intact and p16 is upregulated in the later senescence phase but not in the initial death phase. This may suggest that HOSE-Ras^{V12} cells depend solely on p16 as an effector of the senescence program which may contribute to the delay in reaching the senescence phase. This speculation however remains to be verified.

3.3.7 Autophagy-senescence interplay

Autophagy and senescence are long thought to be two different and independent processes that the suggestion that they might be linked is a surprise. Gosselin and co-workers suggested a link between autophagy and senescence and proposed that senescent keratinocytes ultimately die by autophagic cell death (Gosselin *et al.*, 2009) indicating therefore that senescence occurs upstream of autophagy and the subsequent autophagic cell death. Although the study was rather descriptive and lacked quantification in many occasions it was probably the first study to describe a possible crosstalk between senescence and autophagy. In another recent study linking the two processes, Young and co-workers demonstrated that oncogenic Ras expression evokes autophagy that they found mediates the transition from the initial mitotic burst to the later senescence phase. In this study, Young and co-workers found a subset of autophagy-related genes (ATG) is up-regulated in senescence, including ULK3, LC3B, BNIP3, and BNIP3L. Strikingly, knockdown of autophagy-essential genes Atg 5 and Atg7 reduced or delayed the appearance of the senescence phenotype in response to mutational Ras activation (Young *et al.*, 2009) suggesting a causative role for autophagy in the establishment of senescence and therefore suggesting that autophagy acts upstream of senescence. Although at first sight these two studies seem conflicting in regard to the relative positioning of autophagy and senescence and which of the two acts upstream of

the other, they both suggest that a complex crosstalk between the two processes exists. The potential role for autophagy in preventing senescence bypass can be further assessed by comparing cancer incidence following oncogene activation and induction of senescence in mice with an autophagy wild-type and defective genetic background.

These studies raised further questions about how autophagy and senescence are linked mechanistically. To date, very little is known about the molecular mediators which link the two processes. Young and co-workers reported what might be the first insight into that matter. They showed that autophagy plays an important role in the rapid protein remodelling required to make the efficient transition from a proliferative to a senescent state. The dramatic phenotypic change during the transition phase is accompanied by increased secretion of several key cytokines that play a prominent role in senescence. These secretory proteins include, among others, IL-6 and IL-8, which are critical components of the senescence-associated secretory phenotype (SASP) that reinforces senescence through autocrine and paracrine signalling. Strikingly, knockdown of ATG5 or ATG7 delays synthesis of IL6 and IL8. This effect was not observed by knockdown of Rb, suggesting that the inhibition of IL6/8 production is not a consequence of delayed senescence. The mRNA levels of IL6/8 were often even higher in shATG5/7-expressing cells, indicating that the repression of IL6/8 is post-transcriptional (Young *et al.*, 2009). It is thus possible that rapid protein turnover by autophagy, coupled with active translation, could handle the sudden demand for these abundant secretory protein. Autophagy therefore may mediate senescence establishment, at least in part, through promoting senescence-associated secretory phenotype. Further work will possibly shed more light into this matter.

Autophagy is induced by a variety of stress conditions. An intriguing question to ask is whether oncogene-associated autophagy is a specialized form of autophagy or in general terms whether the autophagy program in response to

different stresses is the same or distinct autophagy programs are customized to specific responses. In this sense it would be interesting to see if the autophagy program accompanying oncogenic stress that mediates senescence or death has a different machinery and genetic signature than for instance autophagy triggered by starvation or hypoxia.

In conclusion, our results suggest a model in which mutational Ras activation initially triggers an immediate autophagic response. Depending on the intensity of Ras activation and therefore the intensity of the subsequently-induced autophagy, cells either die as a consequence of severe autophagic stress or senesce if the level of autophagy is not severe enough to cause cell demolition (Figure 3.18) While the link between excessive autophagy and cell death has been described previously, the mechanism through which mildly elevated levels of autophagy can lead to cell cycle exit and senescence remains to be investigated.

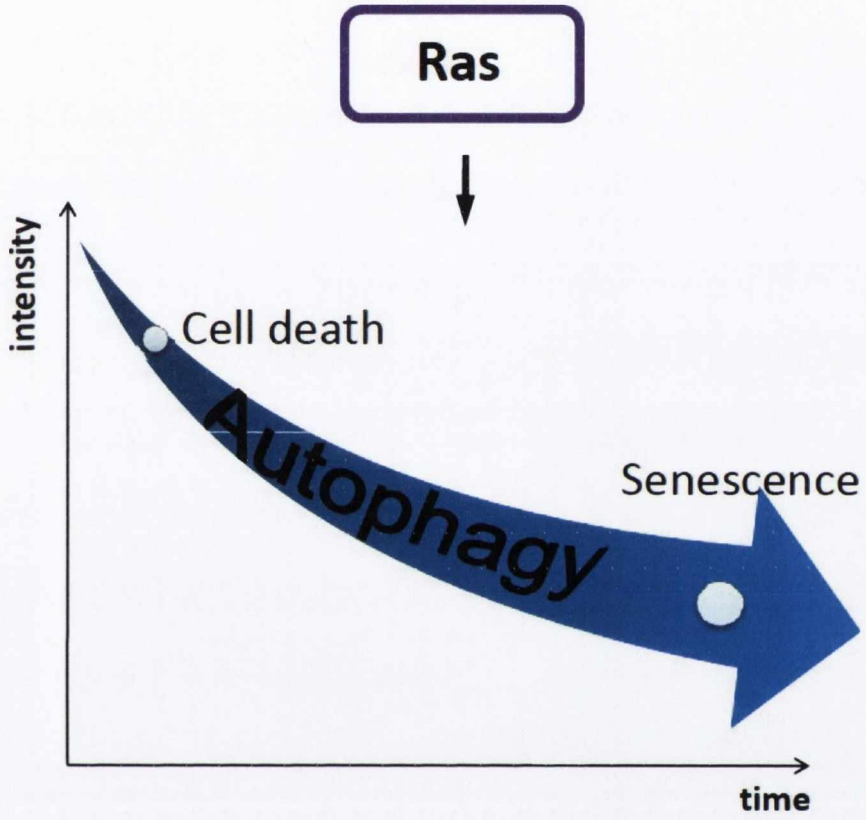


Figure 3.18 Interplay between Ras-induced cell death and senescence

The anti-proliferative response triggered by mutational activation of Ras varies depending on the intensity and duration of Ras activation. Higher levels of activation trigger an initial cell death response, while lower sustained levels induce senescence.

Chapter IV

The Role of the BH3-only Protein Noxa in Mediating Oncogenic H-Ras-Induced Autophagy and Cell Death

4.1 Introduction

As discussed in chapter one, Bcl-2 family members are essential regulators of programmed cell death. The crucial role of Bcl-2 family proteins in positive (Bax, Bak, Bad, Bim, Puma, Noxa, Bid, Hrk, etc) and negative (Bcl-2, Bcl-xL, Bcl-B, Bcl-w, Mcl-1 and A1) regulation of apoptosis is well established. Recently, the role of Bcl-2 family members in the regulation of another form of programmed cell death (so called autophagic cell death or type II programmed cell death) has emerged as a topic of intense investigation and a matter of hot debate at the same time. The discovery that Bcl-2 and Bcl-xL can bind Beclin-1 and inhibit Beclin-1-driven autophagy suggested that anti-apoptotic Bcl-2 proteins can act as anti-autophagic as well (Pattingre *et al.*, 2005). Beclin-1 was later described as a novel BH3-only protein and was shown to bind Mcl-1 and Bcl-w in addition to Bcl-2 and Bcl-xL (Erlich *et al.*, 2007). BH3-only proteins and BH3 mimetics have been shown to disrupt this interaction setting Beclin-1 free and therefore inducing autophagy (Maiuri *et al.*, 2007).

As discussed earlier, we observed that the expression of oncogenic Ras triggers autophagy and autophagic cell death. In order to gain further insight into the mechanism of Ras-induced autophagy and cell death, and given the crucial role of Bcl-2 family members in the regulation of programmed cell death and their emerging role in the regulation of autophagy, we decided to explore the involvement of the Bcl-2 family in Ras-induced autophagy and autophagic cell death.

4.2 Results

4.2.1 Oncogenic H-Ras expression induces Noxa upregulation

Cell death induced by various stimuli is often linked with modulation of one or more of the Bcl-2 family proteins. We, therefore, sought to examine if oncogenic Ras-induced cell death was associated with modulation of the expression of any of the Bcl-2 family proteins. To this end, total cell lysates prepared from HOSE-Ras^{V12} cells treated with or without Dox over a time course of 7 days were analysed for expression profiling of various members of the Bcl-2 family. Immunoblotting revealed that oncogenic H-Ras^{V12} expression is associated with strong upregulation of the levels of the BH3-only protein Noxa, slight upregulation of Puma and downregulation of Bim (Figure 4.1). The levels of the other BH3-only proteins examined were found to be unaffected by H-Ras^{V12} expression. Bim has been described previously to be phosphorylated by ERK which facilitates its degradation (Sheridan *et al.*, 2008). Concomitant with Noxa upregulation, the levels of Mcl-1 were found to go down over time (Figure 4.1). Noxa has been suggested to bind Mcl-1 and drive it to the proteasome for degradation (Nijhawan *et al.*, 2003). It is thus plausible to think that Mcl-1 downregulation may be a consequence of Noxa upregulation. To confirm that Noxa upregulation in HOSE-Ras^{V12} cells is due to H-Ras^{V12} induction rather than Dox- treatment of the cells *per se*, control HOSE cells (not transfected with Dox-inducible H-Ras^{V12}) and HOSE-Ras^{V12} cells were treated with Dox. Dox-treatment of HOSE-Ras^{V12} but not control HOSE cells resulted in Noxa upregulation in a time-dependent manner over the course of 72 hr (Figure 4.2 A). Ras-induced Noxa upregulation in HOSE-Ras^{V12} cells was also found to be dependent on the level of Ras induction as shown by treating HOSE-Ras^{V12} cells with titrated Dox doses (Figure 4.5). Expression of oncogenic B-Raf^{V600E} which similarly activates ERK pathway was also found to upregulate Noxa in HeLa cells. (Figure 4.2 B)

4.2.2 Noxa upregulation by Ras is comparably- profound

To assess the magnitude of Noxa induction by Ras, we compared the relative intensities of Noxa upregulation by comparable DNA plasmids amounts of H-Ras.V12 and other factors that have been previously described to induce Noxa expression. As figure 4.3 illustrates, in HeLa cells, Noxa upregulation induced by H-Ras^{V12} was considerably stronger than that induced by p53, p73α, p73β or c-myc, which were efficiently expressed in the cells and their activity was confirmed by probing for some of their known targets.

4.2.3 Ras-induced cell death is Noxa-dependent

As discussed earlier, BH3-only proteins act as sentinels of stress or damage. Elevation of the expression of some of the BH3-only proteins often correlates with the induction of apoptosis. Ras induces a non-apoptotic form of cell death as shown in the previous chapter. The finding that Ras induces Noxa upregulation therefore raised an intriguing question whether Noxa plays a role in Ras-induced non-apoptotic cell death. To answer this question, HOSE-Ras^{V12} cells were nucleofected with shRNA constructs targeted against Noxa or the other BH3-only proteins Puma, Bad, Bim or Bmf as control followed by Dox treatment. Visual inspection of the cells revealed that while the cells nucleofected with control shRNA and treated with Dox looked stressed, flattened, vacuolated or dying, cells nucleofected with Noxa shRNA proliferated normally and exhibited less of those morphological changes upon Dox treatment (Figure 4.4 A). Assessment of cell death by flow cytometer seven days post Dox-treatment showed that ablation of Noxa markedly impeded Ras-induced cell death while ablation of Bad, Bim or Bmf did not modulate cell death to any significant extent suggesting that Noxa is an important regulator of Ras-induced cell death (Figure 4.4 B and C). Notably, Puma knockdown also seemed to inhibit Ras-induced cell death to some extent, which probably suggests a role for Puma in mediating Ras-induced cell death. Noxa however, seems to be the main player in this context.

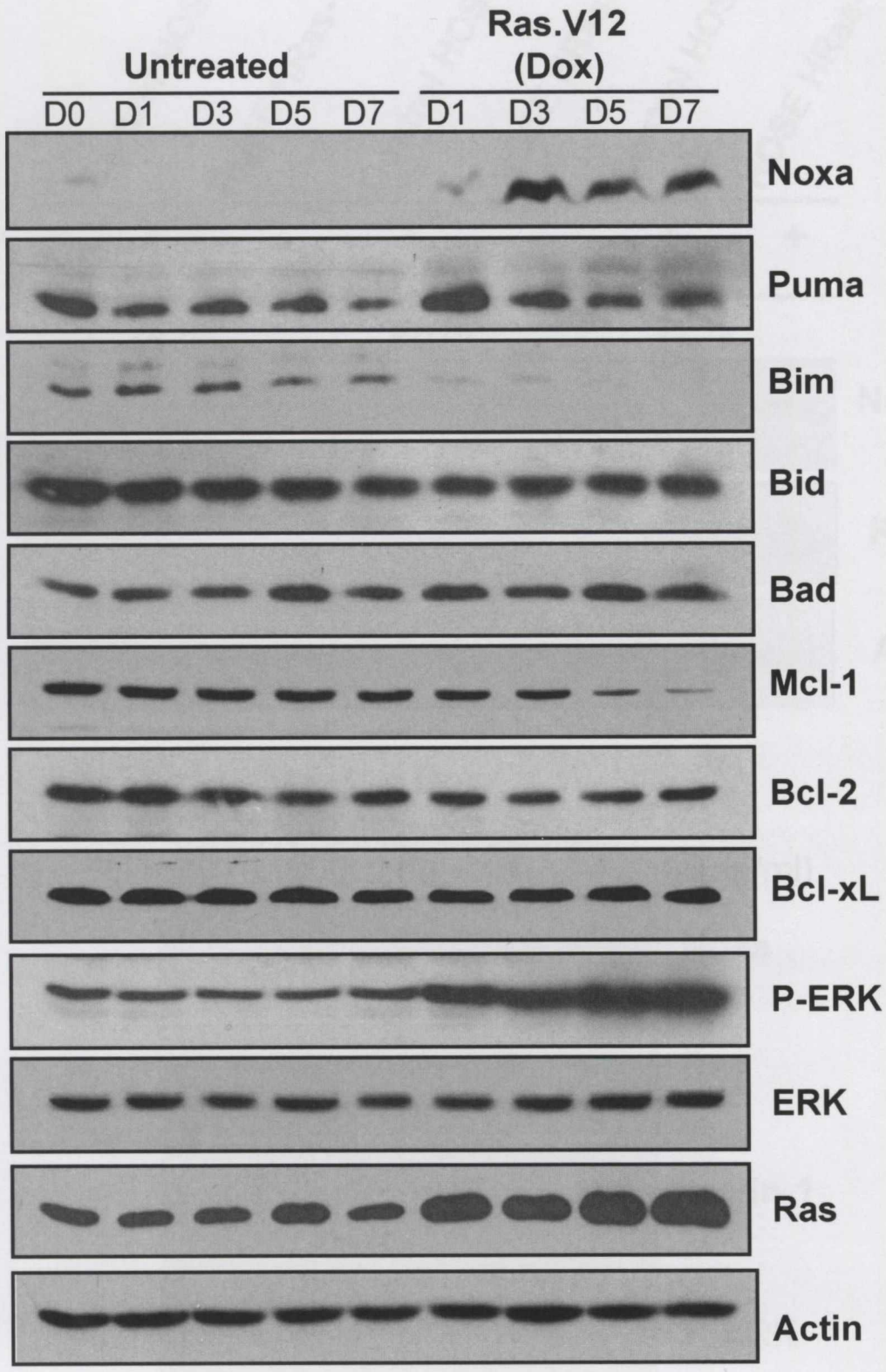


Figure 4.1 Expression of oncogenic H-Ras induces Noxa upregulation

HOSE.RasV12 cells either left untreated or treated with Dox (100 ng/ml) over a time course of 7 days were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies. For re-probing, blots were incubated in 0.2% NaOH for 5 min to kill the signal followed by washing in TBST wash buffer for 30 min.

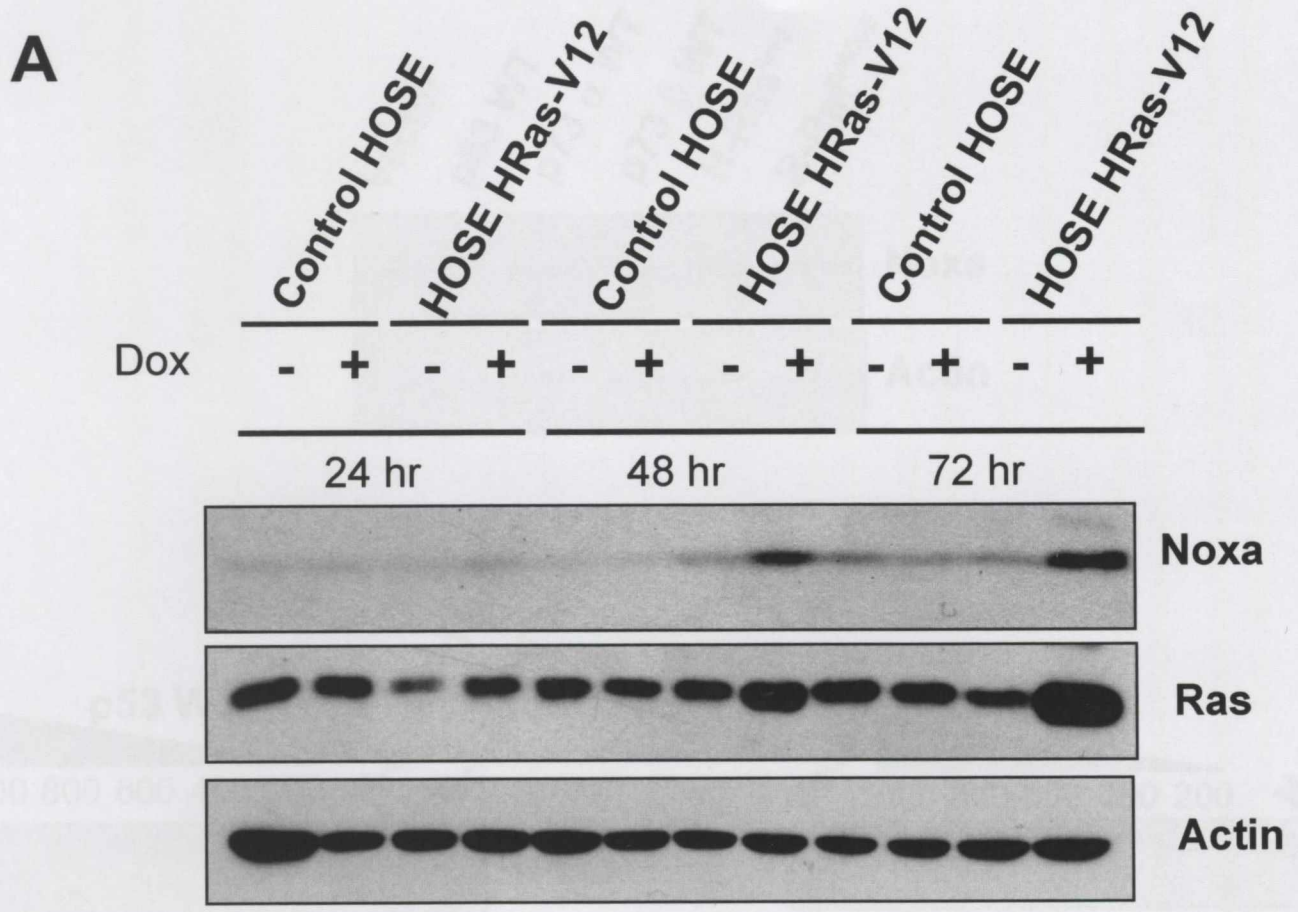


Figure 4.2 Oncogenic H-Ras induces Noxa upregulation in a time- and dose-dependent manner

Control HOSE or HOSE.RasV12 cells were treated with or wthout Dox for 24, 48 or 72 h (A). HOSE. RasV12 cells were treated with various concentrations of Dox for 72 hours (B). Cells were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies.

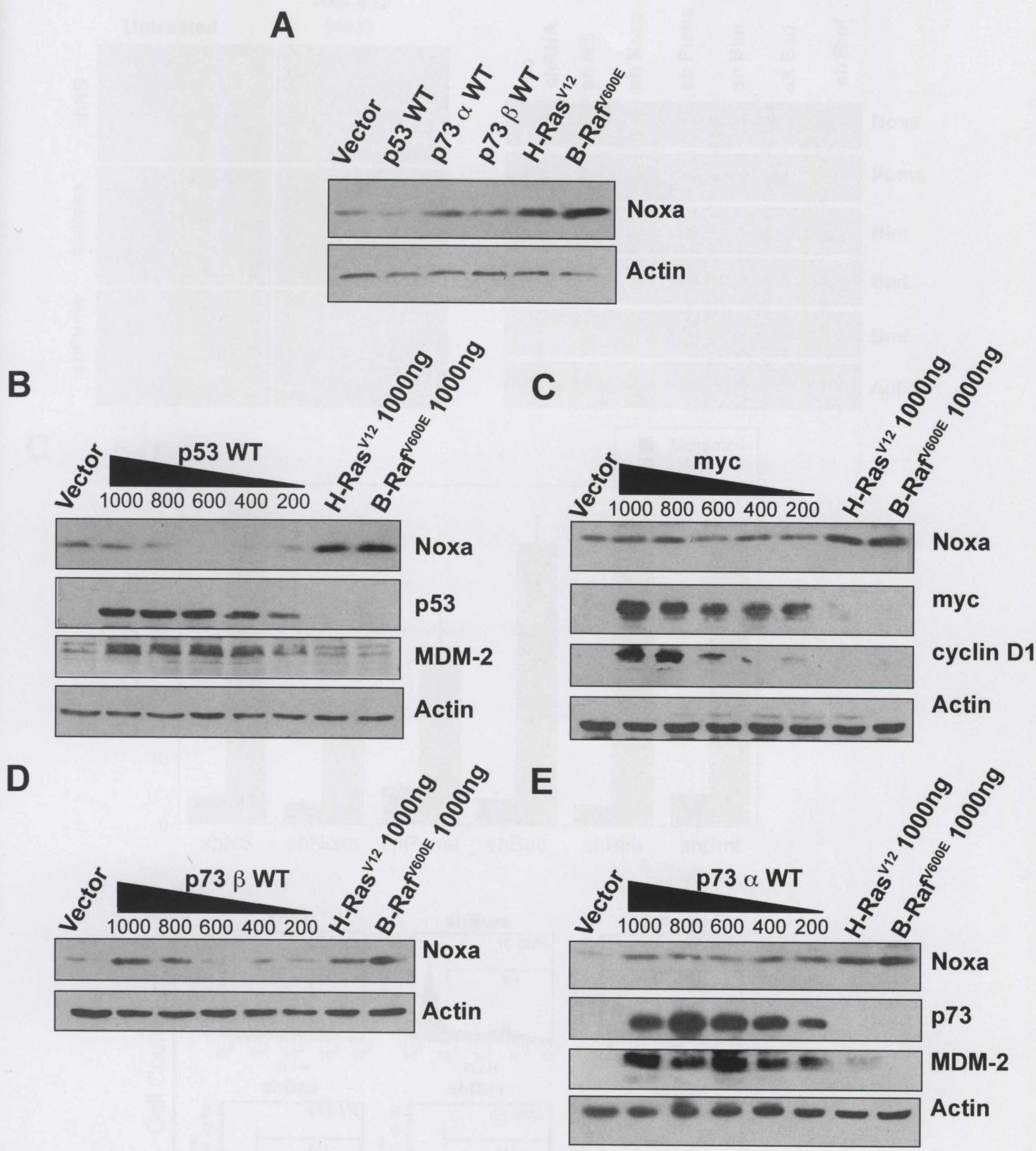


Figure 4.3 Comparison of mangnitude of Noxa induction

HeLa cells were plated at 10^5 cells/well in a 6 well plate and transfected 24 h later with the indicated plasmids . Cell were left 48 h for full expression then lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies.

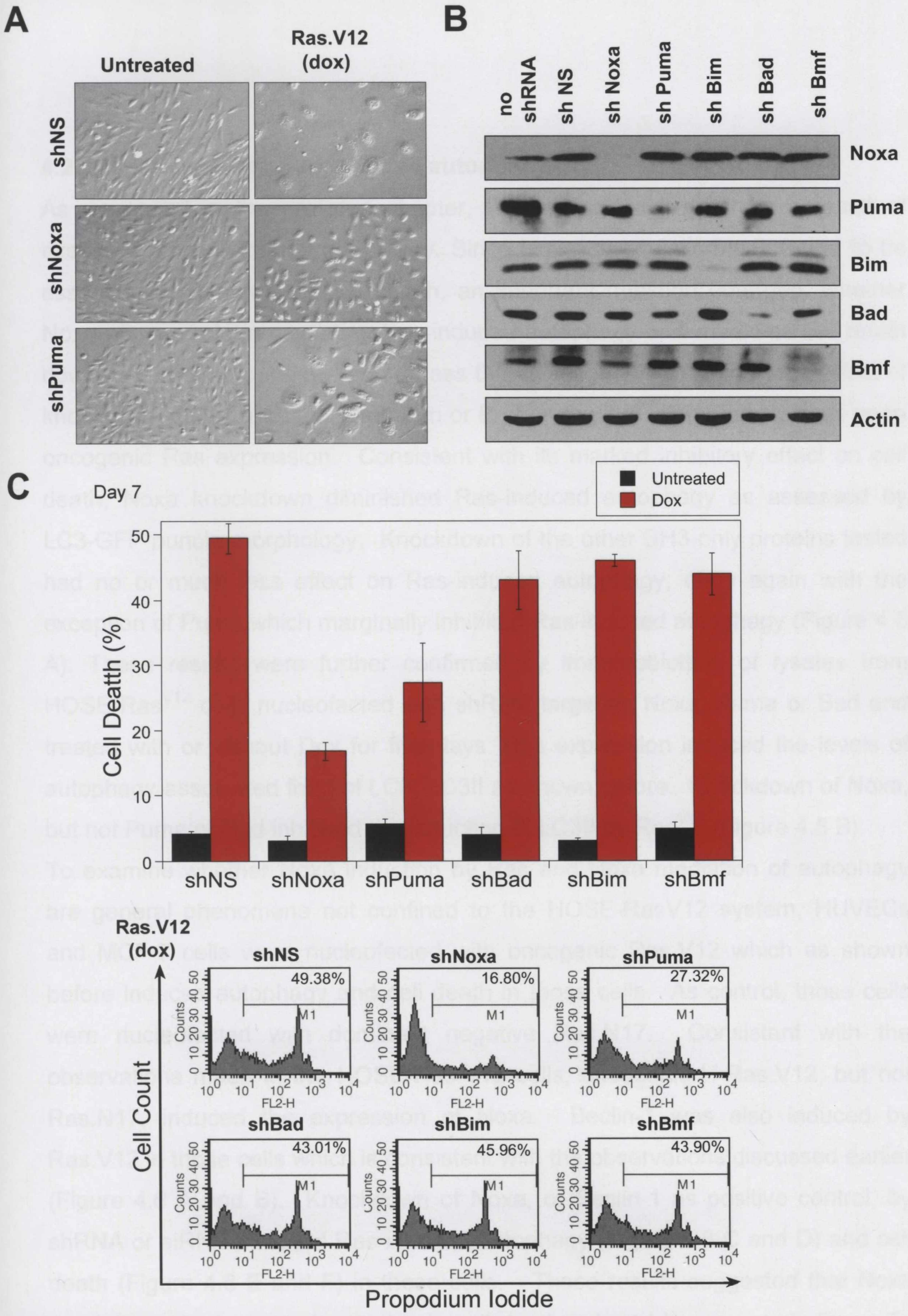


Figure 4.4 Ras-induced cell death is Noxa-dependent

HOSE.Ras.V12 cells were nucleofected with shRNA targeted against the indicated proteins followed 72 h later by treatment with or without Dox (100ng/ml). Cells were followed up for morphological changes. Pictures were taken on a light microscope with 10X magnification. Samples of the cells nucleofected with different shRNA's were lysed in 1x SDS-PAGE buffer and the efficiency of the knockdown was checked by immunoblotting. (B) At day 7, cells were harvested and percentage of cell death in each condition was assessed by flow cytometer analysis of PI uptake. The percentage of PI-positive cells in each treatment is indicated in the top right-hand corner of each panel (C).

4.2.4 Noxa mediates Ras-induced autophagy

As discussed in the previous chapter, Ras-induced cell death is a result of excessive and prolonged autophagy. Since Noxa upregulation was found to be essential for Ras-induced cell death, an important question emerged: whether Noxa plays a role in mediating Ras-induced autophagy or it mediates cell death downstream of autophagy. To address this question, we examined the effect of knockdown of Noxa, Puma, Bad, Bim or Bmf on the induction of autophagy upon oncogenic Ras expression. Consistent with its marked inhibitory effect on cell death, Noxa knockdown diminished Ras-induced autophagy as assessed by LC3-GFP puncta morphology. Knockdown of the other BH3-only proteins tested had no or much less effect on Ras-induced autophagy, once again with the exception of Puma which marginally inhibited Ras-induced autophagy (Figure 4.5 A). These results were further confirmed by immunoblotting of lysates from HOSE-Ras^{V12} cells nucleofected with shRNA targeting Noxa, Puma or Bad and treated with or without Dox for five days. Ras expression induced the levels of autophagy-associated form of LC3; LC3II as shown before. Knockdown of Noxa, but not Puma or Bad inhibited the induction of LC3II by Ras^{V12} (Figure 4.5 B).

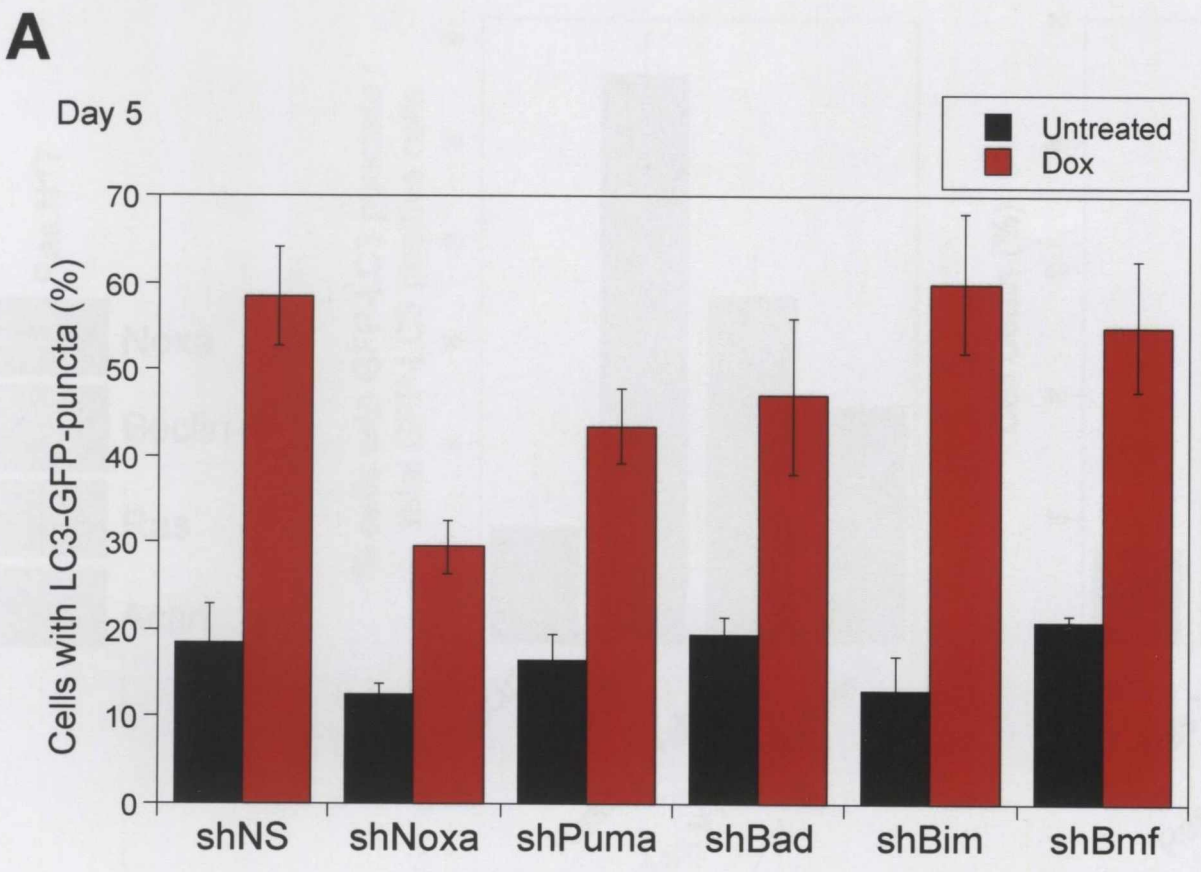
To examine whether Noxa induction by Ras and Noxa mediation of autophagy are general phenomena not confined to the HOSE-RasV12 system, HUVECs and MCF-7 cells were nucleofected with oncogenic Ras.V12 which as shown before induces autophagy and cell death in those cells. As control, those cells were nucleofected with dominant negative Ras.N17. Consistent with the observations made in the HOSE-Ras.V12 cells, oncogenic H-Ras.V12, but not Ras.N17, induced the expression of Noxa. Beclin-1 was also induced by Ras.V12 in those cells which is consistent with the observations discussed earlier (Figure 4.6 A and B). Knockdown of Noxa, or Beclin-1 as positive control, by shRNA or siRNA inhibited Ras-induced autophagy (Figure 4.6 C and D) and cell death (Figure 4.6 E and F) in these cells. These results suggested that Noxa mediates autophagy and autophagic cell death induced by oncogenic Ras. To further confirm this suggestion, we compared phenotypic consequences of H-Ras^{V12} expression in Wild type versus *NOXA* knockout Mouse Embryonic

Fibroblasts (MEFs). Similar to HOSE-Ras^{V12} cells and the other cell lines tested, expression of H-Ras^{V12} in wild type MEFs resulted in autophagic vacuolation and GFP-LC3 punctate morphology when GFP-LC3 was co-expressed with Ras^{V12}. Strikingly, expression of H-Ras^{V12} in *NOXA*$^{-/-}$ MEFs did not trigger similar autophagic vacuolation or GFP-LC3 punctate morphology. Furthermore, reconstitution by Noxa expression, but not Bad expression, together with H-Ras^{V12} in *NOXA*$^{-/-}$ MEFs restored to a great extent the autophagic vacuolation phenotype and GFP-LC3 punctate morphology seen in the wild type MEFs (Figure 4.7 A and B). These results confirm that Noxa is essentially required for the induction of autophagy downstream of Ras.

To test whether Noxa on its own is sufficient to induce autophagy, we nucleofected HOSE cells with Noxa or Bad as control along with GFP-tagged LC3 and scored the percentage of cells undergoing autophagy in each treatment. Noxa expression exhibited weak apoptotic effect as previously reported and although initially showed a weak autophagy-promoting effect over control, that effect did not seem to persist or abrogate as in the case of Ras-induced autophagy (Figure 4.7 C). Noxa, therefore, is not sufficient on its own to induce autophagy. These results suggest that while Noxa is essentially required for Ras-induced autophagy, it requires the co-operation of other factors.

4.2.5 Noxa upregulation limits clonogenic survival of cells expressing oncogenic Ras

The observation that Noxa mediates autophagy and autophagic cell death upon expression of oncogenic Ras led us to postulate that Noxa upregulation in response to mutational activation of Ras may be an intrinsic tumor suppressor response triggered to limit Ras-induced transformation. To test this possibility, HOSE-Ras^{V12} cells nucleofected with various shRNA targeting BH3-only proteins: Noxa, Puma, Bad, Bim or Bmf were subjected to clonogenic assay. Cells nucleofected with control shRNA and treated with Dox were not able to survive the clonogenic assay and thus did not give rise to colonies. Similarly, cell nucleofected with shRNA targeting Bad, Bim or Bmf did not seem to acquire



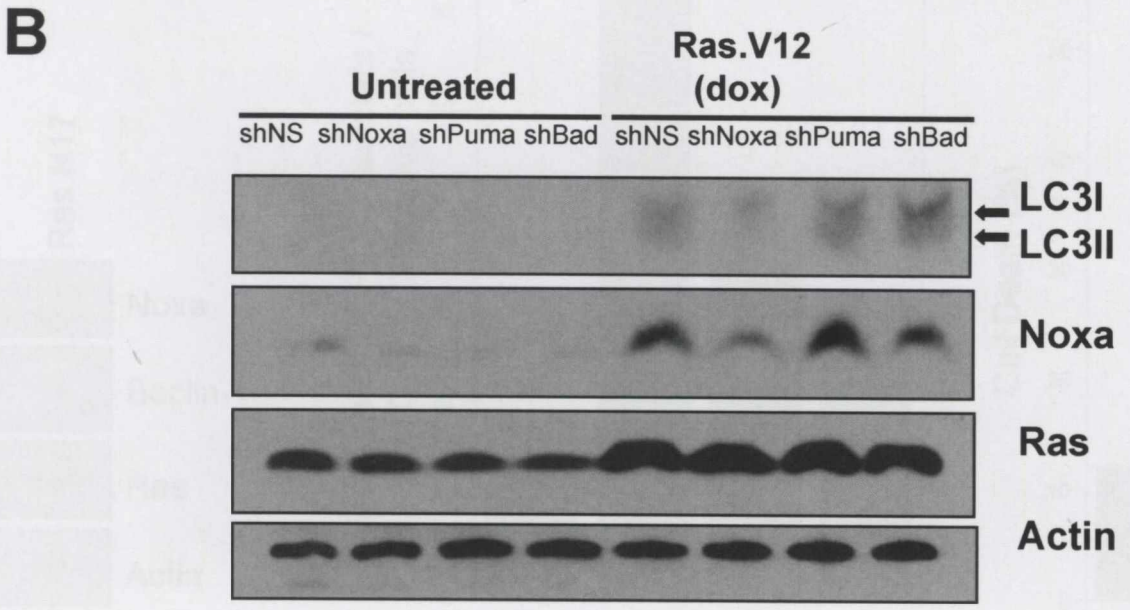


Figure 4.5 Ras-induced autophagy is Noxa-dependent

HOSE.Ras.V12 cells were nucleofected with shRNA targeted against the indicated proteins followed 72 h later by treatment with or without Dox (100ng/ml). At day 7, the percentage of cells exhibiting LC3-GFP puncta in each treatment was scored (A). Cells were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (B).

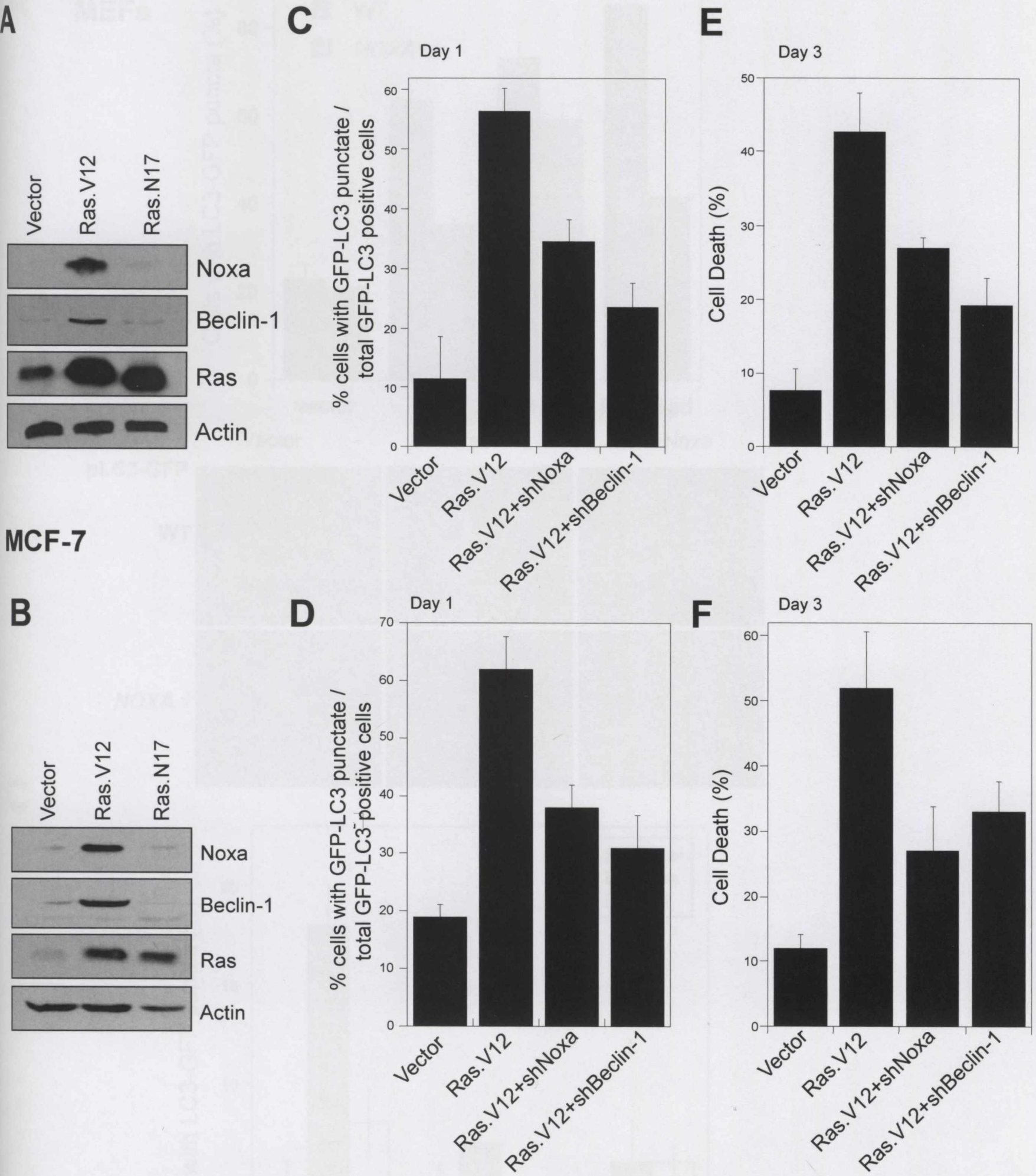


Figure 4.6 Ablation of Noxa or Beclin-1 inhibits Ras-induced autophagy and cell death in HUVECs and MCF-7 Cells

HUVECs (A) or MCF-7 cells (B) were nucleofected with the indicated plasmids using Amaxa nucleofection system (Program T20). 48 hours after nucleofection, cells were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies. HUVECs or MCF-7 cells were nucleofected with the indicated plasmids and the percentage of cells exhibiting LC3-GFP puncta in each condition was scored 24 h later (C and D respectively). 72 hours later, cell death in each condition was enumerated using PI staining (E and F).

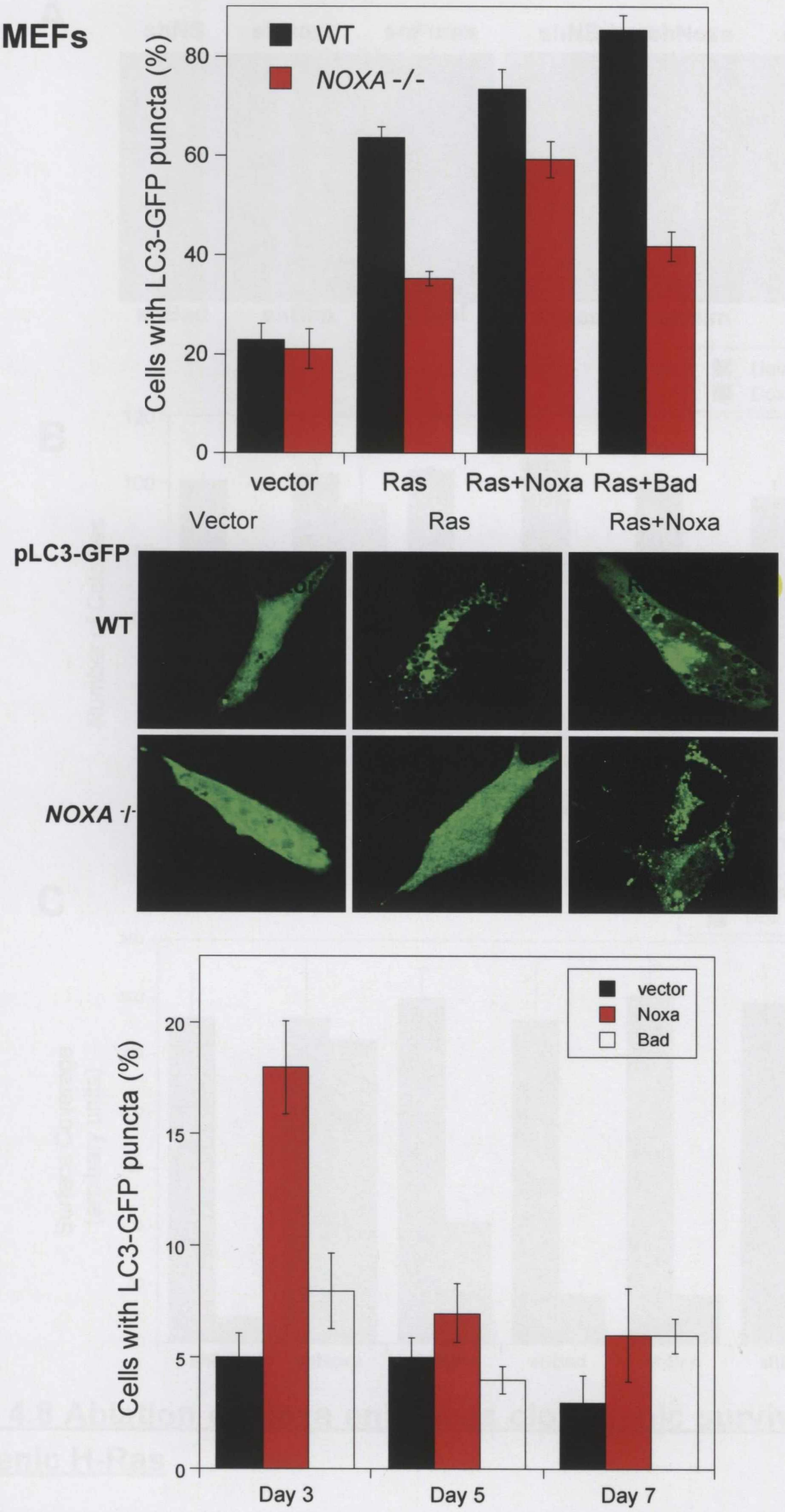


Figure 4.7 Noxa is essentially required but not sufficient on its own for the induction of autophagy

Wild type (WT) or *NOXA* knockout (*NOXA -/-*) MEFs were nucleofected with the indicated plasmids in combination with pLC3-GFP using Amaxa nucleofection system (Program A23). 48 h later, the percentage of cells exhibiting LC3-GFP puncta in each treatment was scored (A) and pictures were taken on a confocal microscope with 60X magnification (B). HOSE.RasV12 cells were nucleofected with the indicated plasmids along with pLC3-GFP. Cells exhbiting LC3-GFP puncta were enumarted at the indicated time points (C).

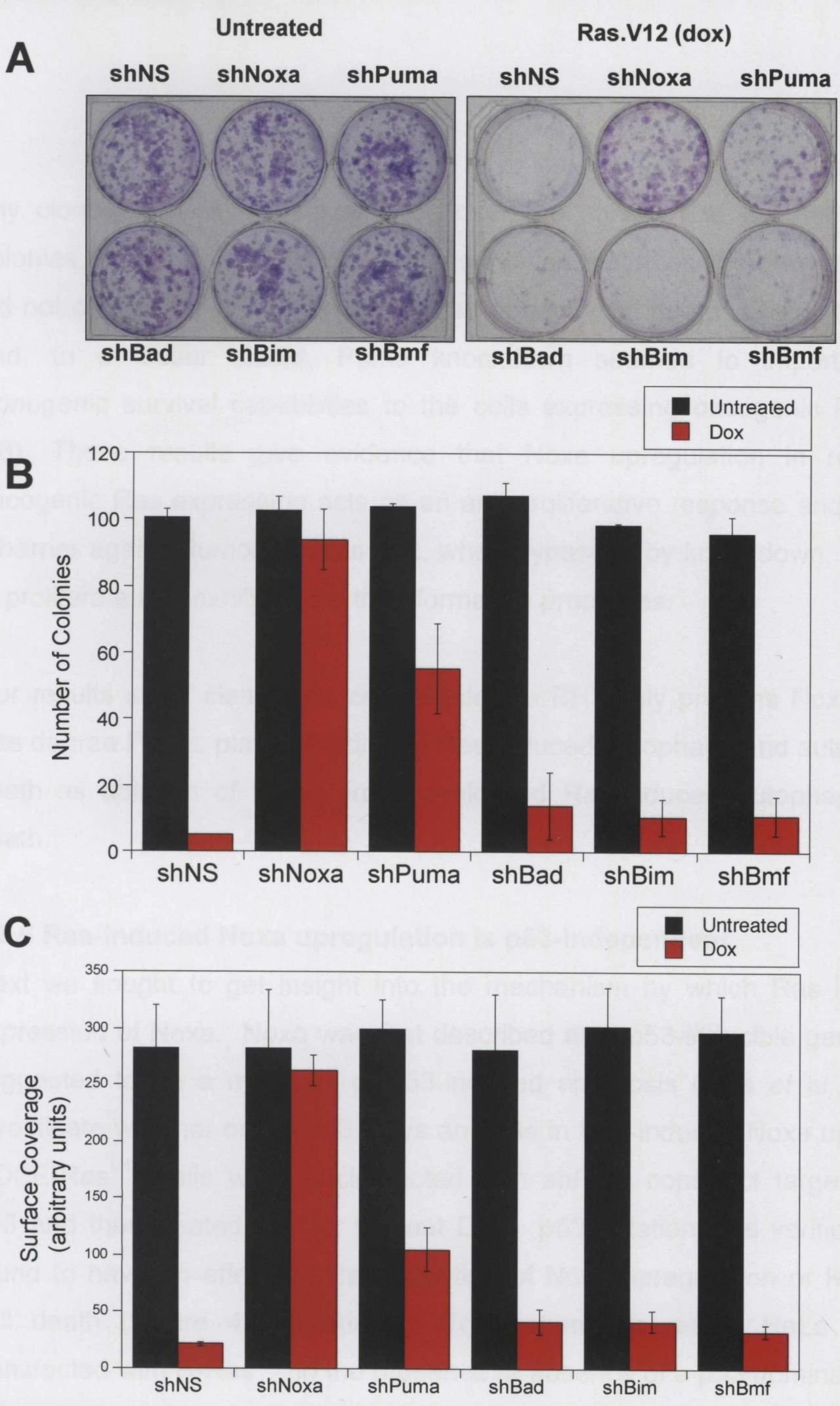


Figure 4.8 Ablation of Noxa enhances clonogenic survival of cells expressing oncogenic H-Ras

HOSE.RasV12 cells were nucleofected with shRNA constructs targeted against various members of the BH3-only proteins. Cells were allowed 72 h for full expression followed by treatment with selection with 300ng/ml puromycin for 7 days then cells were grwon in a medium containing a maintainance dose of 150 ng/ml puromycin for 7 days followed by treatment with or without 100 ng/ml Dox for 3 days. Cells were then harvested by trypsinization, counted and 2000 cells/ well were plated in a 6 well plate. Dox-treatment was continued for 3 weeks. Pictures were taken with Canon G9 digital camera (A). Colonies were stained with 0.5% crystal violet in 20% methanol and colonies bigger than 1mm were counted (B). Ratio of well surface covered by colonies in each treatment was analyzed by image analysis of plates using ImageJ software (C).

any clonogenic survival advantages over the control and did not form many colonies. This is consistent with the observation that knocking down those genes did not confer any protection against autophagic cell death. Dramatically, Noxa and, to a lesser extent, Puma knockdown seemed to impart significant clonogenic survival capabilities to the cells expressing oncogenic Ras (Figure 4.8). These results give evidence that Noxa upregulation in response to oncogenic Ras expression acts as an anti-proliferative response and represents a barrier against tumorigenesis that, when bypassed by knockdown, allows cells to proliferate and exhibit more transformation properties.

Our results show clearly the crucial role the BH3-only proteins Noxa and, to a less degree Puma, play in mediating Ras-induced autophagy and autophagic cell death as ablation of those proteins blocked Ras-induced autophagy and cell death.

4.2.6 Ras-induced Noxa upregulation is p53-independent

Next we sought to get insight into the mechanism by which Ras induces the expression of Noxa. Noxa was first described as a p53-inducible gene and was suggested to be a mediator of p53-induced apoptosis (Oda *et al.*, 2000). To investigate whether or not p53 plays any role in Ras-induced Noxa upregulation, HOSE-Ras^{V12} cells were nucleofected with shRNA construct targeted against p53 and then treated with or without Dox. p53 ablation was verified and was found to have no effect on Ras-induction of Noxa upregulation or Ras-induced cell death (Figure 4.9 A and B). To confirm this result, HeLa cells were transfected with H-Ras^{V12} in the presence or absence of a p53 dominant negative construct in various ratios. In agreement with the result obtained with p53 knockdown, expression of dominant negative p53 did not have any effect on Ras induction of Noxa (Figure 4.9 B). Furthermore, expression of Ras^{V12} was found to induce Noxa upregulation in SAOS-2 cells which are known to be p53 deficient (Figure 4.9 C). Taken all together, these results rule out any role for p53 in Ras-induced Noxa upregulation.

4.2.7 Ras-induced Noxa upregulation is ERK-dependent

Ras protein signals through multiple downstream pathways (reviewed in chapter one). In order to gain further insight into the mechanism(s) by which expression of H-Ras^{V12} leads to Noxa upregulation and because Noxa upregulation tended to happen in a close correlation with ERK activation as indicated by the levels of phosphorylated ERK, we wanted to test whether Ras induces Noxa upregulation in an ERK-dependent manner. To this end, HOSE-Ras^{V12} cells were treated with Dox alone or in combination with inhibitors of different effector pathways activated downstream of Ras. Co-treatment with UO126, a widely used MEK inhibitor, completely abolished induction of Noxa by Ras. Inhibition of PI3K, JNK or p38 by LY294002, SP600129 and SB202190 respectively did not modulate Noxa induction to any extent (Figure 4.10 A and B). Furthermore, expression of different Ras isoforms, which as shown before, differentially activate the ERK pathway led to differential Noxa upregulation that positively correlated with the extent of ERK activation (Figure 4.10 C).

These results suggest that Ras induces Noxa upregulation through an ERK-dependent pathway.

Inhibition of MEK by UO126 also inhibited Ras-induced expression of Beclin-1 as shown before and consistently inhibited Ras-induced autophagy as indicated by the level of p62 (Figure 4.10 A). Strikingly, inhibition of MEK almost completely abolished Ras-induced cell death (Figure 4.11 A and B) and induced the proliferation rate to a level much greater than untreated control cells (Figure 4.11 C). Of note, we also observed that the inhibition of PI3K and JNK marginally attenuated Ras induced autophagy and cell death but to a much lesser extent than MEK/ERK inhibition and did not promote cell growth as in the case of MEK/ERK inhibition. This may suggest that other mechanisms of Ras-induced autophagy may co-operate with ERK-regulated pathways. MEK/ERK pathway however seems to be the main player downstream of Ras in this context.

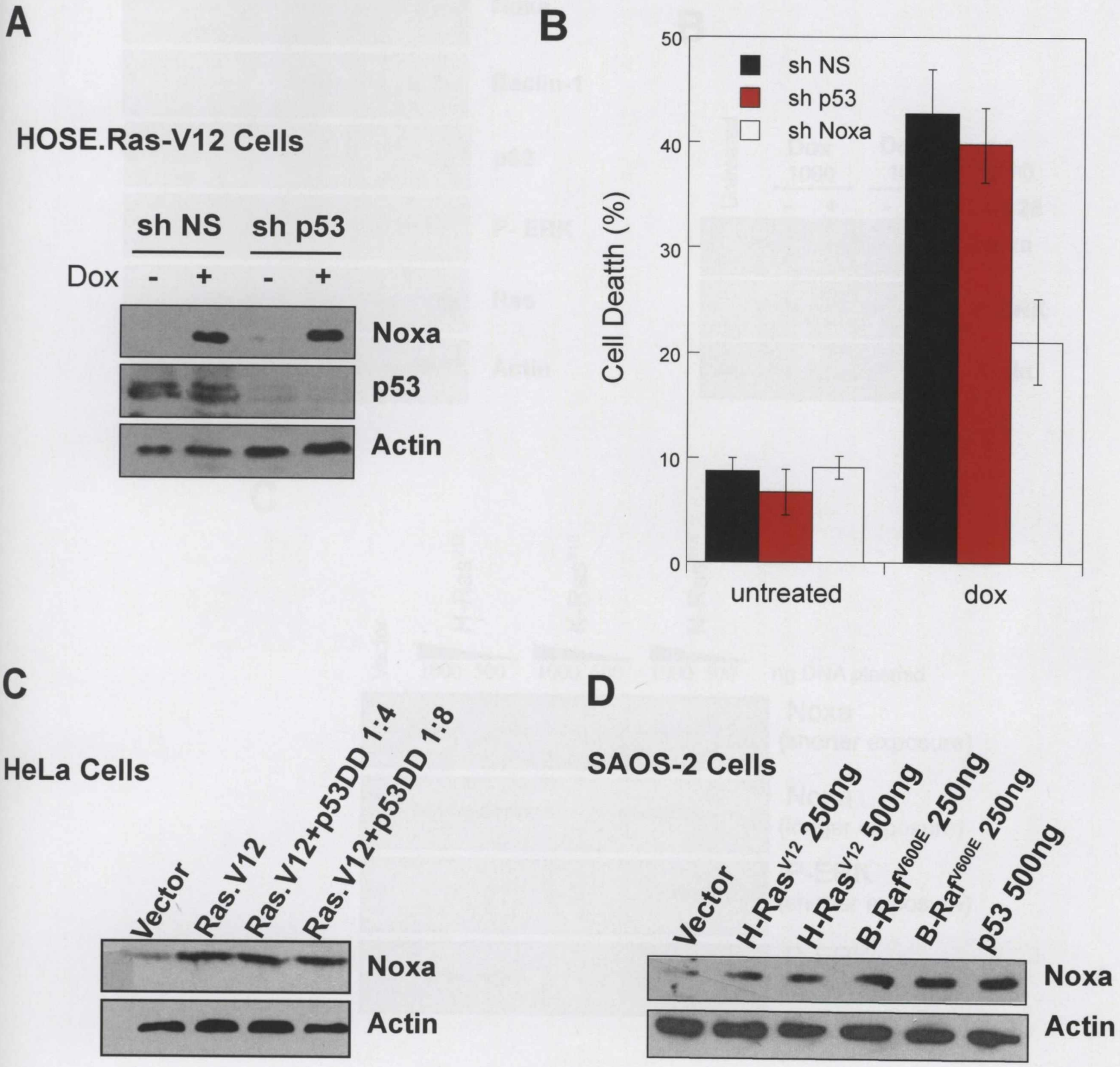


Figure 4.9 Noxa upregulation by Ras is p53-independent

HOSE.Ras.V12 cells were nucleofected with shRNA targeted against p53 followed 72 h later by treatment with or without Dox (100ng/ml). Samples of the cells in each treatment were lysed in 1X SDS-PAGE lysis buffer 3 days after Dox treatment and protein levels were analysed by western immunoblotting with the indicated antibodies (A). Cells nucleofected as above were followed up and cell death in each condition was enumerated on day 7 days after Dox treatment using PI uptake assay (B). HeLa cells were transfected with the indicated plasmids. 48 hr later, cells were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (C). SAOS-2 cells were transfected with the indicated plasmids 48 hr later, cells were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (D)

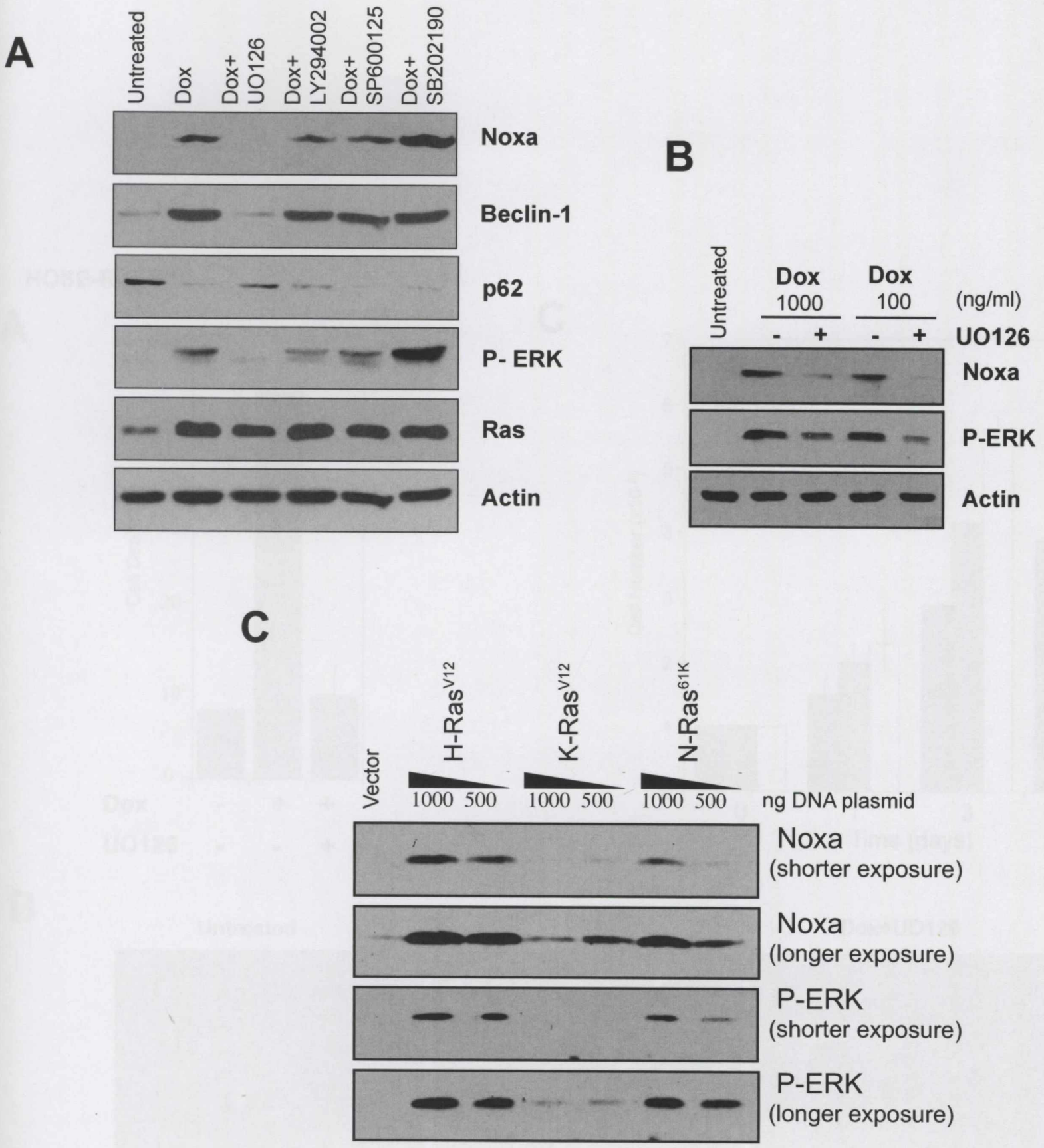


Figure 4.10 Ras induces Noxa expression through an ERK-dependent pathway

HOSE.RasV12 cells were either left untreated or were treated with 100 ng/ml Dox either alone or in combination with MEK inhibitor UO126 (20μM), PI3K inhibitor LY294002 (20μM), JNK inhibitor SP600125 (20μM) or p38 inhibitor SB202190 (20μM). Three days later, cells were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (A). HOSE.RasV12 cells were treated with Dox (1000 or 100 ng/ml) either alone or in combination with MEK inhibitor UO126 (20μM). Three days later, cells were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (B). HeLa cells were transfected with the indicated amounts of each of different Ras isoforms using Genejuice transfection reagent. Follwoing 48h of expression, cells were lysed 48 h after transfection in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (C).

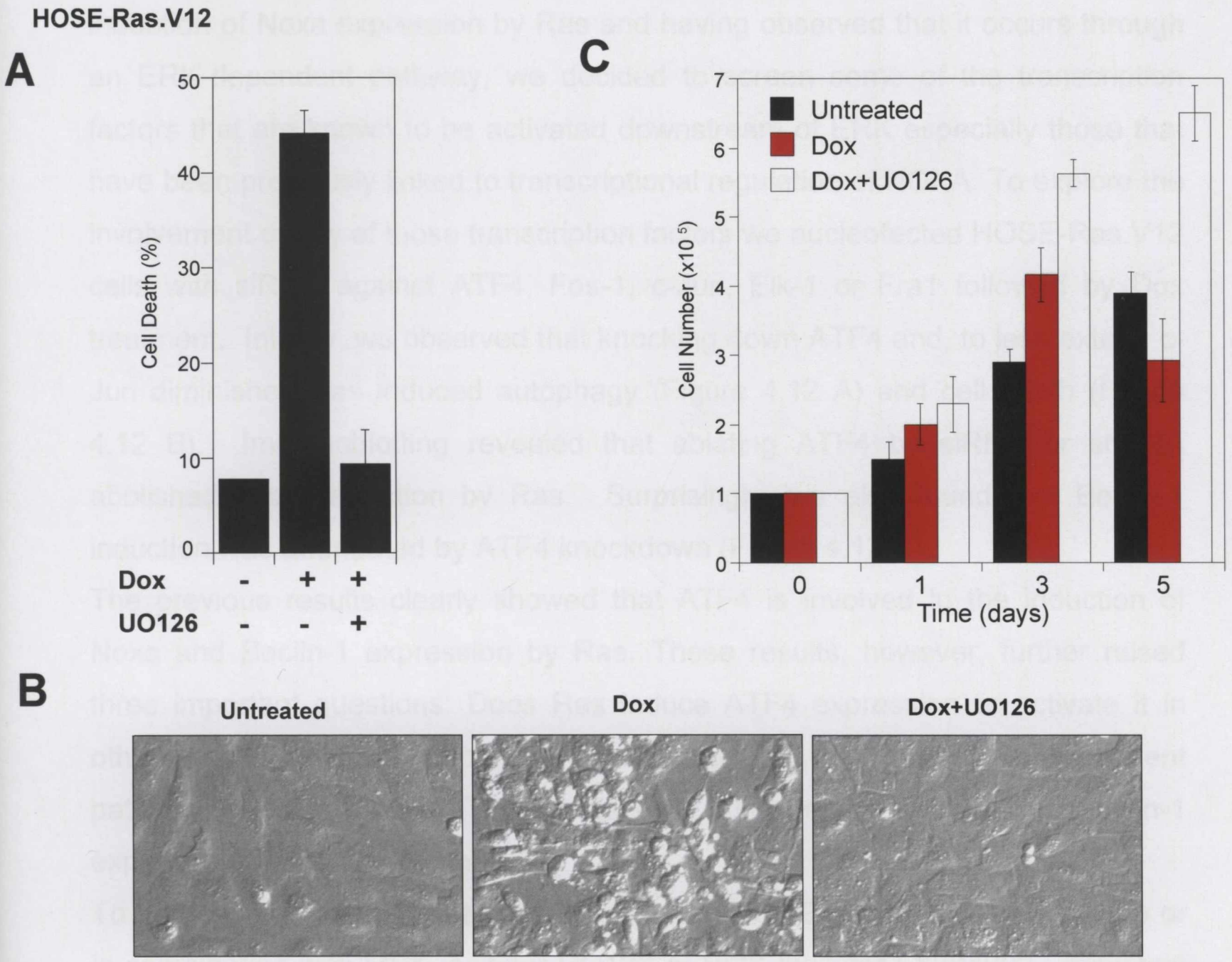


Figure 4.11 Inhibition of MEK/ERK pathway abolishes Ras-induced cell death

HOSE.RasV12 cells were either left untreated or were treated with Dox (100 ng/ml) either alone or in combination with MEK inhibitor UO126. Medium was replenished every 2-3 days. Cell death in each condition was enumerated on day 7 days after Dox treatment using PI uptake assay (A) and pictures were taken on an Olympus light microscope with 20X magnification. Cells treated as above were counted at the indicated time points (C)

4.2.8 Involvement of ATF4 in the induction of Noxa and Beclin-1 expression downstream of ERK

Having found no role for p53, the well described Noxa inducer, in mediating the induction of Noxa expression by Ras and having observed that it occurs through an ERK-dependent pathway, we decided to screen some of the transcription factors that are known to be activated downstream of ERK especially those that have been previously linked to transcriptional regulation of NOXA. To explore the involvement of any of those transcription factors we nucleofected HOSE-Ras.V12 cells with siRNA against ATF4, Fos-1, c-Jun, Elk-1 or Fra1 followed by Dox treatment. Initially, we observed that knocking down ATF4 and, to less extent, c-Jun diminished Ras induced autophagy (Figure 4.12 A) and cell death (Figure 4.12 B). Immunoblotting revealed that ablating ATF4 by siRNA or shRNA abolished Noxa induction by Ras. Surprisingly, we also found that Beclin-1 induction was attenuated by ATF4 knockdown (Figure 4.12 C).

The previous results clearly showed that ATF4 is involved in the induction of Noxa and Beclin-1 expression by Ras. These results, however, further raised three important questions: Does Ras induce ATF4 expression or activate it in other ways? And if that is found to be the case, is it through an ERK-dependent pathway? And finally is ATF4 on its own sufficient to induce Noxa/Beclin-1 expression and/or cell death?

To address the first two questions, we treated HOSE-RasV12 with Dox alone or in combination with MEK inhibitor UO126 or PI3K inhibitor LY294002. Induction of H-Ras.V12 expression by Dox induced ATF4 expression. Co-treatment with UO126 completely abolished the induction of ATF4 by Ras. Co-treatment with LY294002 however did not modulate ATF4 induction (Figure 4.13 A). This suggests that Ras induces ATF4 expression through an ERK pathway. This is consistent with the previous observations that showed that Noxa and Beclin-1 are induced downstream of ERK. To test whether ATF4 was sufficient alone to induce the expression of Noxa or Beclin-1, ATF4 or ATF3, as control, were overexpressed in HOSE cells. Immunoblotting showed that ATF4 expression alone, but not ATF3, was sufficient to profoundly induce the expression of Noxa.

Beclin-1 induction, however, was not as profound (Figure 4.13 B). Other co-operating factor(s) might therefore be required for the induction of Beclin-1 by ATF4. Finally, overexpression of ATF4 induced massive cell death in HOSE cells, which suggests that ATF4 might indeed be playing an important role in Ras-induced cell death (Figure 4.13 C).

4.2.9 A possible link between Ras-induced autophagy and ER stress

Interestingly, the implication of ATF4 in the induction of Noxa and Beclin-1 by ERK suggested a possible link between ER stress and Ras-induced autophagy and autophagic cell death. ATF4 is known to be a key regulator of ER stress. We thus decided to examine if Ras induces any other of the classical markers of ER stress. Immunoblotting of lysates from HOSE-Ras.V12 cells treated with dox or left untreated over a time course of seven days showed that induction of Ras.V12 expression correlated with time-dependent induction of the expression of ATF4, and Grp78 and the phosphorylation of eIF2 alpha, all typical markers of ER stress (Figure 4.14 A). These results further hint to a role for ER stress in mediating Ras-induced autophagic cell death.

To further explore this possibility, we knocked down another major regulator of ER stress, ATF6. Like ATF4, knockdown of ATF6 inhibited Ras-induced autophagy (Figure 4.14 B) and cell death (Figure 4.14 C). Taken altogether these results suggest a possible link between ER stress and Ras-induced autophagy and cell death.

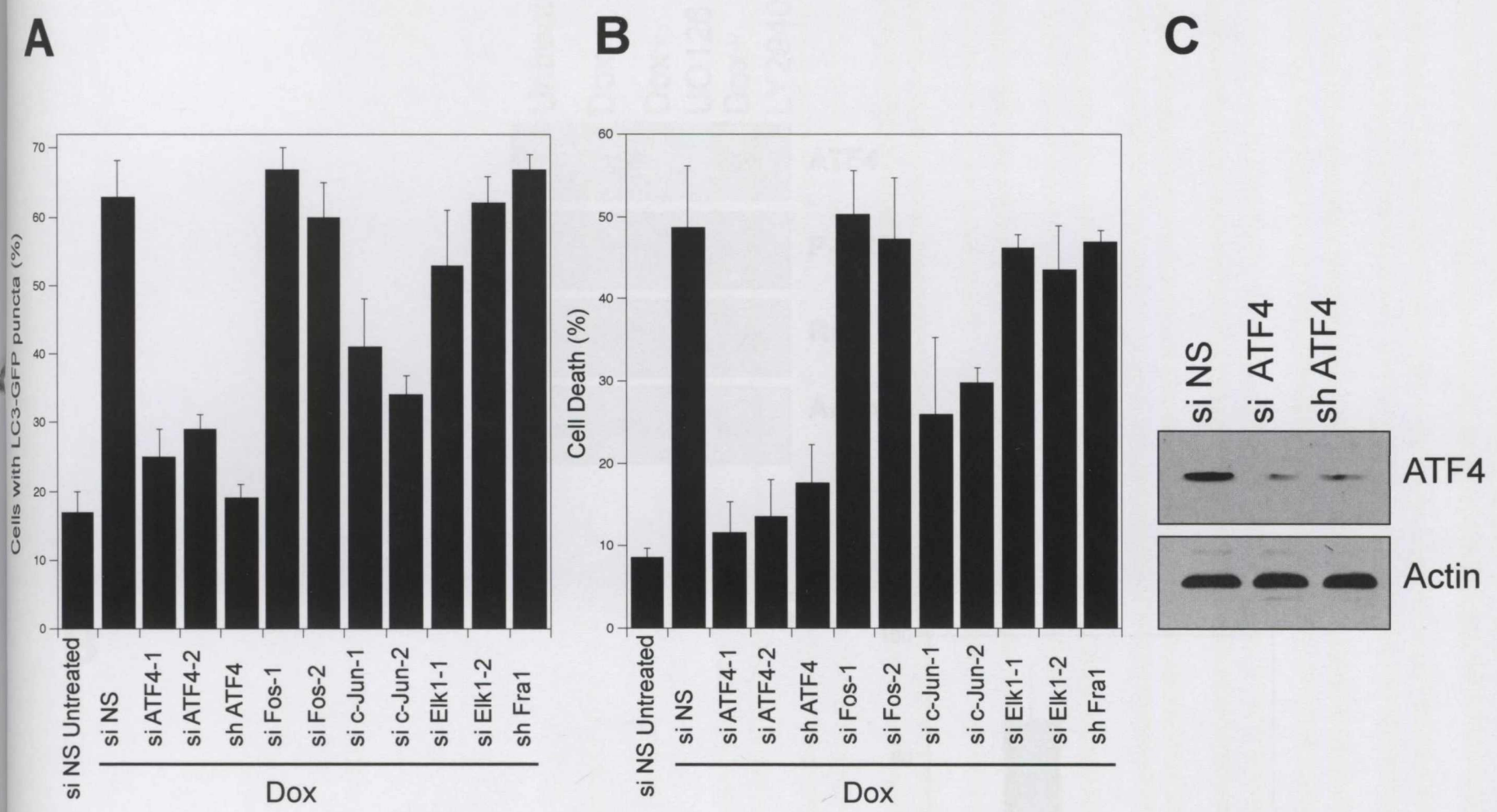
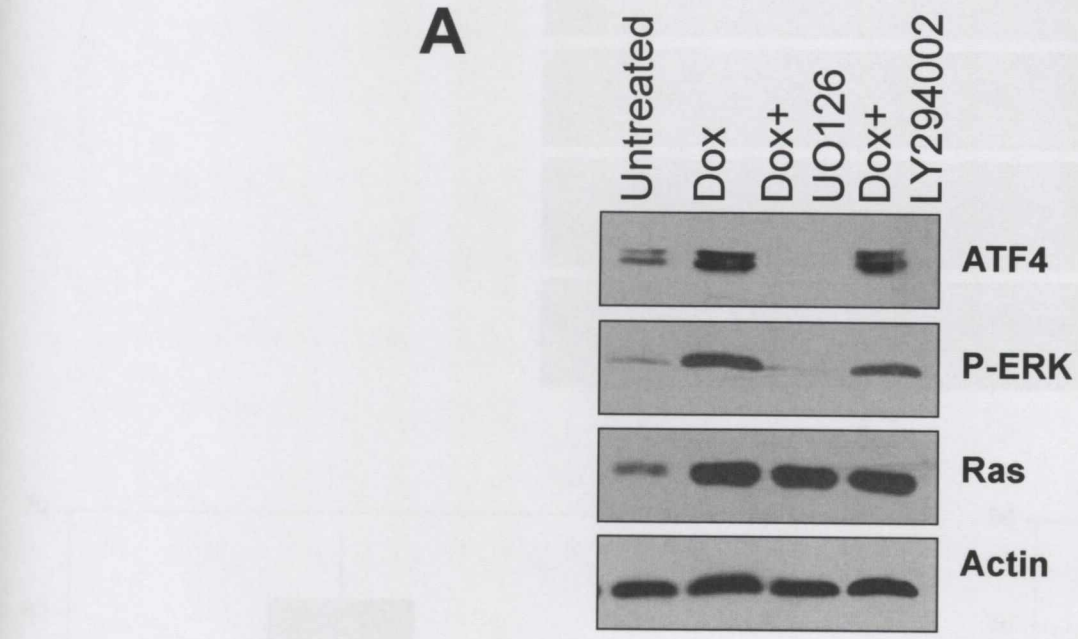
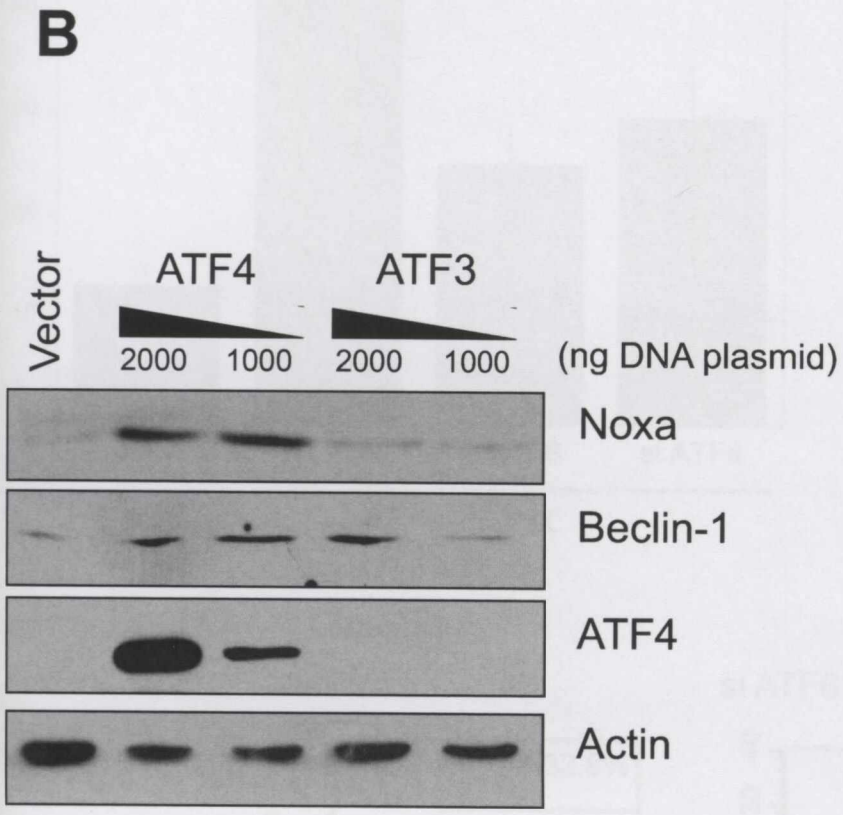


Figure 4.12 ATF4 mediates Ras-induced autophagy and expression of Noxa and Beclin-1

HOSE.RasV12 cells were nucleofected with the indicated siRNA's (200nM). 48h later, cells were treated with Dox (100ng/ml). Percentage of cells exhbiting LC3-GFP puncta (A) and cell death (B) in each condition was scored 5 and 7 days respectively after Dox treament. Efficiency of silencing ATF4 was confirmed by immunoblotting of lysates prepared from HOSE.RasV12 nucleofected as indicated (C). HOSE.Ras.V12 cells nucleofected with the indicated siRNA's and treated with Dox (100ng/ml) for 5 days were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (D).





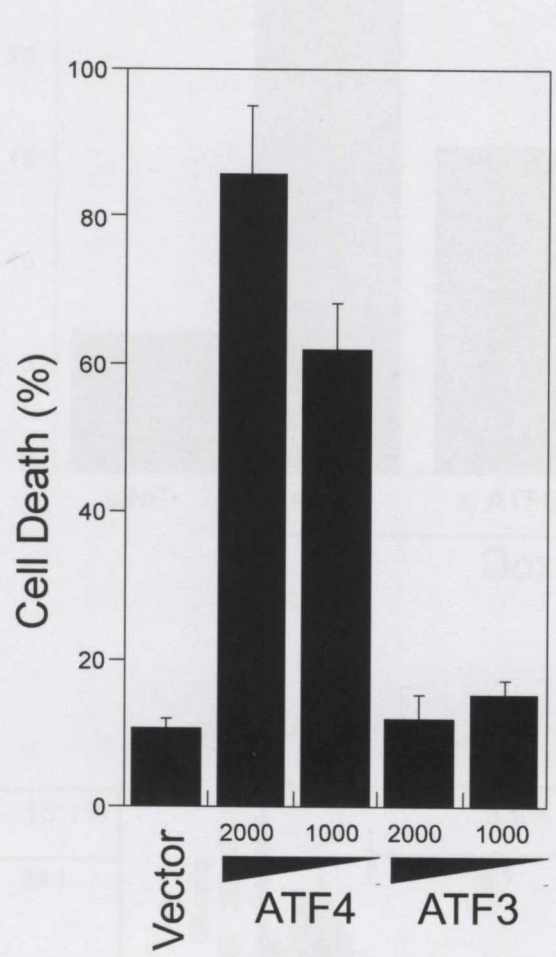
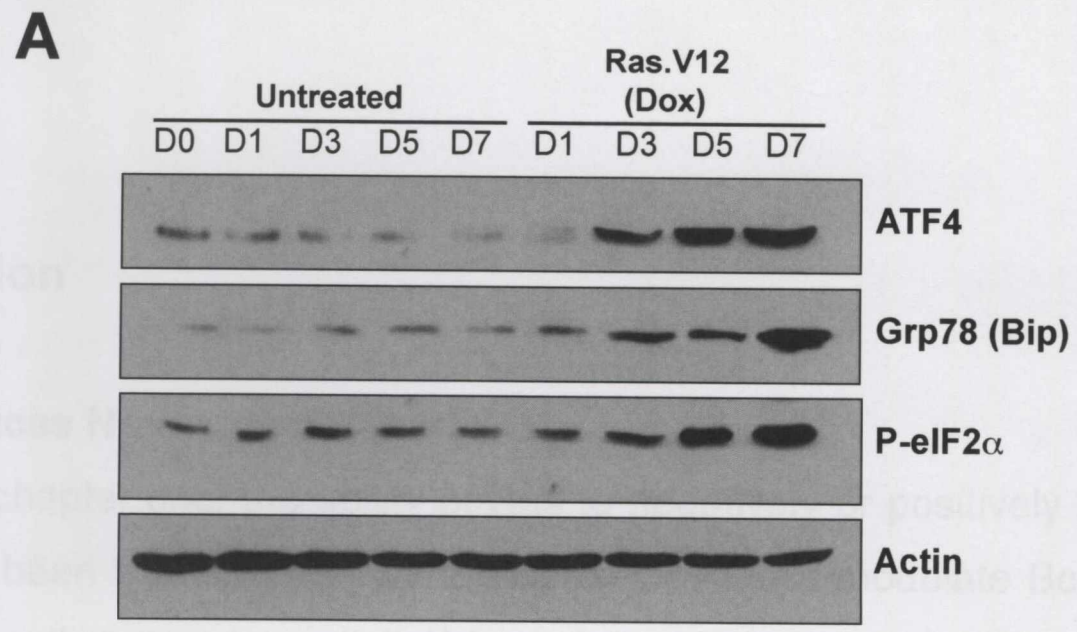
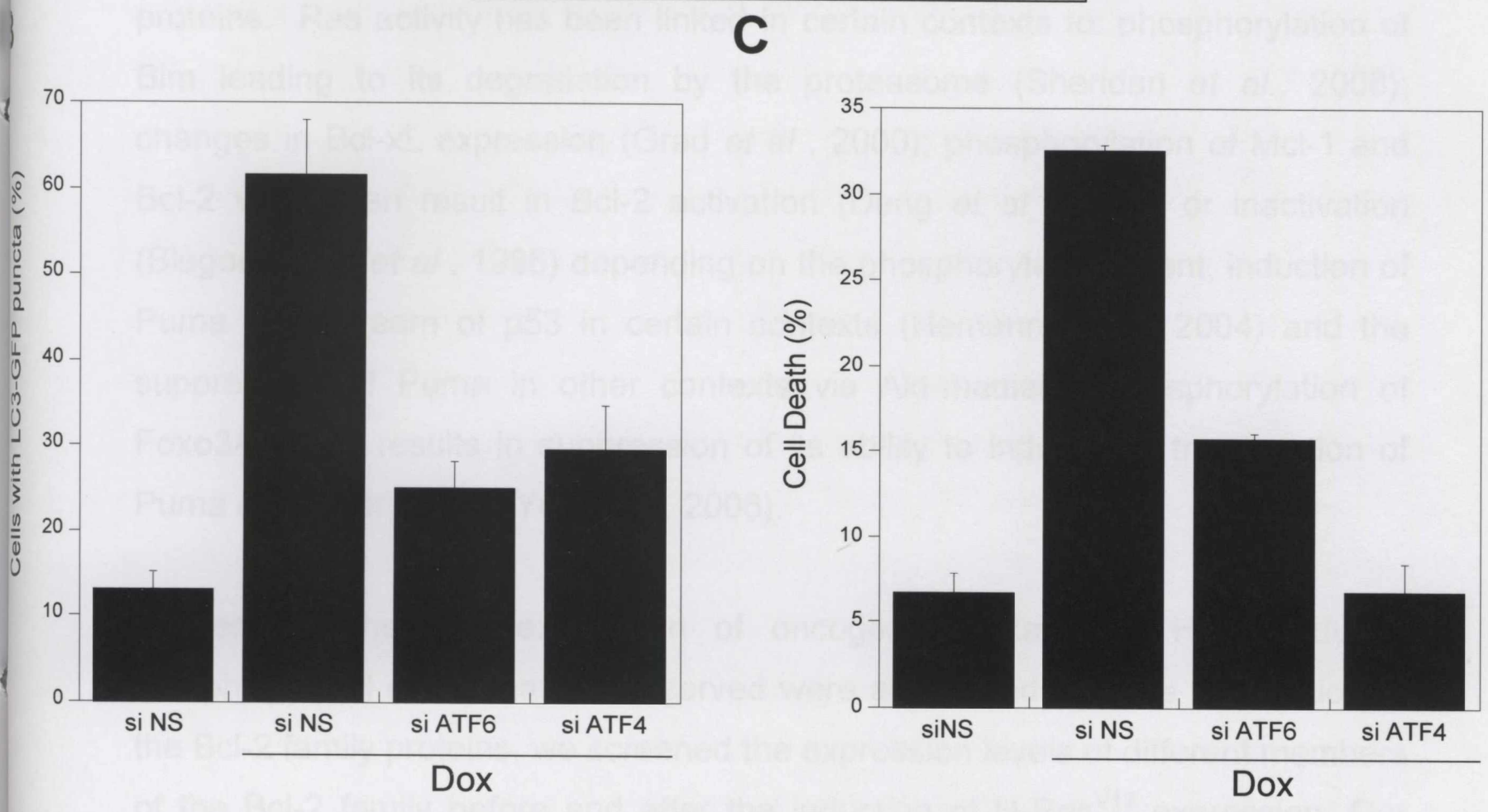


Figure 4.13 The regulation of Noxa expression by ATF4 downstream of ERK

HOSE.RasV12 cells were either left untreated or were treated with 100 ng/ml Dox either alone or in combination with MEK inhibitor UO126 (20μM) or PI3K inhibitor LY294002 (20μM). Three days later, cells were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies.

HOSE.RasV12 cells were nucleofected with the indicated plasmids. 48h later, samples of cells were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (B). Cell death in HOSE.RasV12 cells nucleofected with the indicated plasmids was assessed by PI uptake assay 48 hr after nucleofection (C).





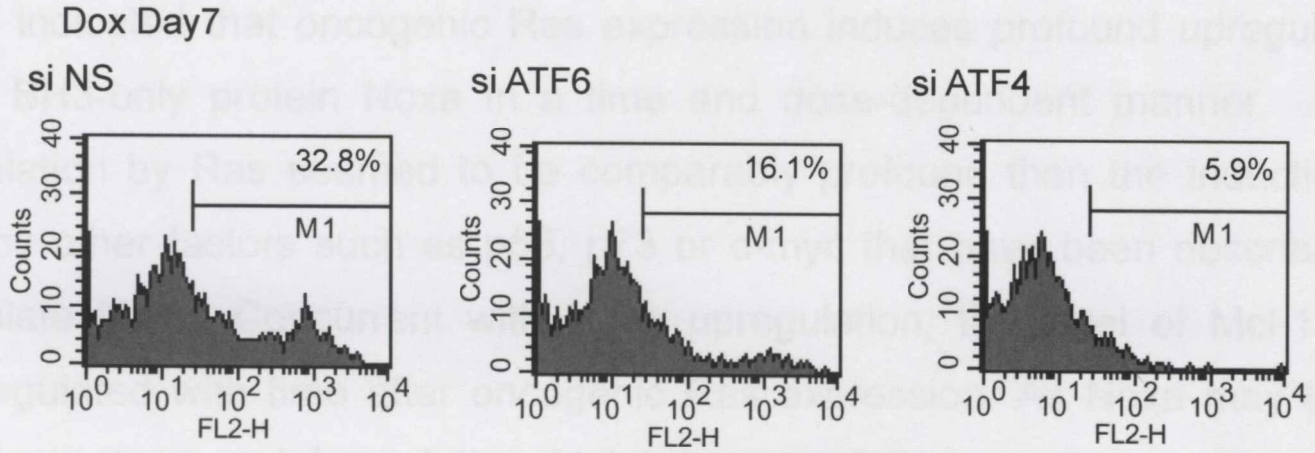


Figure 4.14 Ras induced autophagy is linked to ER stress

HOSE.RasV12 cells either left untreated or treated with Dox (100 ng/ml) a time course of 7 days were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (A). HOSE.RasV12 cells were nucleofected with the indicated siRNA's (200nM). 48h later, cells were treated with Dox (100ng/ml). Percentage of cells exhbiting LC3-GFP puncta (B) and cell death (C) in each condition was scored 5 and 7 days respectively after Dox treament.

4.3 Discussion

4.3.1 Ras induces Noxa upregulation

As outlined in chapter one, the ability of Ras to negatively or positively modulate cell death has been linked in part by the ability of Ras to modulate Bcl-2 family proteins. Ras activity has been linked in certain contexts to: phosphorylation of Bim leading to its degradation by the proteasome (Sheridan *et al.*, 2008); changes in Bcl-xL expression (Grad *et al* , 2000); phosphorylation of Mcl-1 and Bcl-2 which can result in Bcl-2 activation (Deng *et al* , 2000) or inactivation (Blagosklonny *et al* , 1996) depending on the phosphorylation event; induction of Puma downstream of p53 in certain contexts (Hemann *et al.*, 2004) and the suppression of Puma in other contexts via Akt-mediated phosphorylation of Foxo3A which results in suppression of its ability to induce the transcription of Puma and other genes (You *et al.*, 2006).

To test whether the expression of oncogenic H-Ras and H-Ras-induced autophagic cell death that we observed were associated with the modulation of the Bcl-2 family proteins, we screened the expression levels of different members of the Bcl-2 family before and after the induction of H-Ras^{V12} expression. Our results indicated that oncogenic Ras expression induces profound upregulation of the BH3-only protein Noxa in a time and dose-dependent manner. Noxa upregulation by Ras seemed to be comparably profound than the induction of Noxa by other factors such as p53, p73 or c-myc that have been described to upregulate Noxa. Concurrent with Noxa upregulation, the level of Mcl-1 was downregulated with time after oncogenic Ras expression. As Noxa may target Mcl-1 for proteasomal degradation, Mcl-1 downregulation may be a consequence of Noxa upregulation.

4.3.2 Noxa mediates Ras-induced non-apoptotic cell death

Besides their role in apoptosis, BH3-only proteins such as Puma (Yee *et al.*, 2009) Bad (Maiuri *et al.*, 2007) and Bik (Rashmi *et al.*, 2008) have been linked to

the induction of autophagy and autophagic cell death under the conditions when apoptosis is blocked for some other reasons. Bad has been shown to induce autophagy under starvation conditions by competitively disrupt the interaction between Bcl-xL and Beclin-1.

Here we described the novel observation that Noxa, in co-operation with Beclin-1, mediate autophagy and non-apoptotic cell death in response to oncogenic Ras activation.

Our results demonstrate the involvement of Noxa in mediating Ras-induced autophagy and cell death as Noxa knockdown inhibited both Ras-induced autophagy and autophagic cell death. Noxa is known to be a weaker inducer of apoptosis among the BH3-only proteins which suggests that Noxa may be playing a role in other cellular processes. While Noxa is associated with induction of apoptosis in certain contexts e.g. proteasome inhibitors, Noxa upregulation downstream of Ras did not elicit an apoptotic response perhaps because apoptosis is blocked by a panel of anti-apoptotic signals induced by Ras (reviewed in chapter one). With apoptosis pathways blocked, Noxa may be mediating the switch to another mode of non-apoptotic programmed cell death (autophagic cell death) and hereby represents a switch key that is able to finely tune the decision between apoptosis and autophagic cell death.

The interplay between apoptosis and autophagy has been a matter of debate. It has been suggested that autophagic cell death may represent an alternative means to cell demolition under the conditions when the classical apoptosis is blocked. Bim is an essential mediator of apoptosis. Our results showed that induction of oncogenic Ras expression was accompanied by downregulation of Bim. Bim phosphorylation and subsequent degradation in response to ERK activation has been described before (Sheridan *et al.*, 2008) and has been shown to confer resistance against apoptosis (Gillings *et al.*, 2009). Many oncogenes evoke arrays of signals to pro-survival signals to block apoptosis and enhance clonogenic survival. It is thus tempting to assume that Ras-induced Bim downregulation, possibly via the MEK/ERK pathway, may represent one of the

mechanisms elicited by oncogenic Ras to impede the apoptotic machinery. Blockage of apoptosis by Bim downregulation may contribute to the cell's decision to switch to an alternative form of cell death. Such decision may be finely tuned by Noxa. It would thus be interesting to see if in other contexts Noxa overexpression combined with the ablation of Bim or other essential apoptosis regulators such as Bad to impede apoptosis could drive the cell towards the alternative autophagic cell death. This however remains to be tested.

Ras-induced autophagy and cell death was essentially blocked by all anti-apoptotic Bcl-2 proteins tested (except Bcl-B). Nevertheless, Mcl-1 and A1, which are known to interact with Noxa, had the most efficient inhibitory effect on Ras-induced cell death confirming a particularly important role for Noxa in mediating Ras-induced autophagic cell death

4.3.3 Noxa limits Ras-induced transformation

Earlier studies have failed to establish a direct link between deregulated Noxa expression and tumor development. This study points out for the first time for an important role for Noxa in limiting cell transformation induced by the proto-oncogene Ras. Noxa is highly upregulated in response to oncogenic Ras expression and we found that Noxa plays an important role in evoking an autophagic cell death response as Noxa knockdown diminished Ras-induced cell death and more importantly imparted clonogenic survival advantages to cells expressing oncogenic Ras when subjected to clonogenic survival challenge. We found that while Control HOSE-Ras^{V12} cells formed colonies, Dox-treated HOSE-Ras^{V12} cells expressing Ras^{V12} succumbed to either autophagic cell death or senescence and thus were unable to proliferate and form colonies. Ablation of Noxa in cells expressing H-Ras^{V12} diminished the cell death response and allowed cells to proliferate and form more colonies. This strongly suggests that Noxa upregulation in response to oncogenic Ras expression constitutes an obstacle against Ras-induced transformation and may be a part of innate tumor

suppression responses triggered by deregulated Ras expression that must be overcome in order for cells to develop complete tumorigenic potential.

Notably, Puma knockdown similar to Noxa knockdown, but maybe less efficiently, diminished Ras-induced autophagy and cell death and enhanced long term clonogenic survival maybe. These results suggested a role of Puma in counteracting Ras-induced transformation. However unlike Noxa, which is clearly modulated by Ras, the levels of Puma seemed to be induced only slightly. It is however still possible that Puma gets activated by Ras through another way than the induction of expression such as phosphorylation or could exert its action by binding to certain effectors. These possibilities remain to be examined. It would also be interesting to test whether concomitant perturbation of Noxa and Puma expression could exert a synergistic effect to combat Ras-induced transformation. This could be tested by ablating the expression of both Noxa and Puma either individually or simultaneously by transfecting the cells with a combination of shRNAs or siRNAs against Noxa and Puma followed by the induction of oncogenic Ras expression and clonogenic survival challenge. Comparing the number of colonies in the case Noxa knockdown or Puma knockdown alone or when both were combined would give further insight on whether the two proteins co-operate in limiting Ras-induced transformation.

4.3.4 Regulation of the induction of Noxa expression by Ras

Noxa was first identified in a screening for p53 targets that show increased transcription in response to DNA damage. *NOXA* was initially described as a p53-responsive gene and was suggested to act as a mediator of p53-induced apoptosis (Oda *et al.*, 2000). . Oda and co-workers showed that X-ray irradiation increased Noxa mRNA in wild type MEFs but not in p53-deficient MEFs. Moreover, ectopic expression of p53 resulted in increased expression of Noxa mRNA in p53-deficient MEFs and SAOS-2 cells. Additionally, p53 responsive element was identified in NOXA promoter 195 bp upstream of the transcriptional start site. Although other p53-indpendent mechanisms of *NOXA* regulation have

since been described, p53 is still regarded as the "classical" regulator of *NOXA* expression. Thus, we initially asked whether p53 plays a role in the induction of Noxa expression by Ras. To this end we tested whether p53 knockdown by shRNA or the use of p53 dominant negative construct can attenuate Ras induction of Noxa. In addition, we also tested the induction of Noxa by Ras in p53 null cells SAOS-2. Surprisingly, we could not find any correlation between p53 and the induction of Noxa expression by Ras. p53 knockdown or dominant negative construct did not modulate the induction of Noxa to any extent and Noxa was induced by Ras in the p53-deficient cells SAOS-2. These results ruled out a role for p53 in this process and suggested that Ras regulates Noxa expression through a p53-independent pathway.

To further explore the transcriptional regulation of Noxa downstream of Ras, we next examined the effect of inhibition of different pathways activated downstream of Ras on Noxa induction. We found that inhibition of MEK/ERK pathway almost completely abolished induction of Noxa expression upon Ras activation while inhibition of other pathways did not have any effect. Furthermore, Noxa was differentially induced by different Ras isoforms in a closely correlated manner to the extent of ERK activation. Together, these results confirmed the involvement of ERK in the regulation of NOXA induction and assured any concerns that may arise from questioning the specificity of the pharmacological inhibitors.

Having established a role for ERK in this process, we then looked at the transcription factors that are known to be activated downstream of ERK to see if any of them is involved in the regulation of Noxa. Candidate transcription factors that we tested included c-Fos, c-Jun, Elk-1 and Fra1 and ATF4.
c-Fos, c-Jun and Fra1 are members of the activating protein-1 (AP-1) family of transcription factors. Activation of the AP-1 family is one of the earliest nuclear events induced by growth factors that stimulate ERK. ERK phosphorylates multiple residues within the carboxylterminal transactivation domain (TAD) of c-Fos, thus resulting in its increased transcriptional activity (Monje *et al.*, 2005).

ERK has also been shown to phosphorylate c-Jun on Ser63 and Ser73, and Thr91 and/or Thr93 within the trans-activation domain. Phosphorylation of these residues results in the stabilization of c-Jun, as well as enhanced trans-activation and DNA-binding activity (Vinciguerra *et al.*, 2004). Fra1 is a homologue of c-Fos that has also been shown to be phosphorylated by ERK on Thre231 leading to its activation (Young *et al.*, 2002).

Elk-1 is an extracellular signal-regulated Ets-domain transcription factor that has been shown to be another substrate of ERK. Elk-1 is a member of the Ternary Complex Factors (TCFs) family that includes Elk-1, SAP-1 and NET/SAP-2. Elk-1 has a C-terminal transcriptional activation domain with multiple copies of the MAPK core consensus sequence S/T-P. This region is phosphorylated by MAP kinases in vitro and in vivo. Peptide mapping studies have established that Elk-1 residues S324, T336, S383, S389 and S422 can be phosphorylated following growth factor stimulation in vivo and by ERK2 in vitro (Francisco *et al.*, 1999).

ATF4 is a member of the activating transcription factor family (ATF). Members of the ATF family, including AFT4, can each form homodimers as well as heterodimers. They can also interact with members of the AP-1 family of proteins. ATF4 transiently activates a wide range of downstream targets involved in cellular transport, metabolism and other functions. ATF4 level is rapidly elevated in response to the many conditions, including the amino acid response (AAR) following protein or amino acid limitation and the unfolded protein response (UPR) following endoplasmic reticulum stress, that lead to phosphorylation of eIF2α. There are two short upstream open reading frames (uORFs) within the 5'-leader of the ATF4 mRNA. Under no stress conditions, after translation of uORF1, sufficient eIF2-GTP makes it possible to reinitiate translation from the uORF2, which is out of frame with ATF4, and therefore ATF4 synthesis is minimized. Conversely, stress-induced p-eIF2α leads to limited eIF2-GTP and prolongs the duration for the scanning ribosome to reinitiate following uORF1. Consequently, uORF2 is skipped, and initiation at the ATF4 coding region is increased (Lu *et al.*, 2004). Recently, ATF4 has been suggested to be a direct target of ERK signalling (Thiaville *et al.*, 2008). In another recent

report, ATF4 was shown to regulate the transcription of Noxa. Wang and co-workers showed that ATF4 together with ATF3 form a complex that binds to the NOXA promoter and induces its transcription indicating for the first time the involvement of ATF4 and ATF3 in NOXA transcriptional regulation. The regulation of NOXA expression by ATF4 further supported other observations the linked Noxa to ER stress.

In order to test whether any of these transcription factors activated downstream of ERK is involved in Ras-induced Noxa and/or Beclin-1 upregulation, we silenced the expression of each of these factors and studied the effect of that on the induction of Noxa and Beclin-1 by Ras. Strikingly, we found that silencing ATF4 expression almost completely abolished the induction of Noxa as well as Beclin-1 by Ras. Consistent with these results, we found that Ras induced ATF4 expression in an ERK-dependent manner. We next asked whether ATF4 on its own is sufficient to induce the expression of Noxa or Beclin-1 or it requires another binding partner. We found that ectopic expression of ATF4 on its own was sufficient to markedly induce Noxa expression. However ATF4 overexpression did not seem to have the same profound effect on the induction of Beclin-1 expression suggesting that ATF4 may require another factor to induce Beclin-1 expression. Interestingly, we also observed that ATF4 overexpression induced cell death which further supports previous reports that suggested a pro-death effect for ATF4 (Ohoka *et al.*, 2005; Lange *et al.*, 2008)

Our finding that ATF4 regulates NOXA expression downstream of oncogenic Ras provide further evidence about a tight link between ER stress and the regulation of Noxa expression and function. Noxa has been implicated in mediating cell death triggered by anti-cancer drugs that induce ER stress such as Eayarestatin I (Wang *et al.*, 2009), bortezomib (Nawrocki., 2008), fenretinide (Armstrong *et al.*, 2007) as well as the classical ER stress inducers tunicamycin, brefeldin A and thapsigargin (Jianze *et al.*, 2005).

4.3.5 ER stress and Ras-induced autophagy are linked

The endoplasmic reticulum (ER) is an organelle with crucial biosynthetic and signalling functions in eukaryotic cells. The ER is not only the major intracellular calcium storage organelle critically involved in calcium homeostasis and calcium mediated signalling pathways, but it also provides the environment for the synthesis, folding, and modification of proteins destined to be secreted or embedded in the plasma membrane. Various physiological and pathological conditions, including hypoxia, ER-calcium depletion, oxidative injury, high-fat diet, hypoglycemia, and viral infections may cause an imbalance between ER protein folding load and capacity, leading to the accumulation of unfolded proteins in the ER lumen, a condition referred to as "ER stress". ER stress sets in motion an evolutionary conserved and integrated signal transduction pathway known as the Unfolded Protein Response (UPR). The UPR primarily aims at ameliorating the protein load on the ER by coordinating the temporal shut down in protein translation along with a complex program of gene transcription to increase ER folding capacity. In order to clear the ER from the accumulation of terminally misfolded protein aggregates that cannot be degraded by the proteasome, the UPR may upregulate the autophagy machinery. Autophagy is a mechanism of protein degradation. It is thus not surprising that ER stress and autophagy are linked. Direct evidence for a link between both processes came from the observation that polyglutamine (polyQ) proteins-induced autophagy was mediated by ER stress. Kouroku and co-workers showed that a dominant-negative form of PERK or genetic substitution of Serine 51 of eIF2α by Ala (which prevents the phosphorylation of this protein) prevented polyQ protein-induced autophagy suggesting that PERK-dependent eIF2α phosphorylation plays an important role in the activation of autophagy in response to the accumulation of unfolded proteins. PERK-eIF2α-dependent Atg12 upregulation was further shown to be required for induction of autophagy in response to polyQ protein accumulation which suggests that controlling the expression of autophagy-related genes by eIF2α downstream targets could be one of the mechanisms connecting both events. Furthermore, ATF4 and its downstream

target CHOP have been shown to induce the transcription of the essential autophagy genes LC3 and Atg5 under hypoxic conditions (Rouschop *et al.*, 2010; Rzymski *et al.*, 2010). A further link between autophagy and ER stress comes from the reports that clearly demonstrated that autophagy counterbalances ER expansion during unfolded protein response (Bernales *et al.*, 2006). A recent report dissected the origin and source of autophagosomal membranes using electron microscopy and demonstrated that ER interconnect with early autophagic structures called isolation membranes (Hayashi-Nishino *et al.*, 2009) We have observed that ATF4 was induced by Ras in an ERK-dependent manner. In addition, we have found a key role for ATF4 in mediating Ras-induced autophagy through regulating the expression of Noxa and Beclin-1. Given the established role for ATF4 in ER stress signalling, an intriguing question emerged whether ER stress plays a role in mediating Ras-induced autophagy and autophagic cell death. To approach this issue, we first wanted to examine whether ATF4 plays this role independently or as a component of an orchestrated universal ER stress response triggered by oncogenic Ras. We found that expression of oncogenic H-Ras correlated with the induction of typical markers of ER stress such as Grp78 (Bip) upregulation and phosphorylation of eI2Falpha besides the induction of ATF4 expression indicating that Ras triggers a general ER stress. These results are in line with a previous report that pointed to the induction of ER stress by Ras (Denoyelle *et al.*, 2006). Next, to examine whether ER stress mediates Ras induced autophagy and cell death, we knocked down two essential regulators of ER stress ATF4 and ATF6. Silencing these genes impeded Ras induced autophagy and greatly diminished autophagic cell death upon oncogenic H-Ras expression. Taken together, these results strongly suggest a possible link between ER stress and Ras-induced autophagy in which ER stress may lie downstream of Ras and upstream of autophagy and cell death.

4.3.6 Anti-proliferative roles of the ERK pathway

We have shown that H-Ras induces both Beclin-1 and Noxa through an ERK-dependent pathway. Consistent with the essential role both proteins play in

mediating Ras-induced autophagy and cell death, inhibition of the MEK/ERK pathway allowed the cells expressing oncogenic H-Ras to evade cell death and to proceed to complete transformation manifested by aggressive proliferation rate. These results suggest that MEK/ERK pathway may mediate the activation of some pathways that serve to restrain Ras-induced transformation. These observations are in line with the previous reports that suggested that PI3K/Akt pathway is the main pathway that drives transformation downstream of Ras (Weiguan *et al.*, 2004, Carola *et al.*, 2007). This is important to consider when designing an approach to target Ras for therapeutic intervention.

Chapter V

Regulation of Oncogenic H-Ras- Induced Autophagy and Cell Death by Bcl-2 Family Proteins

5.1 Introduction

The Bcl-2 family of proteins can be divided into anti-apoptotic multi-domain proteins, which contain four BH domains, pro-apoptotic multi-domain proteins, which contain three BH domains, and the pro-apoptotic BH3-only protein family, which contain only one BH domain.

As discussed in chapter four, we found that the BH-3 protein Noxa is essentially required for the induction of autophagy and the subsequent cell death upon expression of oncogenic Ras. The ability of the pro-apoptotic Noxa to mediate autophagy and a non-apoptotic form of cell death is intriguing and raises the question about the mechanism by which Noxa mediates autophagy and autophagy-associated cell death. To approach this question it is important to view Noxa as a member of the big family of Bcl-2 proteins that engage in multiple protein-protein interactions among each other and with other proteins and their stoichiometry is therefore what ultimately determines the response to a certain stimulus whether to survive or die.

The role of the Bcl-2 family proteins in the regulation of apoptosis is well established and has been a subject of intensive study. In the recent years, another important function of the Bcl-2 family proteins has emerged and attracted ongoing investigations. The discovery of the essential autophagy regulator Beclin-1 in a screening for Bcl-2 binding partners and the subsequent identification of a BH3 domain in Beclin-1 led to the interesting findings of the role of the Bcl-2 family in the regulation of autophagy. The anti-apoptotic Bcl-2 and Bcl-xL have been shown to physically interact with Beclin-1 leading to the inhibition of autophagy. The dissociation of Beclin-1 from its Bcl-2 family binders is thus essential for its autophagic activity. In the light of these reports a plausible explanation for the role of Noxa in mediating autophagy is that Noxa could competitively bind and disrupt the interaction between the anti-apoptotic members of the Bcl-2 family proteins and Beclin-1. In this chapter we set out to test this speculation and to further investigate the role of the Bcl-2 family members in autophagy.

5.2 Results

5.2.1 Anti-apoptotic members of the Bcl-2 family inhibit Beclin1-driven autophagy

As discussed earlier, previous reports have shown that the anti-apoptotic members of the Bcl-2 family can inhibit autophagy by binding Beclin-1. Bcl-2 was the first member of the family to be shown to bind and inhibit Beclin-1 (Pattingre *et al.*, 2005). Later on, Bcl-xL was also shown to bind Beclin-1 (Maiuri *et al.*, 2007). To recapitulate these results, we made use of a system previously described by Pattingre and co-workers. In this system, Beclin-1 overexpression drives autophagy in MCF-7 cells starved for 6 hours (Pattingre *el al*, 2005).

We overexpressed the anti-apoptotic Bcl-2 family proteins Bcl-2, Bcl-xL or Mcl-1 along with Beclin-1 in MCF7 cell followed by starvation for 4-6 hours. We found that Beclin-1 alone induced autophagy under these conditions as assessed by the percentage of cells exhibiting LC3-GFP puncta. Co-transfection with anti-apoptotic Bcl-2 proteins dramatically inhibited Beclin-1 driven autophagy (Figure 5.1 A). Our results are thus in agreement with the previous studies that showed that Bcl-2 and Bcl-xL inhibit Beclin-1. Furthermore, our results initially suggested that Mcl-1 too was capable to exert the same inhibitory effect on Beclin-1 function. To further confirm this observation, we transfected MCF-7 cells with Beclin-1 alone or in combination with titrated doses of Mcl-1. Twenty-four hours were allowed for full expression of the proteins then the cells were starved for 6 hours and the percentage of cells undergoing autophagy, as assessed by GFP-LC3 puncta, was scored. Similar to Bcl-2 and Bcl-xL, Mcl-1 exerted a very strong inhibitory effect on Beclin1-driven autophagy even in relatively low ratios of Mcl-1 to Beclin-1 encoding plasmids (Figure 5.1 B).

5.2.2 Noxa alleviates the inhibitory effect of Mcl-1 on autophagy

Noxa exhibits a limited binding affinity to anti-apoptotic members of the Bcl-2 family. Noxa binds almost exclusively to Mcl-1 and A1 (Ploner *et al.*, 2009) and probably, with weaker affinity, to Bcl-xL as reported recently (Lopez *et al.*, 2010;

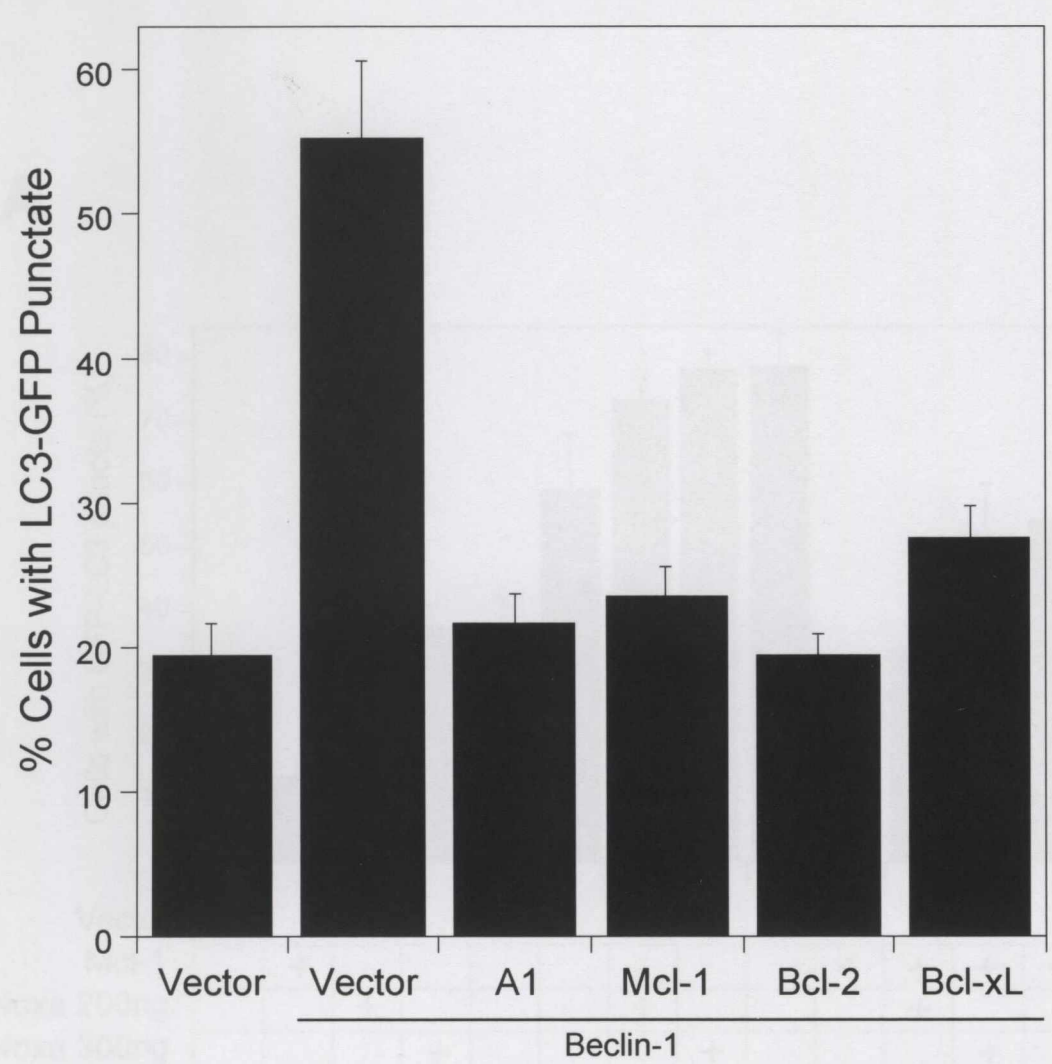
Hagenbuchner *et al.*, 2010). Having observed an efficient inhibitory effect for Mcl-1 on Beclin1-driven autophagy, we next wished to test if co-expression of Noxa with Mcl-1 can modulate this inhibitory effect to any extent. Using the same system described above, we found that co-expression of Noxa with Mcl-1 restored the ability of Beclin-1 to drive autophagy (Figure 5.2 A and B) possibly by binding to Mcl-1 and thus relieving the inhibitory effect of Mcl-1 on Beclin-1-driven autophagy.

5.2.3 Ras-induced autophagy and autophagic cell death can be blocked by anti-apoptotic members of the Bcl-2 family

As discussed in chapter three, Beclin-1 plays an essential role in mediating Ras-induced autophagy. Having observed a strong inhibitory effect for the anti-apoptotic members of the Bcl-2 family on Beclin1-driven autophagy, we next sought to examine whether the anti-apoptotic Bcl-2 family proteins can also inhibit Ras-induced autophagy and autophagic cell death. To this end, various anti-apoptotic members of the Bcl-2 family were overexpressed in HOSE-Ras^{V12} cells followed by treatment with or without Dox. The effect of different Bcl-2 family proteins on Ras-induced autophagy was assessed by scoring the percentage of cells exhibiting autophagic vacuolation (Figure 5.3 A) or GFP-LC3 puncta (Figure 5.3 B). Overexpression of A1, Mcl-1, Bcl-2 and Bcl-xL and, to a much lesser extent, Bcl-B inhibited Ras-induced autophagy.

Next, we examined the effect of Bcl-2 proteins on Ras-induced cell death. Consistent with their inhibitory effect on Ras-induced autophagy, anti-apoptotic members of the Bcl-2 family A1, Mcl1, Bcl-2 and Bcl-xL, but not Bcl-B, blocked Ras-induced cell death (Figure 5.4 A and B). Of note here, although A1, Mcl-1, Bcl-2 and Bcl-xL were all able to modulate Ras-induced cell death, A1 and Mcl-1 were the most efficient inhibitors of Ras-induced cell death among them. The inhibitory effect on Ras-induced autophagy and cell death conferred by the expression of A1, Mcl-1, Bcl-2 and Bcl-xL resulted in enhancement of the long term clonogenic survival of HOSE. Ras^{V12} cells expressing oncogenic H-Ras^{V12}

MCF-7 cells



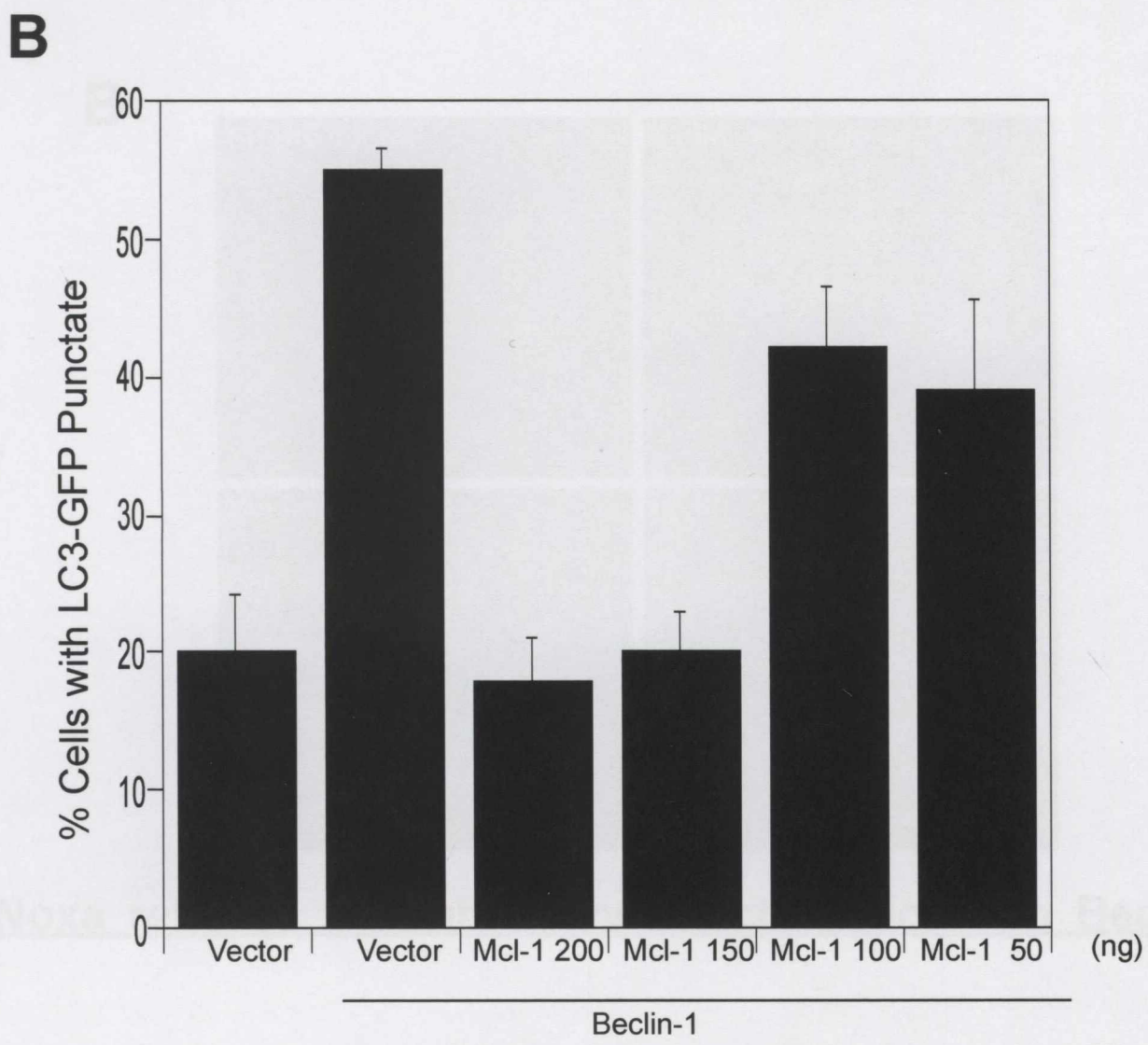
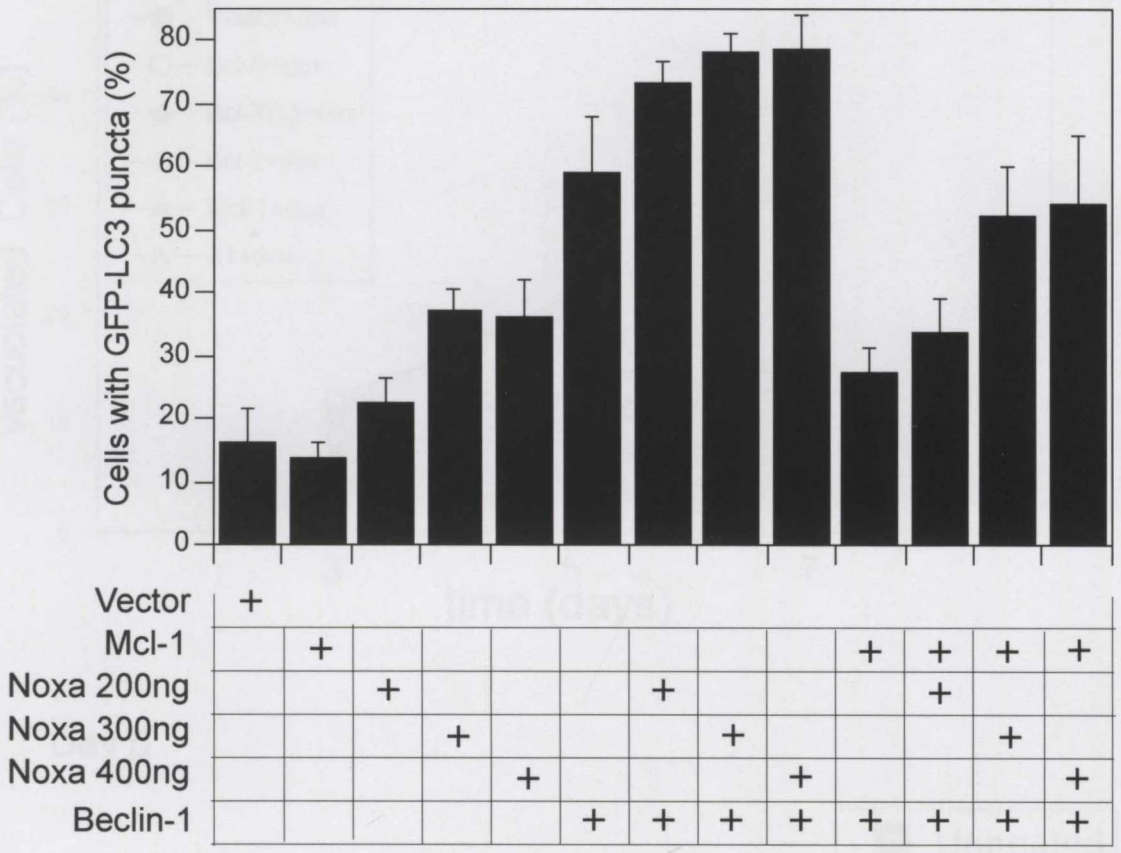


Figure 5.1 Anti-apoptotic Bcl-2 proteins inhibit Beclin1-driven autophagy

MCF-7 cells were nucleofected with the indicated plasmid combinations using Amaxa nucleofection system (Program T20). Cells were staved for 4-6 h in Earle's balanced salt solution (EBSS) medium and the percentage of cells exhibiting LC3-GFP punctate in each treatment was enumerated.

A



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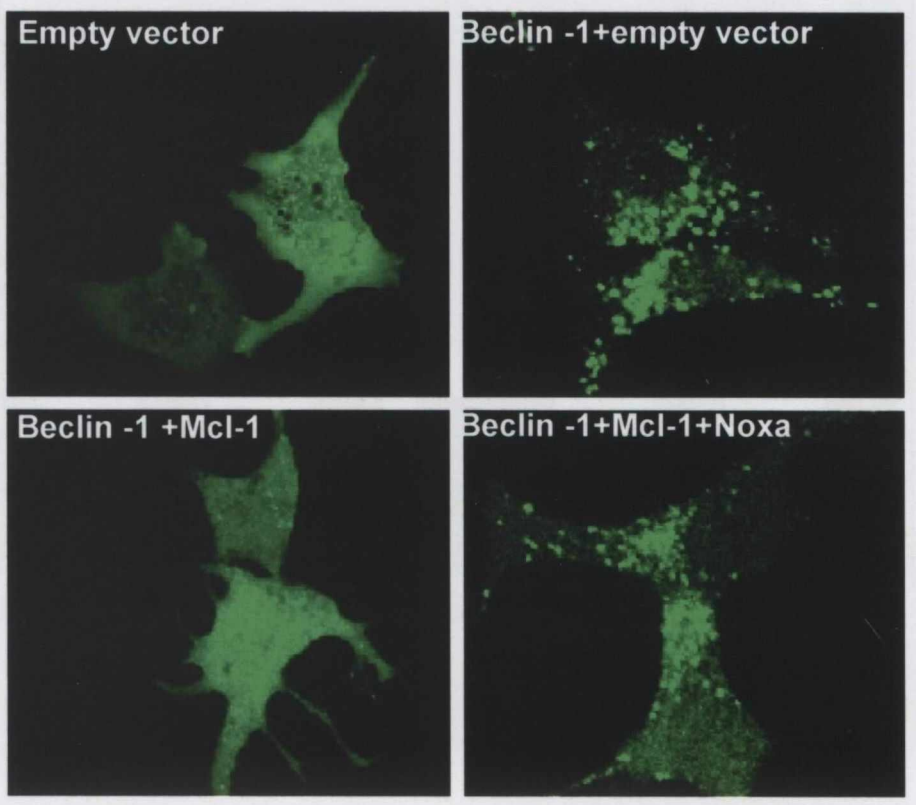
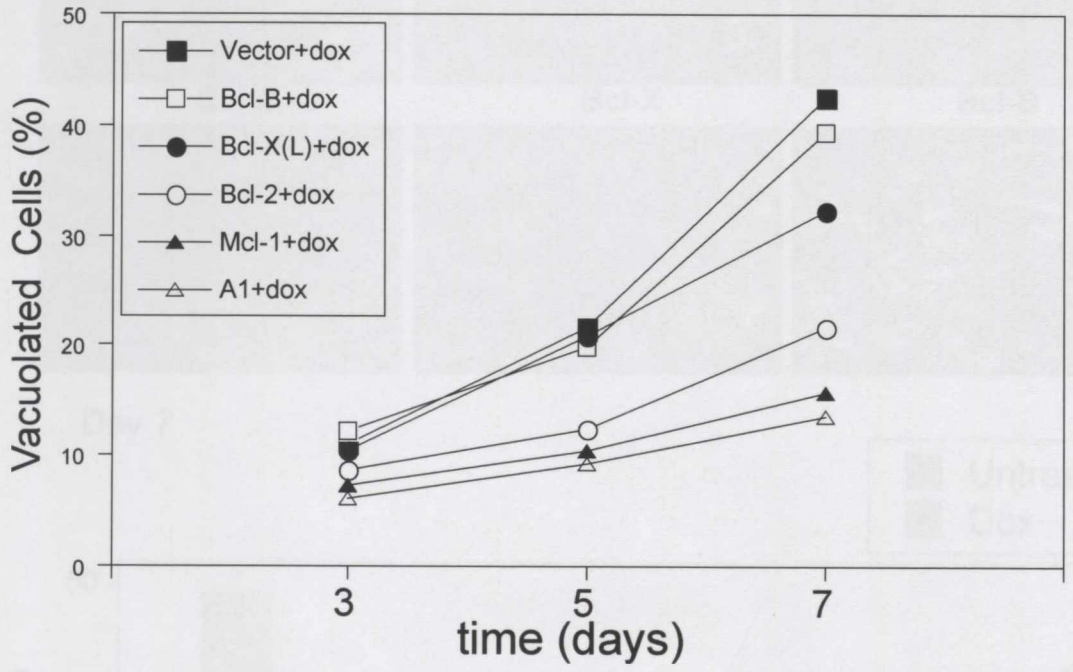


Figure 5.2 Noxa relieves the inhibitory effect of Mcl-1 on Beclin1-driven autophagy

MCF-7 cells were nuceloefected with the indicated plasmid combinations using Amaxa nucleofection system (Program T20). Cells were staved for 4-6 h in Earle's balanced salt solution (EBSS) medium and the percentage of cells exhibiting LC3-GFP punctate in each treatment was enumerated (A). Pictures were taken on a confocal microscope with 40X magnification (B).

A



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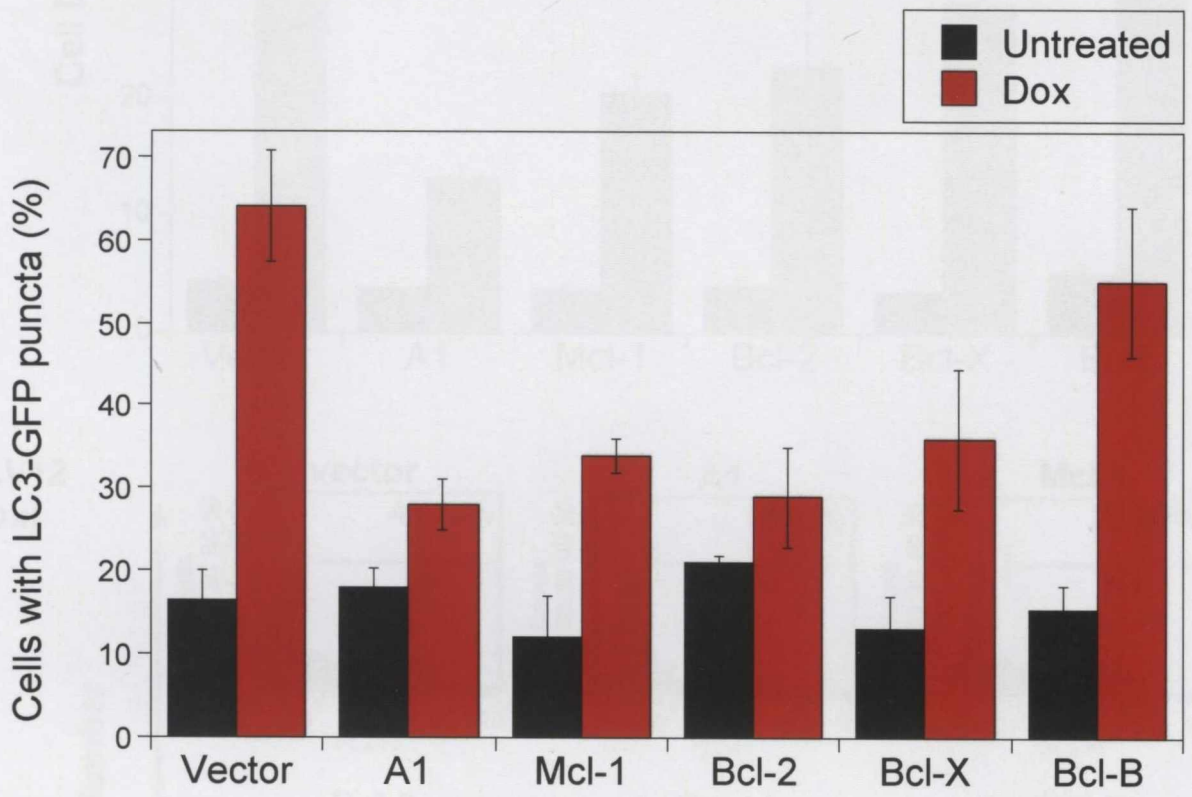


Figure 5.3 Anti-apoptotic Bcl-2 family proteins inhibit Ras-induced autophagy

HOSE.RasV12 cells were nucleofected with various members of the Bcl-2 family proteins using Amaxa nucleofection system (program T20). Cells were allowed 72 h for full expression followed by treatment with or without 100 ng/ml Dox for the indiacted time points. The percentage of cells exhbiting autophagic vacuolation (A) or LC3-GFP puncta (B) was assessed at the indiacted time points.

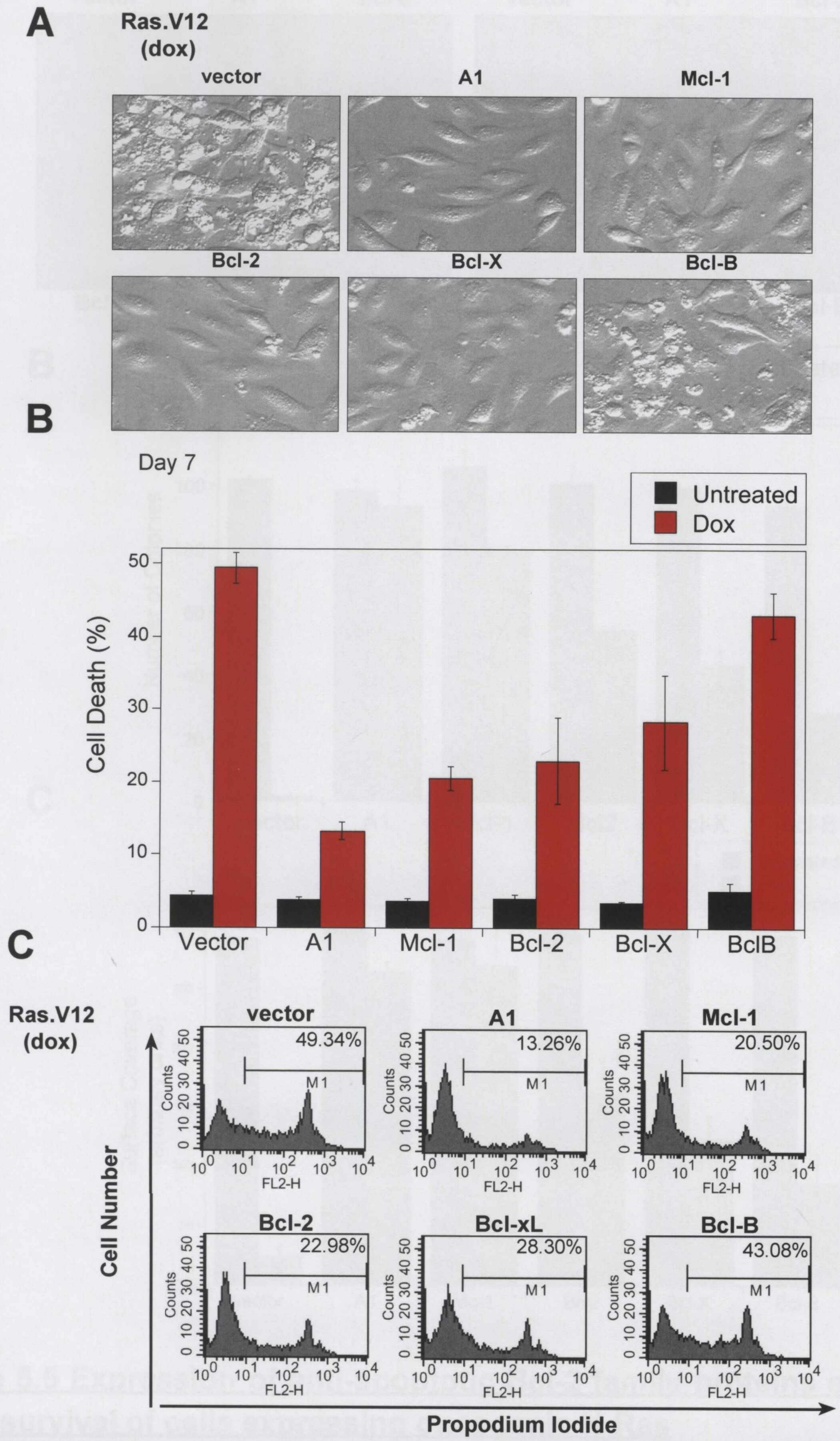


Figure 5.4 Anti-apoptotic Bcl-2 family proteins diminish Ras-induced cell death

HOSE.RasV12 cells were nucleofected with various members of Bcl-2 family proteins.Cells were allowed 72 h for full expression followed by treatment with or without 100 ng/ml Dox for 7 days. Pictures were taken on a light microscope with 40X magnification (A). 7 days after Dox treatment, cells were harvested and the percentage of cell death in each condition was assessed by flow cytometer analysis of PI uptake (B). The percentage of PI-positive cells in each treatment is indicated in the top right-hand corner of each panel (C).

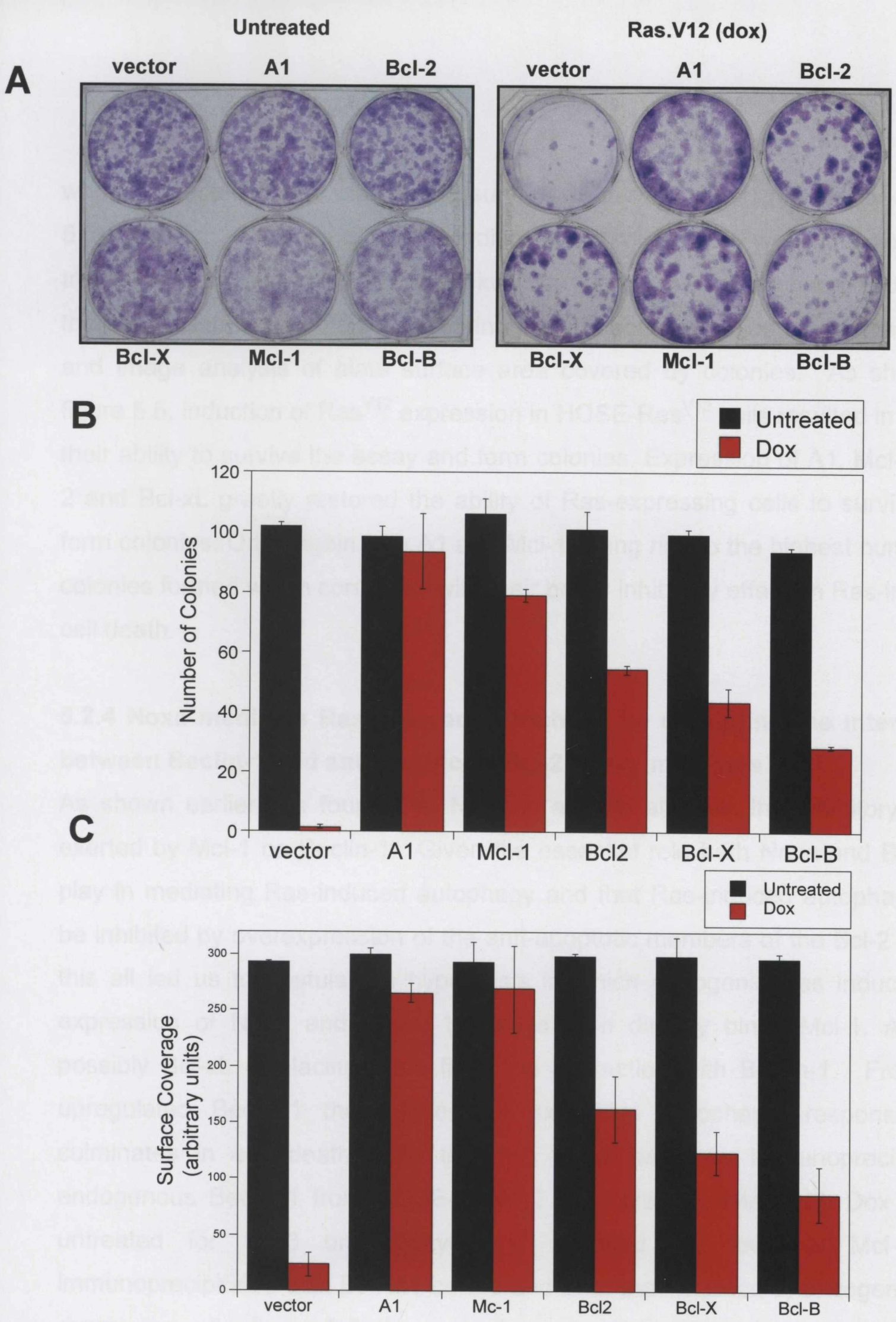


Figure 5.5 Expression of anti-apoptotic Bcl-2 family proteins enhances clonogenic survival of cells expressing oncogenic H-Ras

HOSE.RasV12 cells were nucleofected with various members of Bcl-2 family preteins. Cells were allowed 48 h for full expression followed by treatment with or without 100 ng/ml Dox for 3 days. Cells were then harvested by trypsinization, counted and 2000 cells/ well were plated in 6 well plates. Dox-treatment was continued for 3 weeks. Colonies were stained with 0.5% crystal violet in 20% methanol and pictures were taken with Canon G9 digital camera (A) and colonies bigger than 1mm were counted (B). Ratio of well surface covered by colonies in each treatment was analyzed by image analysis of plates using ImageJ software (C).

when subjected to the clonogenic survival assay described previously (Figure 5.5). Briefly, 2000 cells of each condition were seeded in 6-well plate and were treated with or without Dox for 3 weeks. Formed colonies in each condition were then assessed by two methods: scoring the number of colonies bigger than 1mm and image analysis of plate surface area covered by colonies. As shown in figure 5.5, induction of Ras^{V12} expression in HOSE-Ras^{V12} cells resulted in loss of their ability to survive the assay and form colonies. Expression of A1, Mcl-1, Bcl-2 and Bcl-xL greatly restored the ability of Ras-expressing cells to survive and form colonies. Once again with A1 and Mcl-1 giving rise to the highest number of colonies formed which correlates with their better inhibitory effect on Ras-induced cell death.

5.2.4 Noxa mediates Ras-induced autophagy by disrupting the interaction between Beclin-1 and anti-apoptotic Bcl-2 family members

As shown earlier, we found that Noxa is able to alleviate the inhibitory effect exerted by Mcl-1 on Beclin-1. Given the essential role both Noxa and Beclin-1 play in mediating Ras-induced autophagy and that Ras-induced autophagy can be inhibited by overexpression of the anti-apoptotic members of the Bcl-2 family, this all led us to postulate a hypothesis in which oncogenic Ras induces the expression of Noxa and Beclin-1. Noxa then directly binds Mcl-1, A1 and possibly Bcl-xL displacing them from the interaction with Beclin-1. Free and upregulated Beclin-1 then drives an excessive autophagic response that culminates in cell death. To test this hypothesis, we immunoprecipitated endogenous Beclin-1 from HOSE-RasV12 cells either treated with Dox or left untreated for 1, 3 or 5 days and checked the level of Mcl-1 co-immunoprecipitated with Beclin1 before and after the induction of oncogenic Ras expression. As figure 5.6 shows, in the untreated cells Mcl-1 was readily co-immunoprecipitated with Beclin-1. There was no apparent difference in the amount of Mcl-1 co-immunoprecipitated with Beclin-1 over the time course of 5 days in untreated cells. Conversely, on day 5 after the induction of Oncogenic Ras expression by Dox-treatment we detected significantly less amount of Mcl-1

bound to Beclin-1. The reduced binding between Mcl-1 and Beclin-1 on day 5 post Dox treatment is consistent with the observation that the total level of Mcl-1 expression in those cells is downregulated at that time point after Ras.V12 induction. This may explain, at least in part, why Beclin-1 binds less Mcl-1 despite the total level of Beclin-1 is upregulated at this time point.

To further confirm this result, endogenous Mcl-1 was immunoprecipitated from the cells treated as above. Consistent with the results obtained from endogenous Beclin-1 immunoprecipitation experiment, we detected less Beclin-1 bound to Mcl-1 at day 5 post induction of oncogenic Ras expression. Conversely, we detected increasing amounts of Noxa bound to Mcl-1 after H-Ras^{V12} induction. This is consistent with the greater levels of Noxa being expressed by the cells after H-Ras^{V12} induction (Figure 5.6).

Interestingly, we also found that Bcl-xL is displaced from Beclin-1 after Dox treatment. It is very likely that enhanced binding of Noxa to Mcl-1 and the downregulation of Mcl-1 contribute to the displacement of the latter from interaction with Beclin-1. It, however, remains elusive to determine whether Noxa plays a role in the displacement of Bcl-xL from Beclin-1 as Noxa binds Bcl-xL with weak affinity (Lopez *et al.*, 2010; Hagenbuchner *et al.*, 2010). This weak interaction between Noxa and Bcl-xL might still be sufficient to displace Bcl-xL from Beclin1.

Notably, the observed displacement of Beclin-1 from Mcl-1 and Bcl-xL was clearly evident at day 5 post induction of Ras.V12 expression which is about the time point that significant number of cells start to die at, suggesting that the two events are indeed correlated.

5.2.5 Noxa drives Mcl-1 downregulation upon oncogenic Ras expression

In HOSE-Ras^{V12} cells, Noxa upregulation in response to H-Ras^{V12} expression was concurrent with downregulation of Mcl-1 (Figure 4.1). Binding of Noxa to Mcl-1 has been shown to trigger the degradation of the latter by the proteasome (Ploner *et al.*, 2009). This led us to ask whether elevated level of Noxa plays a role in the downregulation of Mcl-1 upon Ras^{V12} induction. To address this

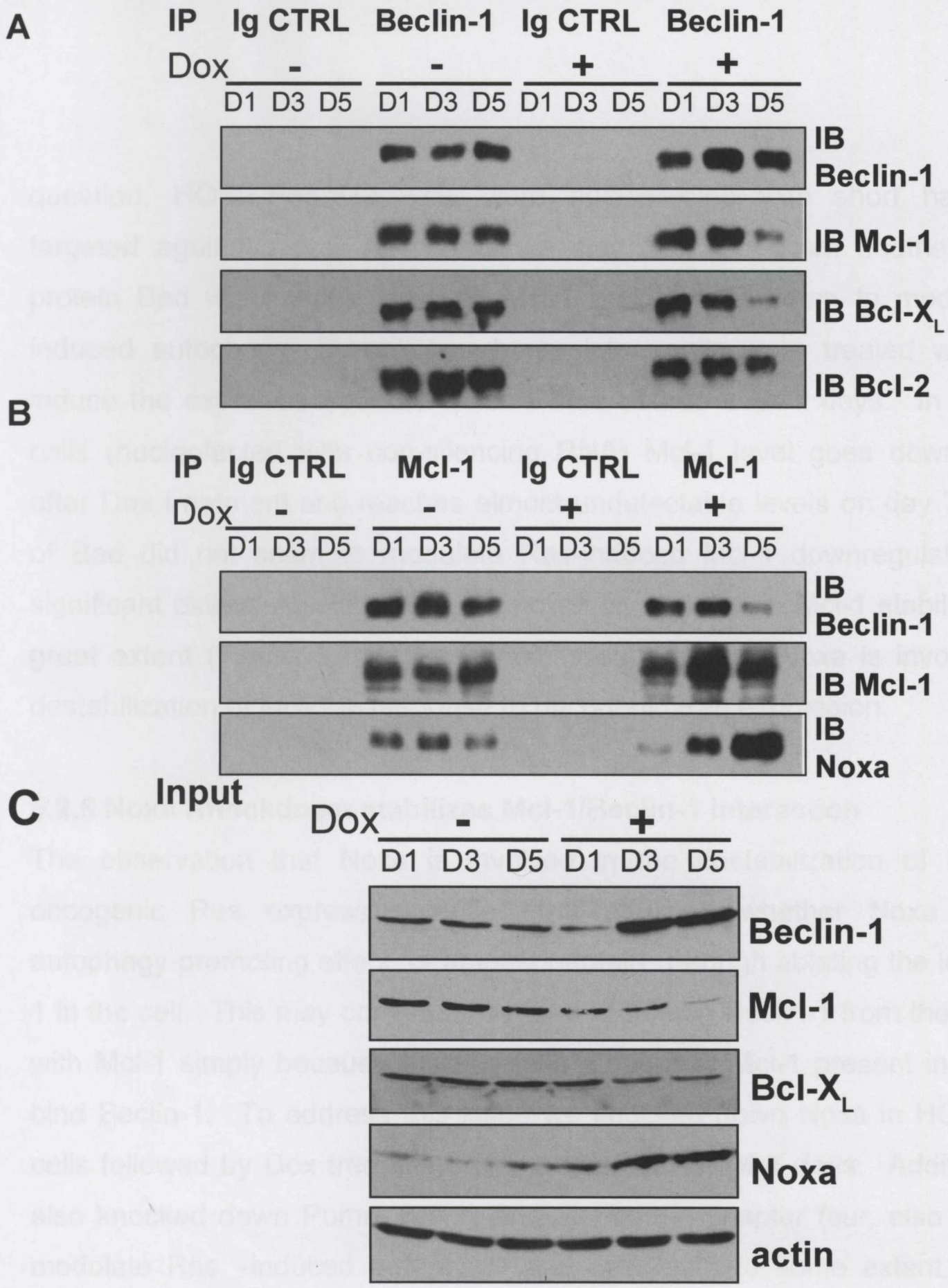


Figure 5.6 Noxa mediates Ras-induced autophagy by disrupting the interaction between Mcl-1 and Beclin-1

HOSE.RasV12 cells treated with or without Dox for 1, 3 or 5 days were harvested by trpsinization, washed in ice cold PBS pH7.2 and lysed in 800 µl per $1x10^7$ cells of IP lysis buffer. Cell lysates were precleared by incubation for 2 hours with pre-immuned serum and 20 µl protein G agarose beads under constant rotation at 4 °C. Beads were precipitated by centrifuging at 2000g for 2 min at 4°C. Supernatants from each treatment were isolated and incubated overnight with 1 µg of Beclin-1 (A) or Mcl-1 (B) or a pre-immuned serum as control and 20 µl protein G agarose under constant rotation at 4 °C. Beads were washed 5 times in ice cold lysis buffer containing 0.1% NP-40. Beads were finally collected by centrifugation at 5000g for 2 min and brought up in SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies. 10% v/v of the total cell lysates used in the IP's above were analyzed by Western immunoblotting for the protein levels with the indicated antibodies.

question, HOSE-Ras.V12 cells were nucleofected with short hairpin RNA targeted against Noxa. As control we also knocked down another BH3-only protein Bad which does not bind Mcl-1 and did not seem to modulate Ras-induced autophagy. Seventy-two hours later, cells were treated with Dox to induce the expression of Ras^{V12} for a time course over 7 days. In the control cells (nucleofected with non-silencing RNA) Mcl-1 level goes down gradually after Dox treatment and reaches almost undetectable levels on day 7. Ablation of Bad did not seem to modulate Ras-induced Mcl-1 downregulation to any significant extent. Ablation of Noxa, however, resulted in Mcl-1 stabilization to a great extent (Figure 5.7). These results suggest that Noxa is involved in the destabilization of Mcl-1 in response to oncogenic Ras expression.

5.2.6 Noxa Knockdown stabilizes Mcl-1/Beclin-1 interaction

The observation that Noxa is involved in the destabilization of Mcl-1 after oncogenic Ras expression raised the question whether Noxa exerts its autophagy-promoting effect, or at least partially, through ablating the level of Mcl-1 in the cell. This may consequently lead to freeing Beclin-1 from the interaction with Mcl-1 simply because there is less amount of Mcl-1 present in the cell to bind Beclin-1. To address this issue we knocked down Noxa in HOSE-Ras^{V12} cells followed by Dox treatment over a time course of 7 days. Additionally, we also knocked down Puma, which as described in chapter four, also seemed to modulate Ras –induced autophagy and cell death to some extent. We then immunopricipitated endogenous Beclin-1 in those cells and compared the levels of Mcl-1 pulled down in each treatment to control cells nucleofected with non-silencing shRNA and treated with or without Dox over the same time course. As observed before, Beclin-1 binds readily to Mcl-1 in untreated cells. However, in cells nucleofected with control shRNA and treated with Dox, the level of Beclin-1 bound to less Mcl-1 greatly diminished five to seven days after Ras^{V12} induction. Noxa knockdown in Dox-treated cells seemed to increase the binding of Beclin-1 to Mcl-1, which is consistent with its effect on Mcl-1 stabilization (Figure 5.7). Interestingly, Puma knockdown in Dox-treated cells also seemed to enhance the

binding between Beclin-1 and Mcl-1 but to a lesser extent than Noxa knockdown. This might help explain the effect of Puma knockdown on Ras-induced autophagy that we discussed earlier.

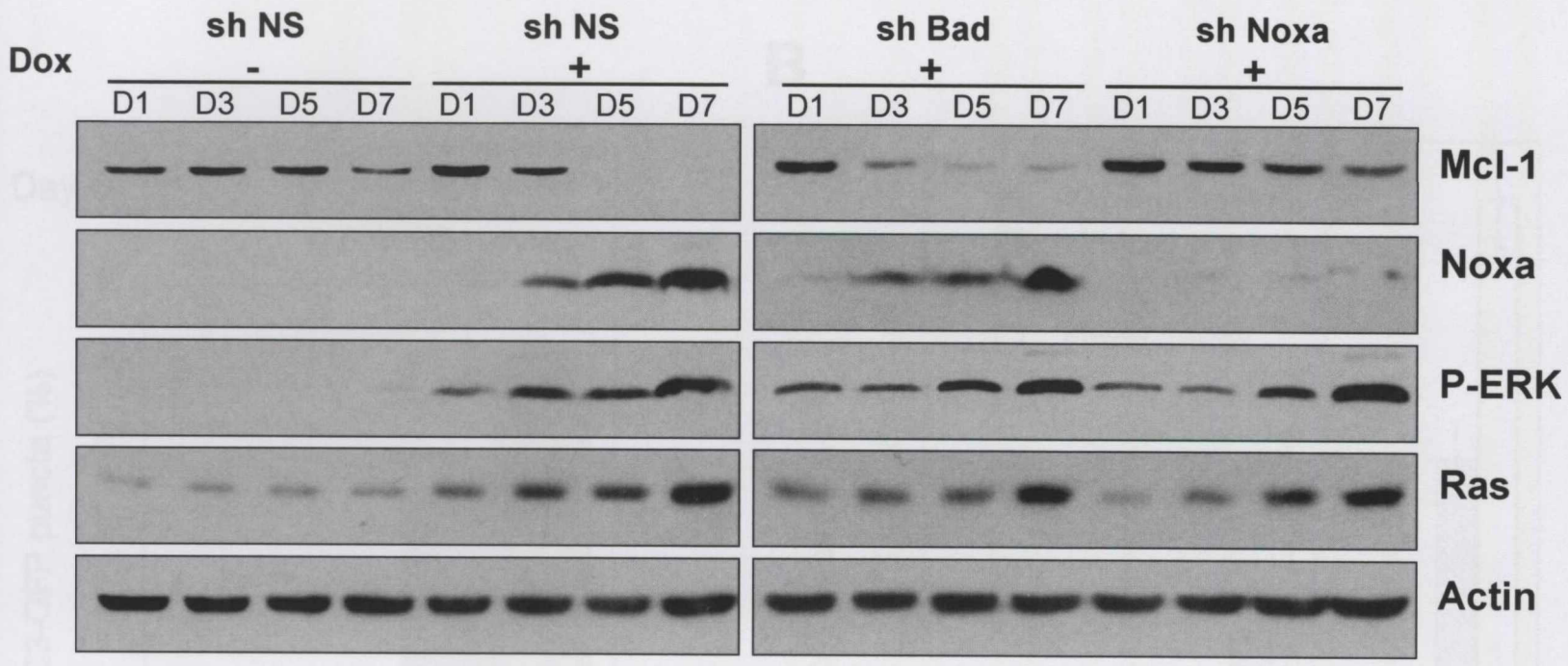
Additionally, as we observed that Ras signalling reduced the interaction between Beclin-1 and Bcl-xL, we wondered whether Noxa or Puma play any role in this. Puma strongly binds Bcl-xL while Noxa has been suggested to bind Bcl-xL with lower affinity. To address this question, we checked the binding of Bcl-xL to Beclin-1 in the above-described immunoprecipitation experiment. Similar to Mcl-1, Bcl-xL is displaced from Beclin-1 five to seven days post Ras.V12 induction. Noxa as well as Puma knockdown seemed to reduce this displacement to a certain extent (Figure 5.7).

5.2.7 Ablation of Mcl-1 sensitizes to Ras-induced autophagy and cell death

As shown earlier, Mcl-1 overexpression imparted a significant protection against Ras-induced autophagy and cell death. Noxa upregulation in response to Ras signalling correlated with Mcl-1 downregulation. To further explore whether Noxa-mediated Mcl-1 downregulation in response to Ras^{V12} expression is important for the induction of Ras-induced autophagy and cell death, we nucleofected HOSE-Ras^{V12} cells with shRNA targeted against Mcl-1 followed by Dox-treatment for a time course of seven days. Mcl-1 knockdown augmented Ras-induced autophagy (Figure 5.8 A) and significantly exacerbated Ras-induced cell death (Figure 5.8 B). Consistently, silencing the expression of Mcl-1 by siRNA also sensitized the cells to Ras-induced cell death to a dramatic effect. Silencing Bcl-xL expression also seemed to slightly enhance Ras-induced cell death but to much less extent than Mcl-1 (Figure 5.8 C). These results further confirm that Noxa/Mcl-1/Beclin-1 axis plays an essential role in mediating Ras-induced autophagy and the subsequent autophagic cell death.

5.2.8 Pharmacological inhibition of Mcl-1 induces autophagic cell death

It is clear from the previous experiments that Mcl-1 plays a crucial role in negative regulation of autophagy through binding and inhibiting Beclin-1. While



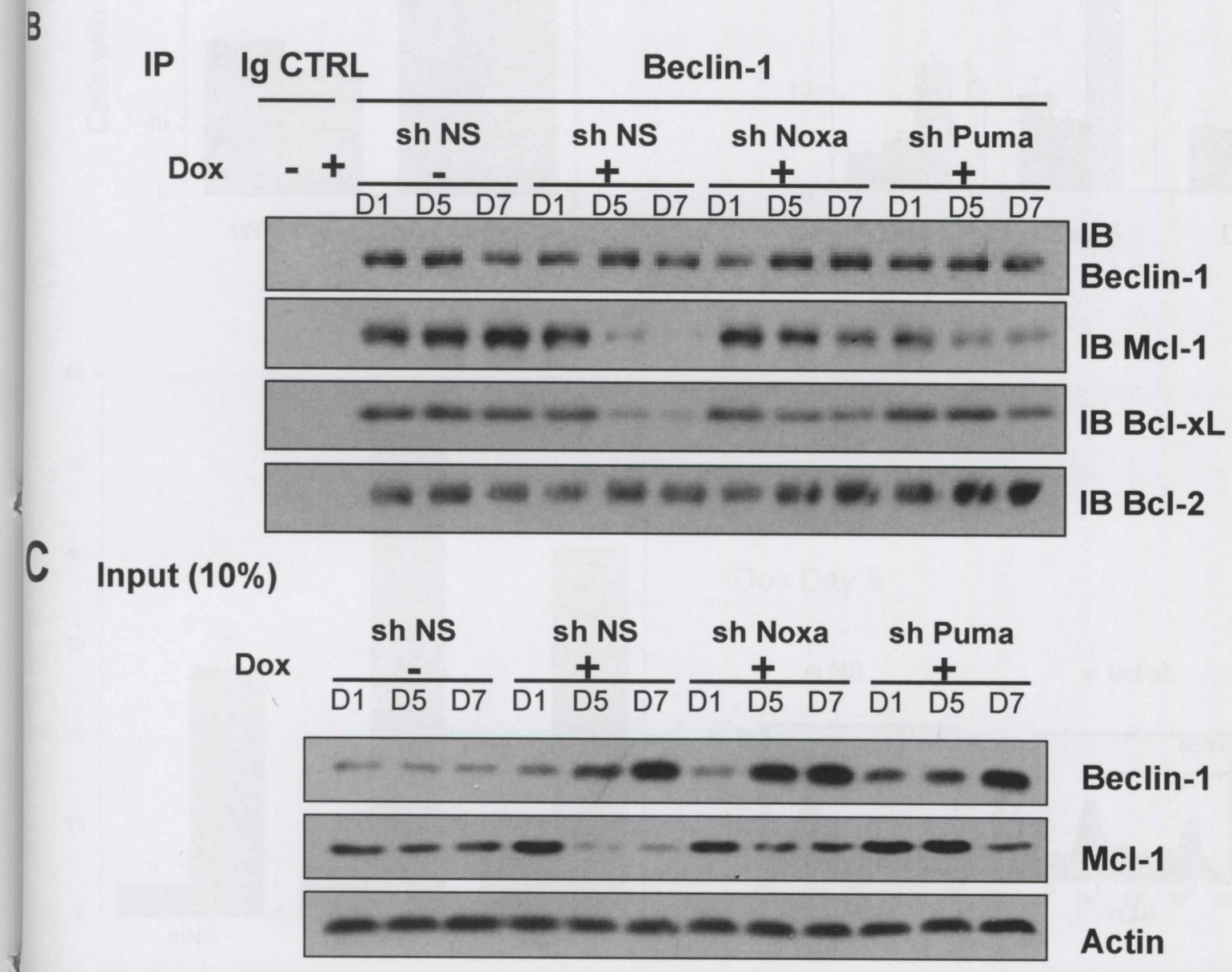


Figure 5.7 Noxa upregulation contributes to Ras-induced Mcl-1 downregulation

HOSE.RasV12 cells nucleofected with non-silencing shRNA or shRNA targeted against either Bad or Noxa were treated with or without Dox (100 ng/ml) for the indicated time points. Cell lysates were prepared in 1x SDS-PAGE lysis buffer and protein levels were analyzed with the indicated antibodies (A). HOSE.RasV12 cells nucleofected with non-silencing shRNA or shRNA targeted against either Noxa or Puma were treated with or without Dox (100 ng/ml) for 1, 5 or 7 days. At the indicated time points, cells were lysed in 800 µl per $1x10^7$ cells of IP lysis buffer and incubated overnight with 1 µg of Beclin-1 and 20 µl protein G agarose under constant rotation at 4 °C. Beads were washed 5 times in ice cold lysis buffer containing 0.1% NP-40. Beads were finally collected by centrifugation at 5000g for 2 min and brought up in SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies (B). 10% v/v of the total cell lysates used in the IP's above were analyzed by Western immunoblotting for the protein levels with the indicated antibodies (C).

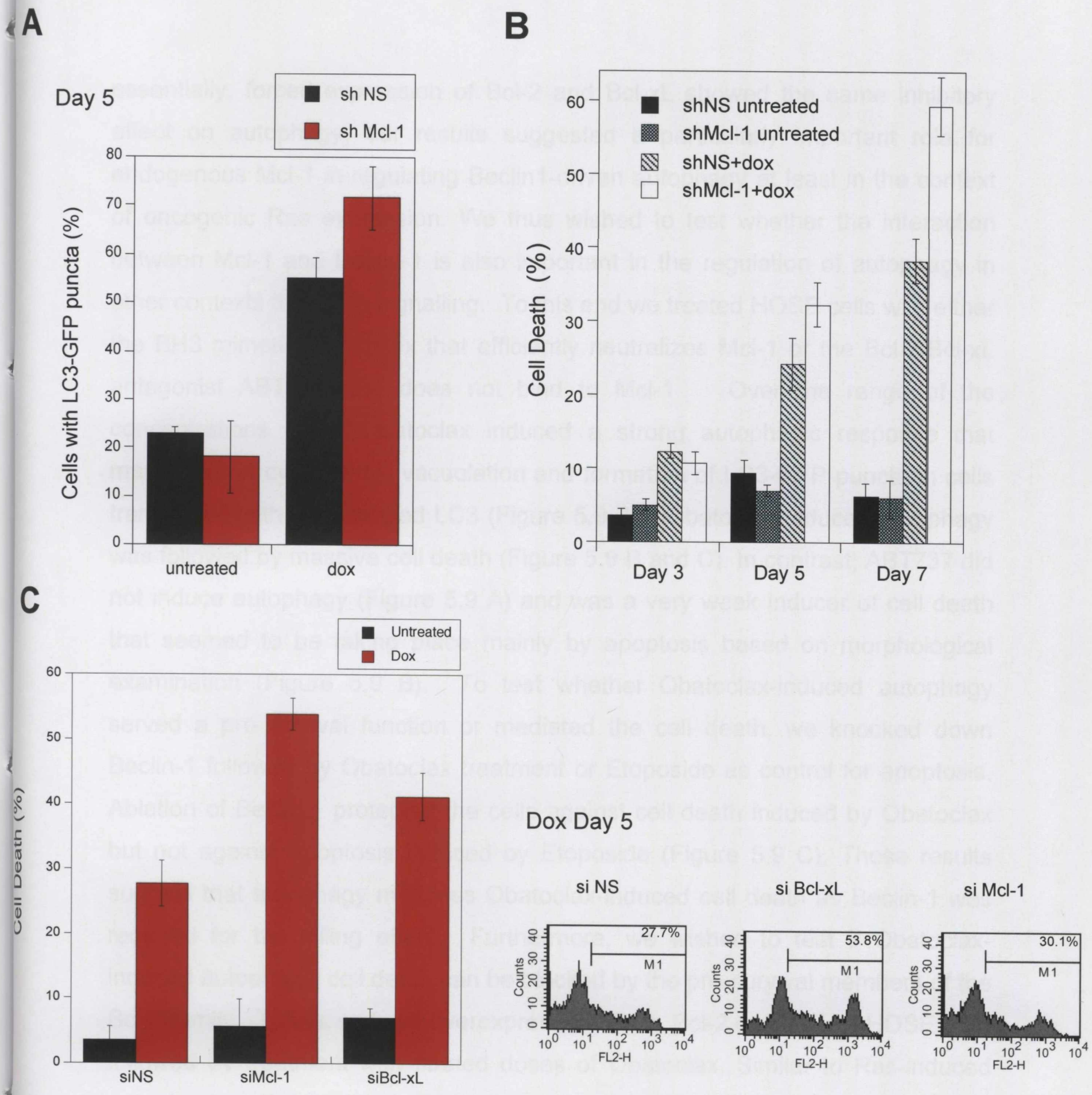


Figure 5.8 Ablation of Mcl-1 enhances Ras-induced autophagy and cell death

HOSE.RasV12 cells were nucleofected with non-silencing shRNA or shRNA construct targeted against Mcl-1. Cells were allowed 72 h for full expression followed by treatment with or without 100 ng/ml Dox. The percentage of cells exhibiting LC3-GFP puncta in each treatment was scored 5 days later (A). At day 7 post Dox-treatment, cells were harvested and percentage of cell death in each condition was assessed by flow cytometer analysis of PI uptake (B). HOSE.RasV12 cells nucleofected with non-silencing siRNA or siRNA targeted against either Mcl-1 or Bcl-xL, were treated with or without Dox (100 ng/ml). At day 5 post Dox-treatment, cells were harvested and percentage of cell death in each condition was assessed by flow cytometer analysis of PI uptake (B). The percentage of PI-positive cells in each treatment is indicated in the top right-hand corner of each panel (C)

essentially, forced expression of Bcl-2 and Bcl-xL showed the same inhibitory effect on autophagy, our results suggested a particularly important role for endogenous Mcl-1 in regulating Beclin1-driven autophagy at least in the context of oncogenic Ras expression. We thus wished to test whether the interaction between Mcl-1 and Beclin-1 is also important in the regulation of autophagy in other contexts than Ras signalling. To this end we treated HOSE cells with either the BH3 mimetic Obatoclax that efficiently neutralizes Mcl-1 or the Bcl-2/Bcl-xL antagonist ABT737 that does not bind to Mcl-1. Over the range of the concentrations used, Obatoclax induced a strong autophagic response that manifested in cytoplasmic vacuolation and formation of LC3-GFP puncta in cells transfected with GFP-tagged LC3 (Figure 5.9 A). Obatoclax-induced autophagy was followed by massive cell death (Figure 5.9 B and C). In contrast, ABT737 did not induce autophagy (Figure 5.9 A) and was a very weak inducer of cell death that seemed to be taking place mainly by apoptosis based on morphological examination (Figure 5.9 B). To test whether Obatoclax-induced autophagy served a pro-survival function or mediated the cell death, we knocked down Beclin-1 followed by Obatoclax treatment or Etoposide as control for apoptosis. Ablation of Beclin-1 protected the cells against cell death induced by Obatoclax but not against apoptosis induced by Etoposide (Figure 5.9 C). These results suggest that autophagy mediates Obatoclax-induced cell death as Beclin-1 was required for the killing effect. Furthermore, we wished to test if Obatoclax-induced autophagic cell death can be blocked by the pro-survival members of the Bcl-2 family. To this end, we overexpressed Mcl-1, Bcl-2 or Bcl-xL in HOSE cells followed by treatment with titrated doses of Obatoclax. Similar to Ras-induced autophagic cell death, overexpression of the pro-survival Mcl-1, Bcl-2 or Bcl-xL protected against Obatoclax-induced cell death (Figure 5.9 D).

Strikingly, we observed that Obatoclax treatment induced upregulation of Beclin-1 levels while treatment with ABT737 did not have any effect in this regard (Figure 5.9 E).

5.2.9 Mcl-1/Beclin-1 interaction modulates the level of Beclin-1

Obatoclax has been shown to disrupt the interaction between Mcl-1 and Beclin-1 (Bonapace *et al.*, 2010). The observation that treatment with Obatoclax, but not ABT737, induced Beclin-1 upregulation raised the question whether the interaction between Mcl-1 and Beclin-1 modulates the levels of the latter. To address this question, we decided to modulate the interaction between Mcl-1 and Beclin-1 by another approach which is simply ablating the levels of Mcl-1 in the cells and examine if this has any subsequent effect on the levels of Beclin-1. HOSE cells were nucleofected with two different siRNA's targeted against Mcl-1. Cells were allowed three days for full knockdown of the protein. Beclin-1 level in those cells were then analysed by immunoblotting and compared to cells that were nucleofected with either non-silencing RNA or siRNA against Bcl-xL as control. Strikingly, ablation of Mcl-1 induced the accumulation of Beclin-1 (Figure 5.10 A). To further confirm this observation, we overexpressed Mcl-1, Bcl-2 or Bcl-xL in HOSE cells and assessed the level of Beclin-1. Consistent with the results obtained with Mcl-1 knockdown, overexpression of Mcl-1 tended to diminish the level of Beclin-1 slightly. These results clearly indicate that Mcl-1 is involved in the regulation of the level of Beclin-1 (Figure 5.10 B). Interestingly, co-transfection of Noxa with Mcl-1 abolished the negative effect of Mcl-1 on Beclin-1 level (Figure 5.10 B).

Next we sought to examine if these observations have implications in the context of Ras-induced autophagy. As shown earlier, Beclin-1 is induced by Ras through ERK/ATF4-depnedent pathway. Having observed that Mcl-1 and Noxa can modulate the basal level of Beclin-1, we then wished to test if in the context of oncogenic Ras, Mcl-1 and Noxa can further impact the level of Beclin-1. To this end, we silenced the expression of Mcl-1 in HOSE-Ras^{V12} cells by two different siRNA's followed by Dox treatment for 3 days. Consistent with previous results, expression of H-Ras^{V12} induced the upregulation of Beclin-1. Ablation of Mcl-1, but not Bcl-xL, further synergized with Ras and further enhanced the elevation in Beclin-1 level (Figure 5.10 C). Conversely, Knockdown of Noxa, and Puma,

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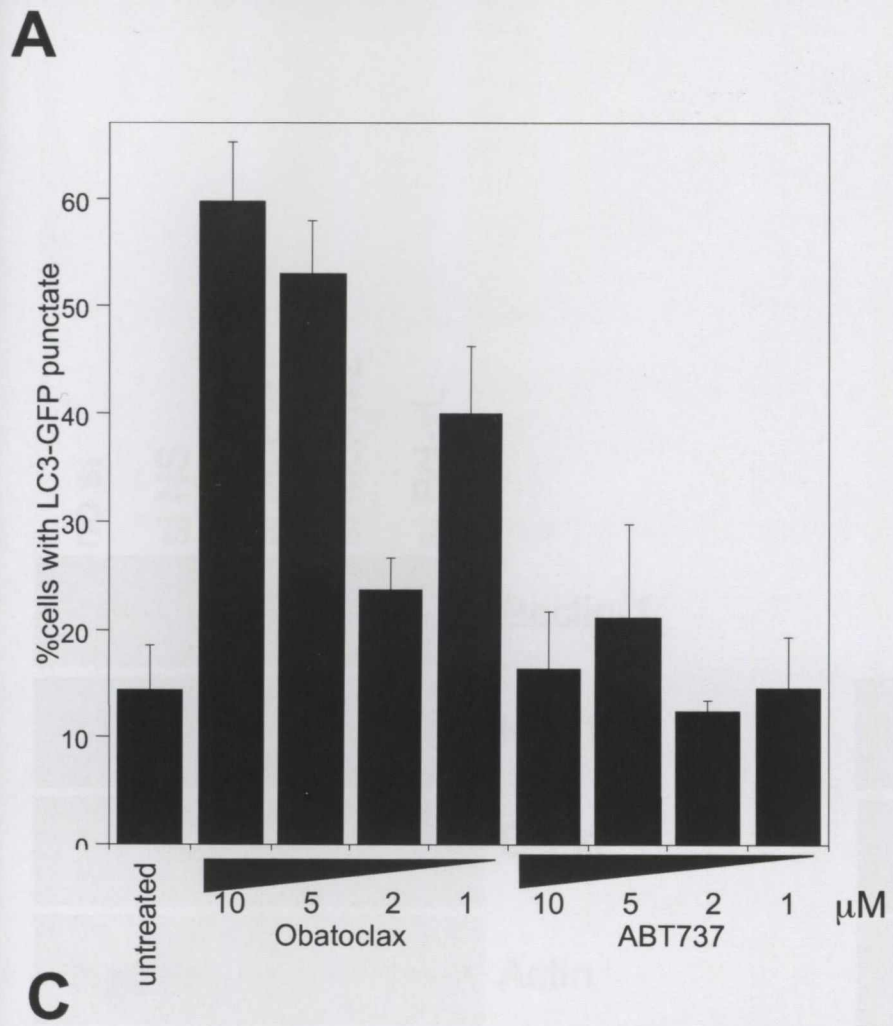

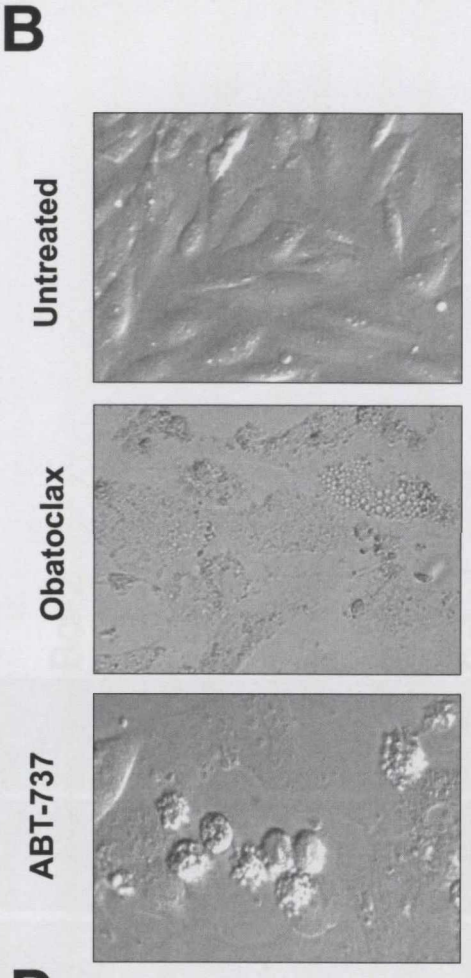

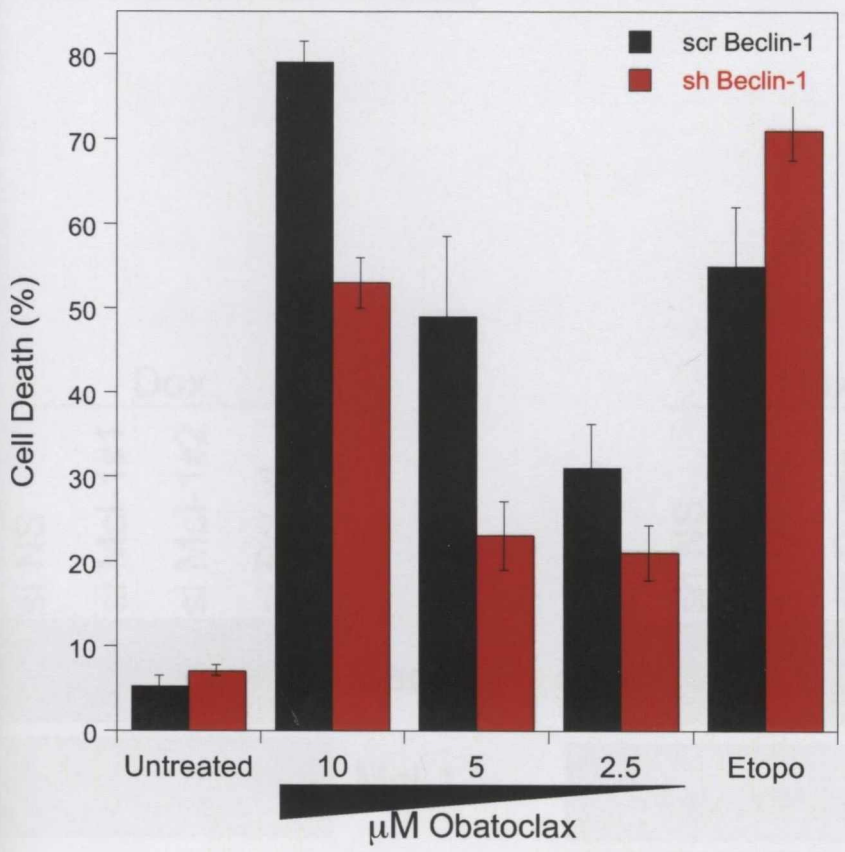

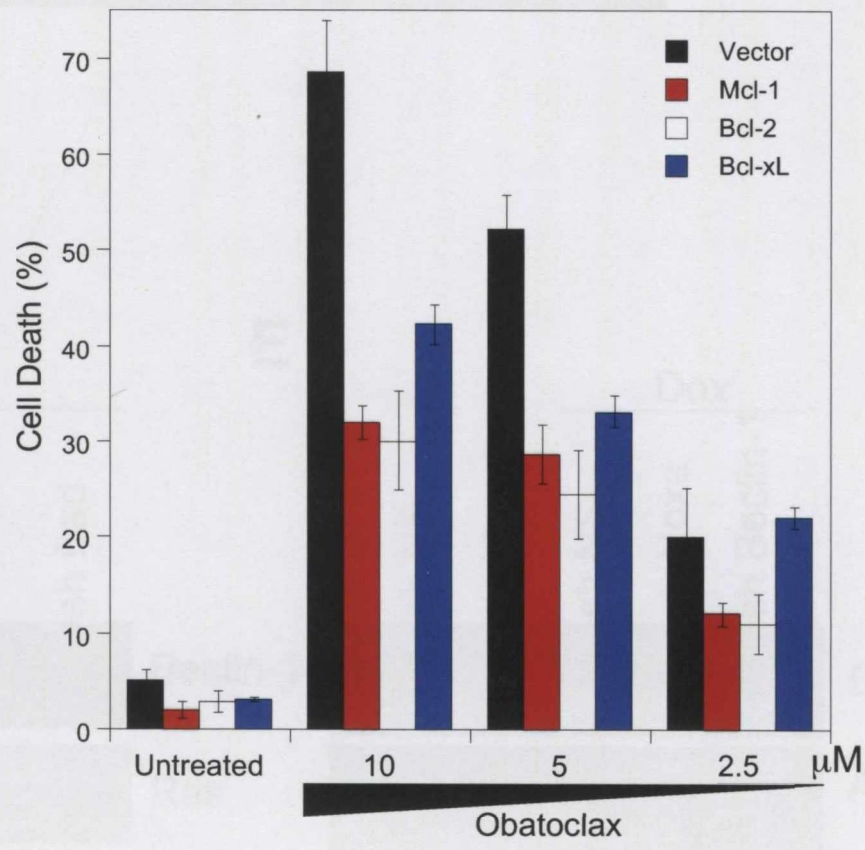

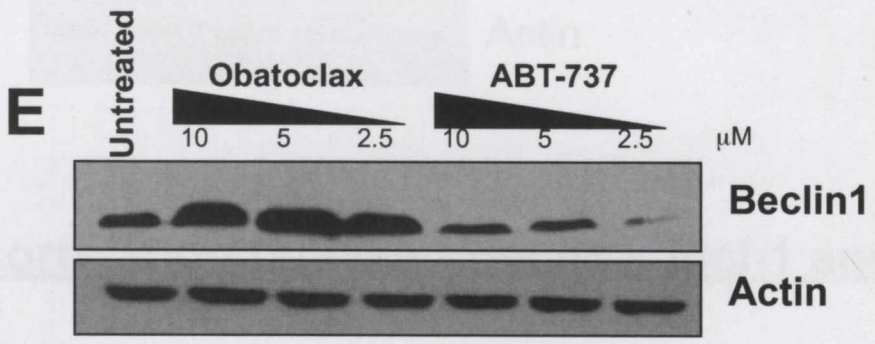

Figure 5.9 Obatoclax induces autophagic cell death

HOSE.RasV12 cells were either left untreated or were treated with the indicated concentrations of either Obatoclax or ABT737. 48 h later, The percentage of cells exhibiting LC3-GFP puncta in each treatment was enumerated (A). Pictures were taken on a light microscope with 20X magnification (B). HOSE.RasV12 cells were nucleofected with either scrambled shRNA or shRNA construct targeted against Beclin-1 (C) or members of the Bcl-2 family proteins (D). Following 24 hr of expression, cells were treated with the indicated concentrations of Obatolcax or Etoposide (100 μM) as control for apoptosis. 72 h later, cell death in each treatment was assessed using PI uptake assay. HOSE.Ras. V12 cell treated as in A were lysed in 1X SDS-PAGE lysis buffer and Beclin-1 levels were analysed by western immunoblotting utilizinf Actin as loading control (E).

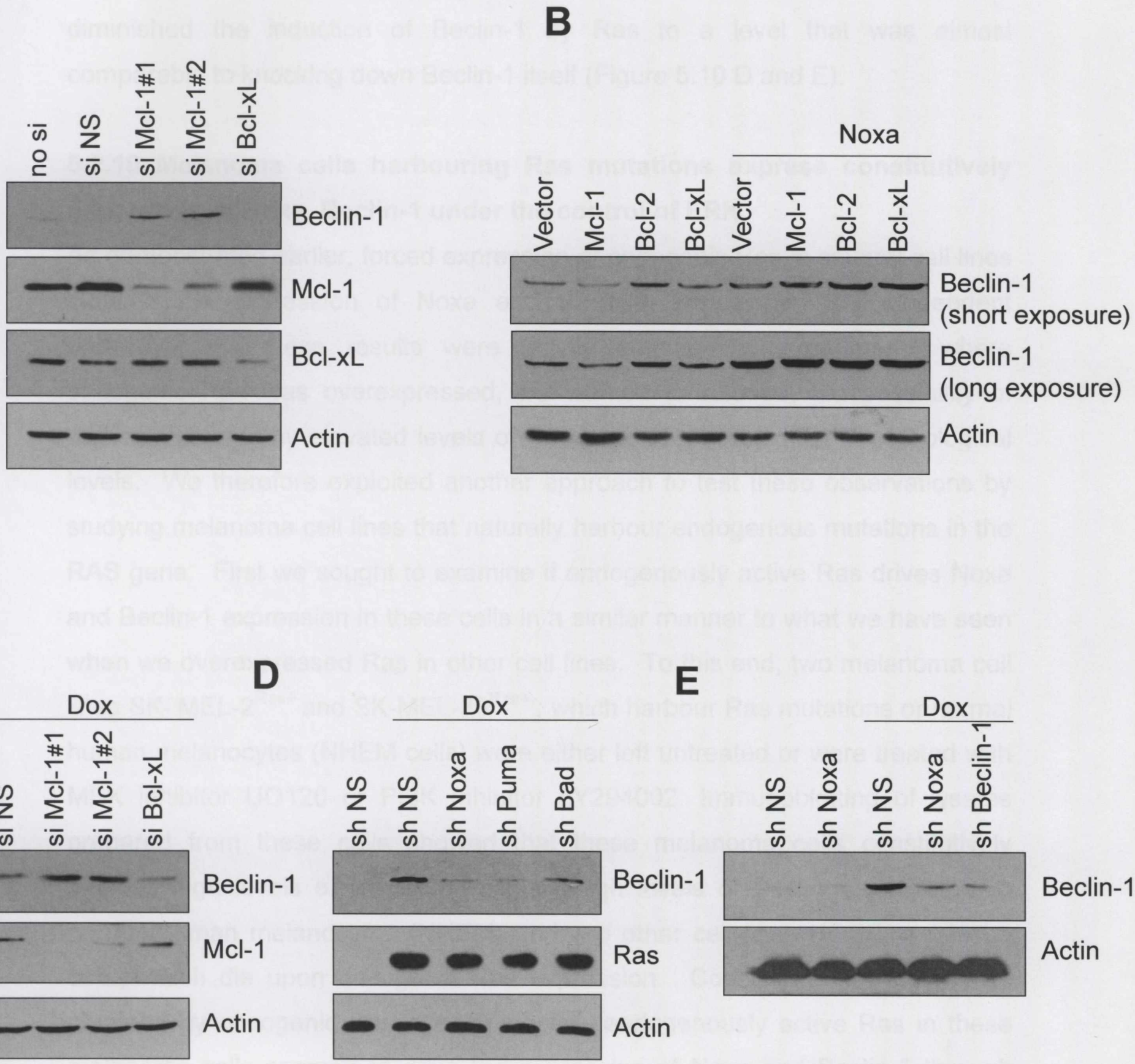

Figure 5.10 Reciprocal correlation between Beclin1, Mcl-1 and Noxa levels

HOSE.RasV12 cells were nucleofected with non-silencing siRNA or siRNA's targeted against either Mcl-1 or Bcl-xL (200nM). 72 hr later, cells were lysed in 1x SDS-PAGE lysis buffer and protein levels were analyzed with the indicated antibodies (A). HOSE.RasV12 cells were nucleofected with different anti-apoptotic members of the Bcl-2 family (1 μg) either alone or in combination with Noxa (1 μg). 72 hr later, cells were lysed in 1x SDS-PAGE lysis buffer and protein levels were analyzed with the indicated antibodies (B). HOSE.RsaV12 cells nucleofected as in A (C) or with shRNA constructs targeting eith Noxa, Puma, Bad or Beclin-1 (D and E), were treated with or without Dox (100 ng/ml). 72 hr later, cells were lysed in 1x SDS-PAGE lysis buffer and protein levels were analyzed with the indicated antibodies (A)

diminished the induction of Beclin-1 by Ras to a level that was almost comparable to knocking down Beclin-1 itself (Figure 5.10 D and E).

5.2.10 Melanoma cells harbouring Ras mutations express constitutively high levels of Noxa, Beclin-1 under the control of ERK

As demonstrated earlier, forced expression of oncogenic Ras in several cell lines induced the expression of Noxa and Beclin-1 through an ERK-dependent pathway. As these results were mainly obtained in experiments where oncogenic Ras was overexpressed, we wanted to rule out the possibility of artefacts caused by elevated levels of Ras beyond physiological or pathological levels. We therefore exploited another approach to test these observations by studying melanoma cell lines that naturally harbour endogenous mutations in the RAS gene. First we sought to examine if endogenously active Ras drives Noxa and Beclin-1 expression in these cells in a similar manner to what we have seen when we overexpressed Ras in other cell lines. To this end, two melanoma cell lines SK- MEL-2^{Ras+} and SK-MEL-30^{Ras+}, which harbour Ras mutations or normal human melanocytes (NHEM cells) were either left untreated or were treated with MEK inhibitor UO126 or PI3K inhibitor LY294002. Immunoblotting of lysates prepared from these cells showed that these melanoma cells constitutively express high levels of Noxa and slightly high levels of Beclin-1 compared to normal human melanocytes (NHEM) and two other cell lines HOSE and HeLa cells, which die upon oncogenic Ras expression. Consistent with the results obtained by oncogenic Ras overexpression, endogenously active Ras in these melanoma cells seemed to drive the expression of Noxa and Beclin-1 through ERK as MEK inhibition by UO126 treatment greatly attenuated the basal expression levels of these proteins (Figure 5.11).

5.2.11 Mcl-1 is crucial for survival of melanoma cell lines harbouring Ras mutations

These results however in agreement with our previous results raised an intriguing question: why Ras-induced elevated levels of Beclin-1 and Noxa in SK- MEL-

2^{Ras+} and SK-MEL-30^{Ras+} melanoma cells did not play the same tumor-restraining role through promoting autophagy that we observed upon oncogenic Ras expression in other cell lines. Instead, these melanoma cells did not show signs of autophagy as indicated by morphological inspection and comparison of p62 levels and they obviously proliferated normally and proceeded to form tumors despite expressing higher levels of Noxa and Beclin-1. A clue to understanding these conflicting observations came from studying the expression levels of anti-apoptotic members of the Bcl-2 family in these cells. In comparison to NHEM, HeLa or HOSE-Ras^{V12} cells which readily die upon oncogenic Ras expression, both SK- MEL-2^{Ras+} and SK-MEL-30^{Ras+} melanoma cells expressed much higher levels of Mcl-1 but comparable levels of Bcl-2 and Bcl-xL (Figure 5.11). Given the crucial role we have demonstrated for Mcl-1 in inhibiting Noxa- and Beclin-1 driven autophagy, it was tempting to postulate that those cells might have acquired a co-operating mutation to elevate the expression of Mcl-1 to a level that is high enough to neutralize and inhibit both arms of Ras-induced autophagy; Noxa and Beclin-1. If this truly was the case then we would expect to sensitize those cells again to autophagy and autophagic cells death driven by Noxa and Beclin-1 by ablating the level of Mcl-1. To test this hypothesis we transfected SK- MEL-2^{Ras+} and SK-MEL-30^{Ras+} melanoma cells with siRNA against Mcl-1 or Bcl-xL and Bcl-2 as control. Strikingly, knockdown of Mcl-1 induced autophagy in both cell lines as assessed by GFP-LC3 puncta (Figure 5.12 A, B, D and E) and the level of p62 (Figure 5.12 C and F). Ablation of Bcl-xL or Bcl-2, however did not seem to evoke similar effect. Interestingly, autophagy triggered in response to Mcl-1 knockdown culminated into massive cell death that became evident three days post transfection (Figure 5.13 A and B). To examine whether autophagy contributed to this cell death, we nucleofected these cells with shRNA against Mcl-1 alone or in combination with another shRNA against Beclin-1. Knockdown of Mcl-1 by shRNA triggered massive cell death. Ablating Beclin-1 together with Mcl-1 reduced cell death to some extent indicating that autophagy accounts for cell death at least in a portion of the cells (Figure 5.14 A and B)

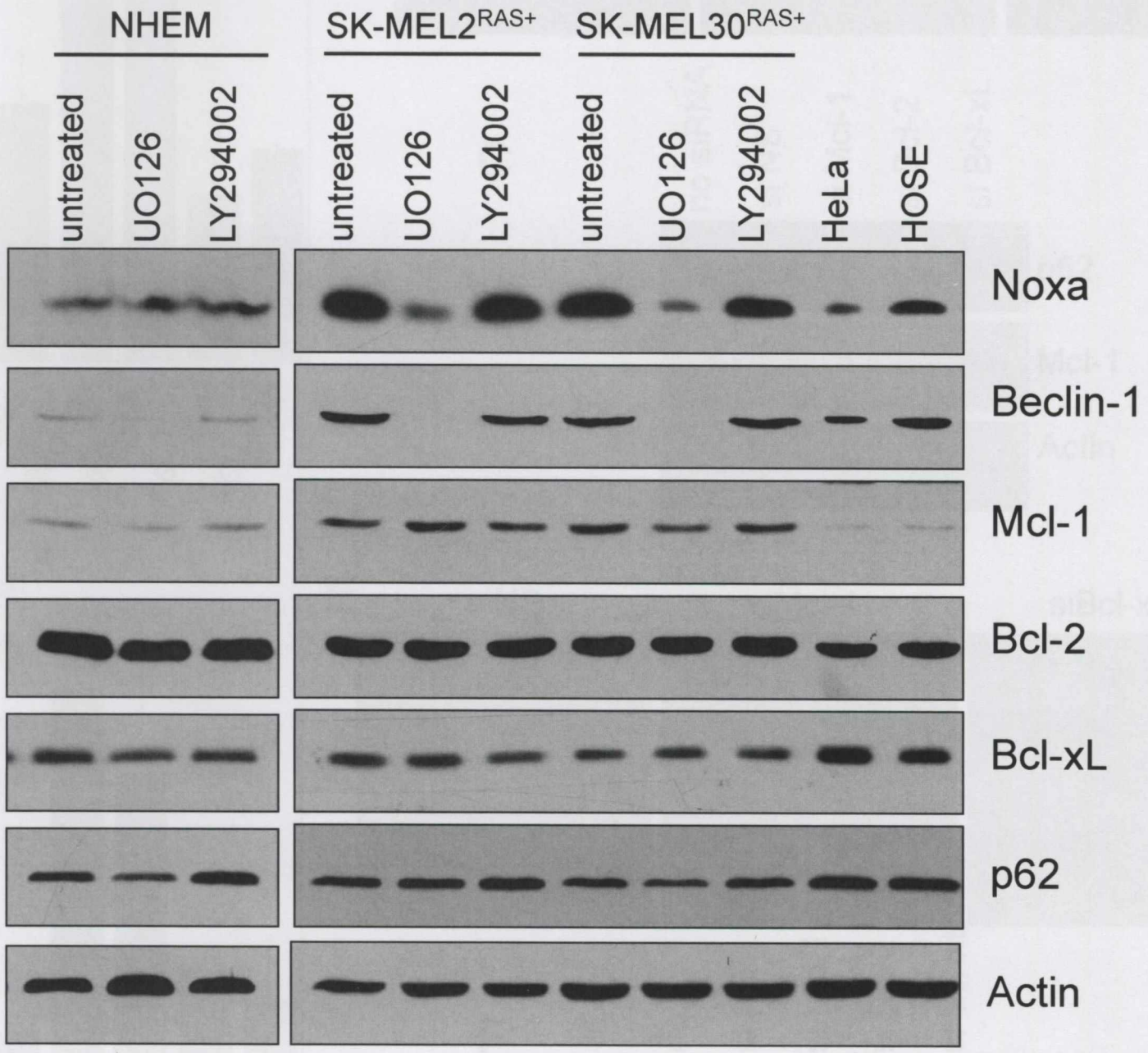

Figure 5.11 Expression levels of Beclin-1, Noxa and Mcl-1 in melanoma cell lines harbouring Ras mutations

NHEM, SK-MEL2^{Ras+} or SK-MEL30^{Ras+} cells were either left untreated or were treated with MEK inhibitor UO126 (20μM) or PI3K inhibitor LY294002 (20μM). 24 h later, cells were lysed in 1X SDS-PAGE lysis buffer and protein levels were analysed by western immunoblotting with the indicated antibodies. Total cell lysates prepared from HeLa or untreated HOSE.RasV12 cells were used for comparison of the expression levels of different proteins.

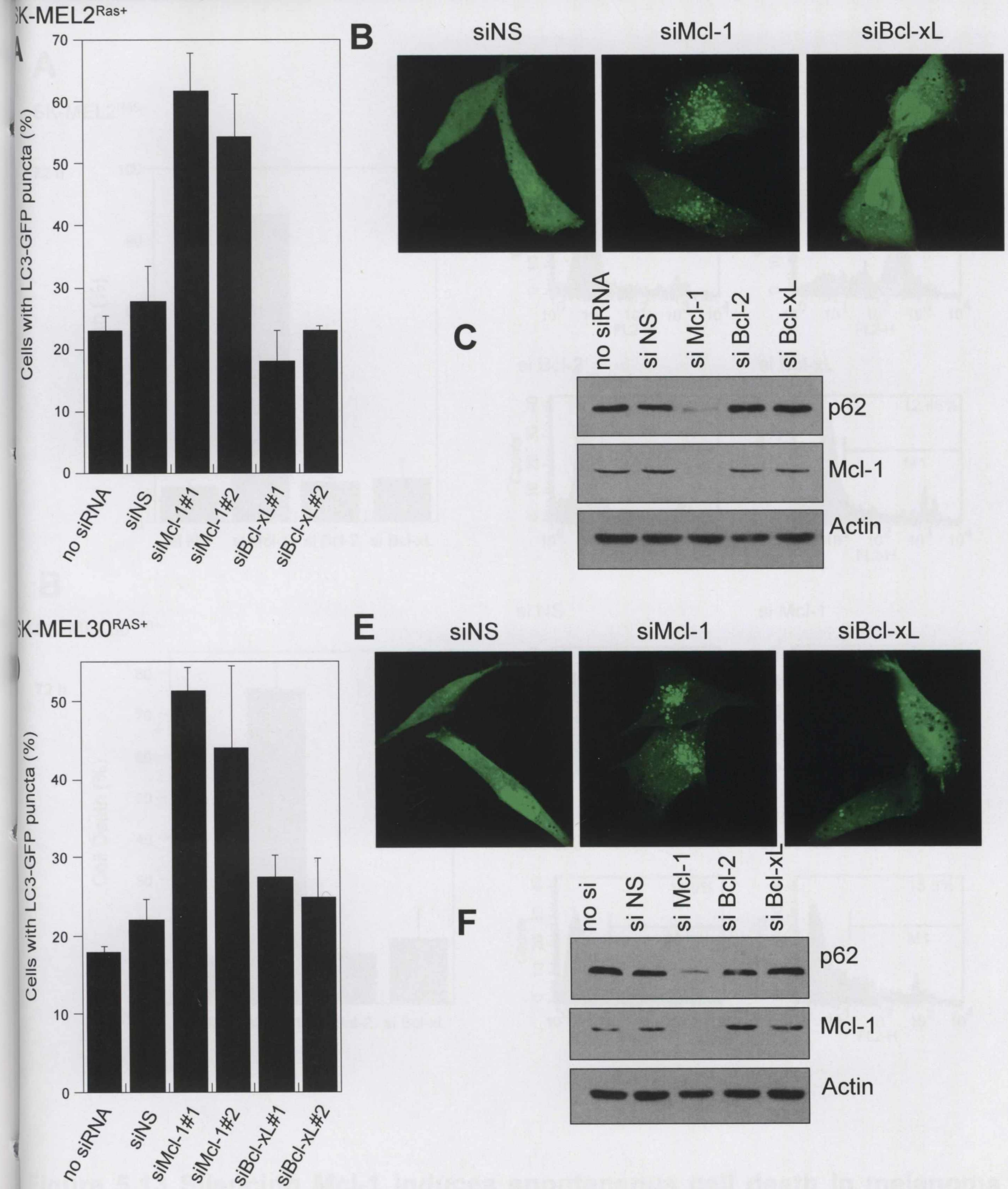

Figure 5.12 Silencing Mcl-1 induces autophagy in melanoma cell lines harbouring Ras mutations

SK-MEL2^{Ras+} or SK-MEL30^{Ras+} cells were nucleofected with non-silencing siRNA or siRNA's targeted against either Mcl-1 or Bcl-xL (200nM) along with pLC3-GFP (250 ng). 48 hr later, cells exhbiting LC3-GFP puncta were enumerated (A and D repectively) and pictures were taken on a confocal microscope with 60 X maginification (B and D). Cells nucleofected as above were lysed in 1x SDS-PAGE lysis buffer 72 h after nucleofection and protein levels were analyzed with the indicated antibodies (C and F).

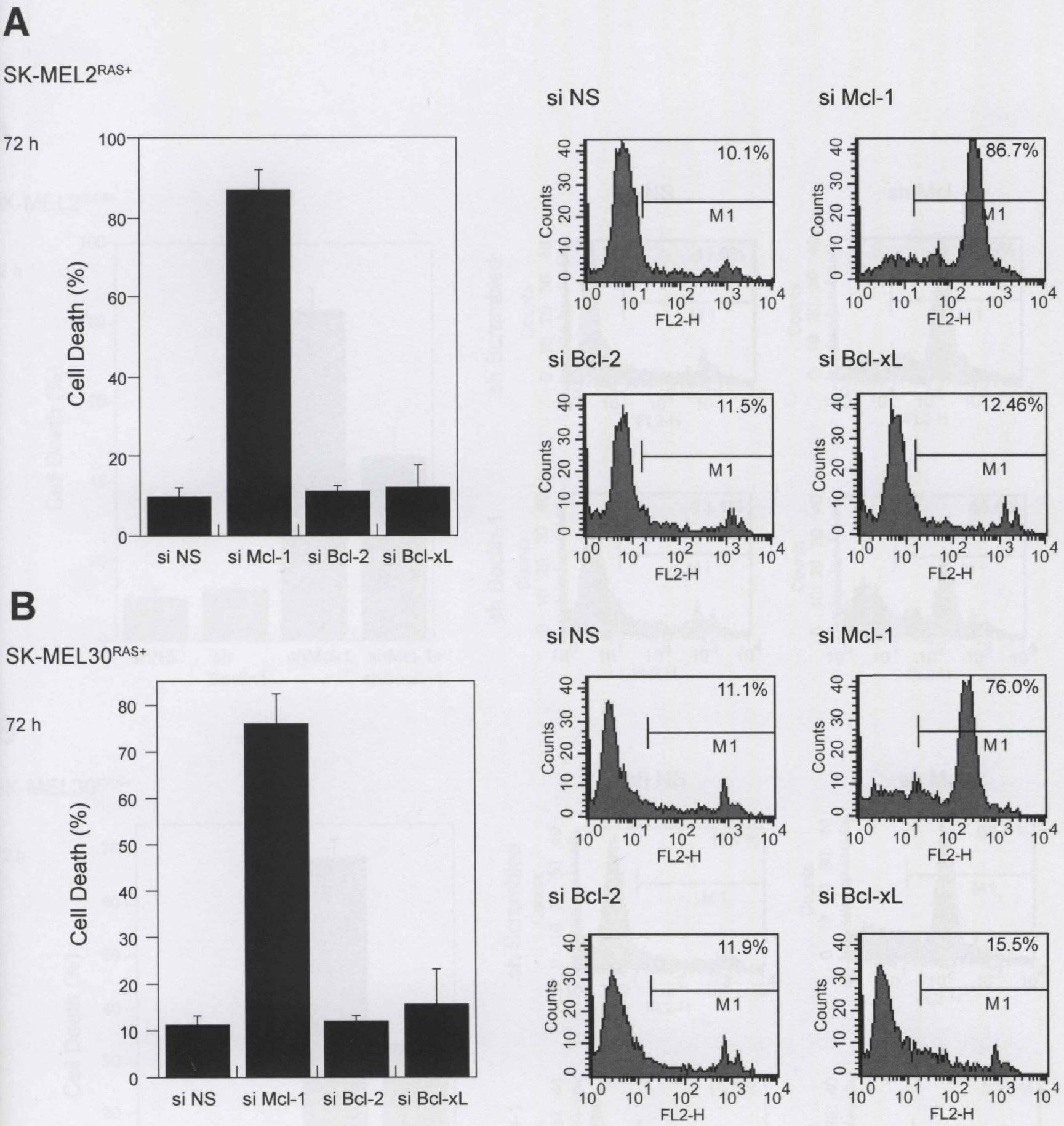

Figure 5.13 Silencing Mcl-1 induces spontaneous cell death in melanoma cell lines harbouring Ras mutations

SK-MEL2^{Ras+} or SK-MEL30^{Ras+} cells were nucleofected with non-silencing siRNA or siRNA's targeted against either Mcl-1, Bcl-2 or Bcl-xL (200nM). 72 h later, cells were harvested and percentage of cell death in each condition was assessed by flow cytometer analysis of PI uptake (A and B). The percentage of PI-positive cells in each treatment is indicated in the top right-hand corner of each panel.

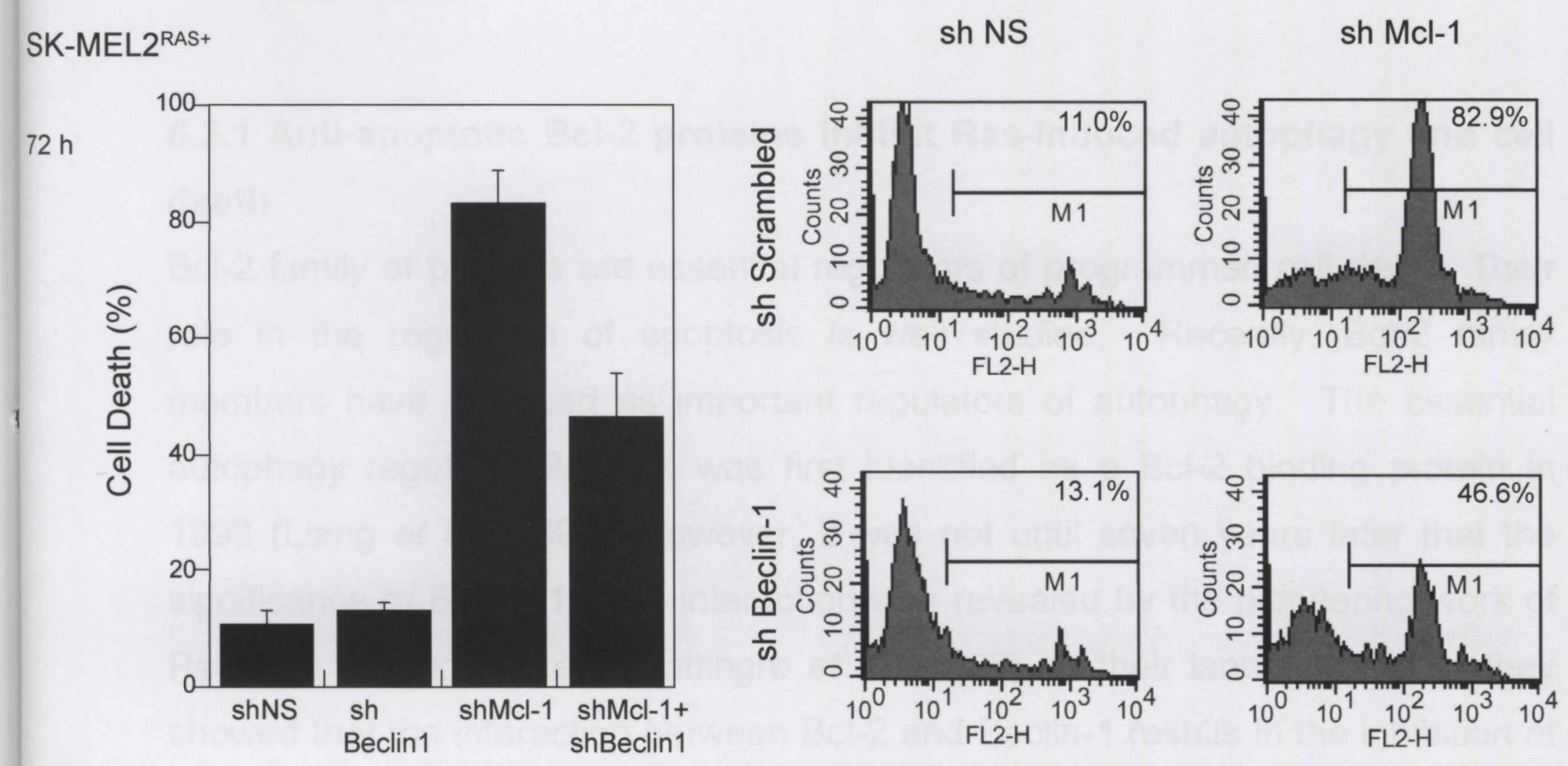

Figure 5.14 Silencing Beclin-1 attenuates spontaneous cell death triggered by Mcl-1 knockdown in melanoma cell lines

SK-MEL2^{Ras+} or SK-MEL30^{Ras+} cells were nucleofected with the indicated shRNA's. After 72 h, cells were harvested and percentage of cell death in each condition was assessed by flow cytometer analysis of PI uptake (A and B). The percentage of PI-positive cells in each treatment is indicated in the top right-hand corner of each panel.

5.3 Discussion

5.3.1 Anti-apoptotic Bcl-2 proteins inhibit Ras-induced autophagy and cell death

Bcl-2 family of proteins are essential regulators of programmed cell death. Their role in the regulation of apoptosis is well studied. Recently, Bcl-2 family members have emerged as important regulators of autophagy. The essential autophagy regulator Beclin-1 was first identified as a Bcl-2 binding protein in 1998 (Liang *et al.*, 1998). However, it was not until seven years later that the significance of Beclin-1/Bcl-2 interaction was revealed by the pioneering work of Pattingre and co-workers (Pattingre *et al.*, 2005). In their landmark study, they showed that the interaction between Bcl-2 and Beclin-1 results in the inhibition of Beclin-1-driven autophagy as Beclin-1 bound to Bcl-2 is unable to associate with PI3K class III to form a complex that is required to promote autophagy. Recently, Beclin-1 has been described to carry a BH3 domain that enables it to bind to the hydrophobic cleft of the anti-apoptotic Bcl-2 proteins. Beclin-1 has also been shown to interact with Bcl-xL. Similar to the effect of Bcl-2 on Beclin-1-driven autophagy, binding of Bcl-xL to Beclin-1 has an inhibitory effect on autophagy which can be relieved by co-expression of the BH3-only protein Bad or treatment with BH3 peptides ABT737 (Maiuri *et al.*, 2007).

As we observed that the expression of oncogenic Ras in HOSE-Ras^{V12} cells induces Beclin-1 upregulation and triggers Beclin-1-driven autophagy and autophagic cell death, we decided to explore whether Bcl-2 family proteins are involved in regulating Ras-induced autophagy and autophagic cell death. Our results demonstrate that the expression of various anti-apoptotic Bcl-2 proteins especially A1 and Mcl-1 can indeed block Ras-induced autophagy and cell death. The inhibitory effect of the anti-apoptotic Bcl-2 proteins was initially suggested to be exerted through diminishing the ability of Beclin-1 to associate with PI3KC3 to initiate autophagy. However, it was later suggested that anti-apoptotic Bcl-2 proteins inhibit Beclin-1-mediated autophagy not by disrupting the interaction

between Beclin-1 and PI3K class III as Beclin-1 does not bind PI3K class III through its BH3 domain. It is instead suggested that Bcl-2/Beclin-1/PI3K class III complex possesses less ability to drive autophagy than free Beclin-1/PI3K class III complex. Beclin-1/PI3K class III complex recruits other proteins (UVRAD, Bif-1 and Ambra-1) to initiate autophagy (Levine *et al.*, 2008). One speculative possibility is that binding of Bcl-2 proteins may sterically hinder the recruitment of other proteins to the complex. In this regard, A1 and Mcl-1 are unique in that they are bigger in size than the rest of the anti-apoptotic Bcl-2 proteins. A1 and Mcl-1 may thus have stereochemical properties that least favour the recruitment of other proteins to the complex and hence possess a more efficient inhibitory effect on Beclin-1-driven autophagy. This hypothesis may explain our observation that while Ras-induced autophagy and autophagic cell death was essentially blocked by all anti-apoptotic Bcl-2 proteins tested (except Bcl-B), Mcl-1 and A1 had the most efficient inhibitory effect on Ras-induced cell death.

Consistent with their inhibitory effect on Ras-induced cell death, anti-apoptotic Bcl-2 proteins enhanced clonogenic survival of cells expressing oncogenic Ras. Anti-apoptotic Bcl-2 proteins may thus co-operate with Ras in the process of tumorigenesis.

5.3.2 The role of Noxa in mediating Ras-induced autophagy

Some BH3-only proteins and BH3 mimetics have been shown to competitively disrupt the interaction between Beclin-1 and the anti-apoptotic members of Bcl-2 family setting Beclin-1 free to promote autophagy (Maiuri *et al.*, 2007). We have demonstrated that Noxa is essentially required for Ras-induced autophagy. We set out to get more insight into the mechanism by which Noxa promotes autophagy. Given the profound inhibitory effect of Mcl-1 on Beclin1-driven autophagy, it was tempting to speculate that Noxa might mediate autophagy by competitively disrupting the interaction between Mcl-1, and possibly Bcl-xL, and Beclin-1. To test this hypothesis, we immunoprecipitated endogenous Beclin-1 and Mcl-1 before and after the induction of oncogenic Ras expression (which as shown earlier, induces Noxa upregulation). We found that Beclin-1 binds Mcl-1

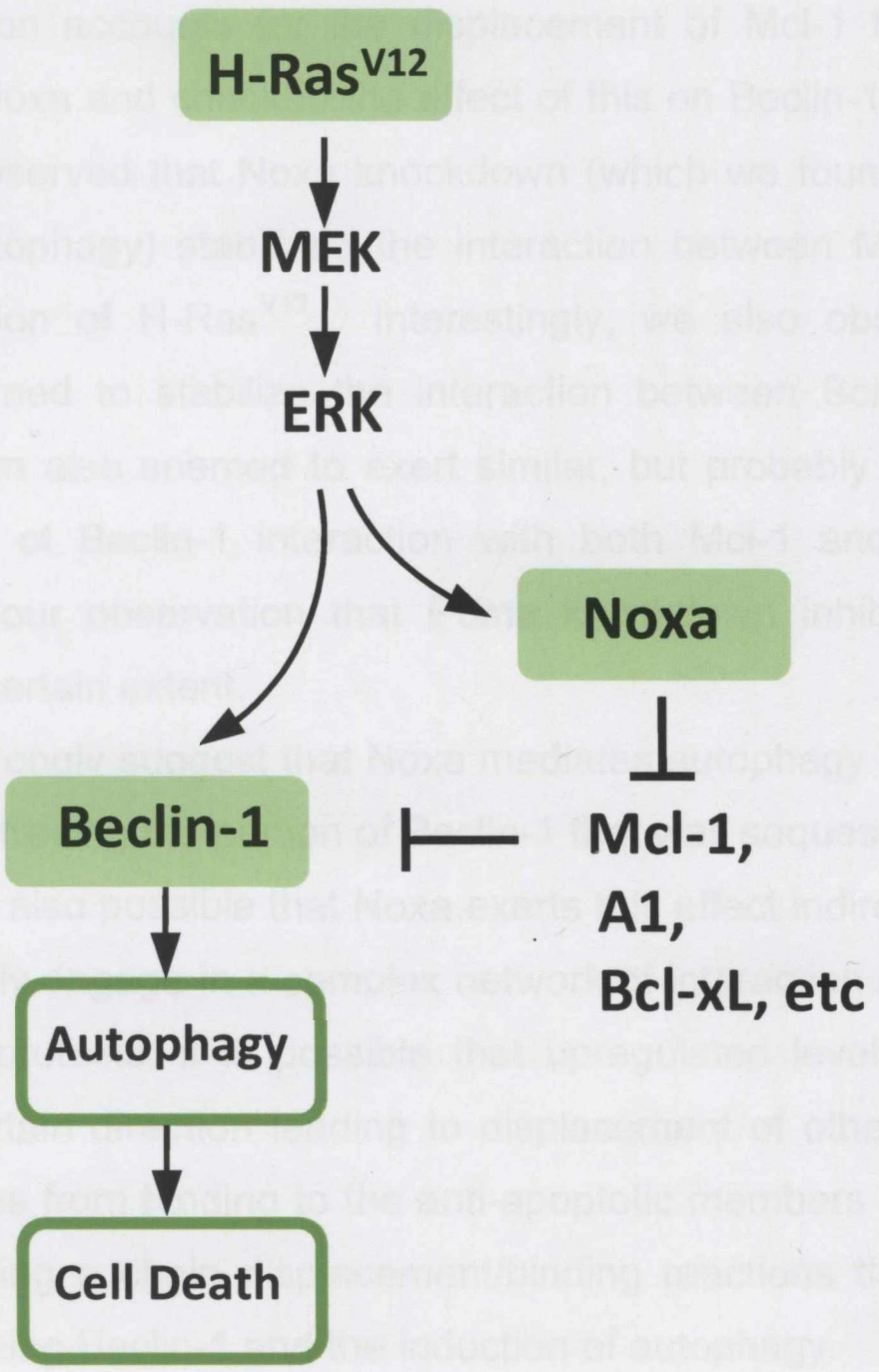

Figure 5.15 Schematic presentaion of mechanism of Ras-induced autophagy.

Upon Ras activation, Beclin-1 and Noxa are induced. Noxa binds Mcl-1 displacing it from binding to Beclin-1. Upregualated and free Beclin-1 promotes autophagy.

and Bcl-xL readily before H-Ras^{V12} induction but becomes displaced from Mcl-1 and Bcl-xL after induction of H-Ras ^{V12}. Conversely, we observed increased binding between Mcl-1 and Noxa after H-Ras^{V12} induction. These results suggest that elevated levels of Noxa engage more Mcl-1 in interaction leading to the displacement of Mcl-1 from Beclin-1 (Figure 5.15). To further confirm that Noxa upregulation accounts for the displacement of Mcl-1 from Beclin-1, we knocked down Noxa and checked the effect of this on Beclin-1/Mcl-1 interaction. Strikingly, we observed that Noxa knockdown (which we found before to inhibit Ras-induced autophagy) stabilized the interaction between Mcl-1 and Beclin-1 after the induction of H-Ras^{V12}. Interestingly, we also observed that Noxa knockdown seemed to stabilize the interaction between Bcl-xL and Beclin-1. Puma knockdown also seemed to exert similar, but probably weaker, effect on the stabilization of Beclin-1 interaction with both Mcl-1 and Bcl-xL which is consistent with our observation that Puma knockdown inhibited Ras-induced autophagy to a certain extent.

These results strongly suggest that Noxa mediates autophagy directly by binding Mcl-1 leading to freeing the portion of Beclin-1 that was sequestered by Mcl-1. Additionally, It is also possible that Noxa exerts this effect indirectly. As members of the Bcl-2 family engage in a complex network of interaction among each other and with other proteins, it is possible that upregulated levels of Noxa tip the balance in a certain direction leading to displacement of other members of the BH3-only proteins from binding to the anti-apoptotic members of the Bcl-2 family and thus triggering a chain displacement/binding reactions that may ultimately contribute to freeing Beclin-1 and the induction of autophagy.

5.3.3 Ras induces Mcl-1 downregulation

An intriguing feature of Mcl-1 is that the interaction between different BH3-only proteins and Mcl-1 can modulate the level of the latter both positively and negatively. Binding of Mcl-1 to Noxa has been shown to destabilize Mcl-1 by targeting it for proteasomal degradation. Conversely, binding to Bim or Puma stabilizes Mcl-1 (Mei *et al.*, 2005; Czabotar *et al.*, 2007; Plonet *et al.*, 2008). The

signature for degradation of the Mcl-1/Noxa appears to be encoded in the C-terminal portion of the BH3 domain of Noxa as replacement of these residues with aa residues found in the same position in Bim led to Mcl-1 stabilization (Czabotar *et al.*, 2007).

The level of Mcl-1 has also been suggested to be regulated by ERK. ERK has been implicated in the regulation of Mcl-1 levels through both transcriptional and post-translational mechanisms. Induction by TPA in hematopoietic cell increased Mcl-1 transcription through an ERK-mediated pathway. This pathway results in the activation of a transcription factor complex that binds Mcl-1 promoter. This complex was shown to contain serum response factor (SRF) and Elk-1 (Townsend *et a.*, 1998). Phosphorylation of Mcl-1 at a conserved MAP kinase phosphorylation site in the PEST region (Thr 163) through an ERK-dependent mechanism also stabilized Mcl-1 (Domina *et al.*, 2004).

However, in the HOSE.H-Ras.V12 system, we found that the induction of oncogenic Ras expression correlated with Mcl-1 downregulation that was concurrent with Noxa upregulation. This is contrast to the other situations such as proteasome inhibition when Noxa accumulation is accompanied by Mcl-1 accumulation due to inhibition of the degradation of both short-lived proteins by the proteasome. It is thus important when studying the consequences of Noxa upregulation in response to Ras to consider the change in Noxa/Mcl-1 axis rather than just the increase in Noxa levels.

As discussed above, Noxa has been shown to drive Mcl-1 degradation. This suggested that elevated levels of Noxa can contribute to the downregulation of Mcl-1. To test this speculation, we knocked down Noxa or Bad as control. Interestingly, while Bad knockdown had no effect, Noxa knockdown reversed Ras-induced Mcl-1 destabilization. These results may suggest that upregulated levels of Noxa upon Ras activation drive Mcl-1 destabilization possibly by binding it and introducing it to the proteasome for degradation.

Additionally, as mentioned above interaction between Bim and Mcl-1 stabilizes Mcl-1. We detected destabilization of Bim after induction of Ras.V12 (Figure 4.1) Whether or not this plays an important role in Mcl-1 downregulation is unclear. It is evident however from our results that Noxa plays a crucial role in this process.

Nevertheless, while it is tempting to think that Mcl-1 downregulation is a direct consequence of Noxa induction, these results need to be interpreted with caution as the gradual drop in Mcl-1 levels after oncogenic Ras expression could be an indirect consequence of the autophagic stress and the subsequent autophagic death. Mcl-1 is a highly unstable protein whose level in the cell is primarily maintained by de novo synthesis. Cells undergoing autophagic stress or subsequently dying after oncogenic Ras expression might not be able to maintain efficient protein synthesis program that is required to maintain the levels of Mcl-1. If this was the case Noxa knockdown could still indirectly lead to Mcl-1 stabilization simply by alleviating the autophagic stress and rescuing the cell death allowing the cells to maintain a more efficient protein synthesis capacity.

5.3.4 Inhibition of Mcl-1 contributes to autophagic cell death

Small-molecule mimetics of BH3-only proteins are emerging as promising anticancer agents by inhibiting anti-apoptotic Bcl-2 family proteins. Obatoclax, (also known as GX015-070), was identified by chemical library screening to bind the hydrophobic groove of anti-apoptotic Bcl-2 family proteins and antagonize their function. In contrast to another BH3 mimetic, ABT737, which disables Bcl-2 and Bcl-XL but does not inhibit Mcl-1, obatoclax can efficiently neutralize Mcl-1. It has been reported that obatoclax can potently kill some types of cancer cells, such as myeloma cells, as a single agent, and enhance apoptosis induced by various apoptotic stimuli such as lapatinib (Martin *et al.*, 2009), proteasome inhibitor bortezomib (Pérez-Galán *e al.*, 2008) and dexamethasone (Bonapace *et al.*, 2010). Obatoclax has been shown to bind Mcl-1 and disrupt the interaction between Mcl-1 and Beclin-1 (Bonapace *et al.*, 2010). Our results suggested that interruption of Mcl-1/Beclin-1 is crucial for induction of autophagy by Ras. To test

whether disruption of Mcl-1/Beclin-1 interaction can lead to autophagy in other contexts or it is a confined phenomenon to Ras-induced autophagy, we treated HOSE-Ras^{V12} cells with Obatoclax or ABT737 which does not bind Mcl-1 as control. Strikingly, Obatoclax but not ABT737 induced autophagy and autophagic cell death that can be diminished by knockdown of Beclin-1 or overexpression of Bcl-2 family members that inhibit Beclin-1. These results suggest that the interaction between Mcl-1 and Beclin-1 is particularly important for the regulation of autophagy. Surprisingly, we found that treatment with Obatoclax correlated with elevation of Beclin-1 level in a dose-dependent manner. It is however unclear whether Obatoclax induced Beclin-1 through transcriptional or post-translational mechanism.

5.3.5 Inverse correlation between Mcl-1 and Beclin-1 levels

The observation that Obatoclax, that has been shown before to disrupt Mcl-1/Beclin-1 interaction, led to enhancement in Beclin-1 level raised the intriguing question whether the interaction between Mcl-1 and Beclin-1 can modulate the level of Beclin-1. To further explore this issue, we found that knocking down Mcl-1, but not Bcl-xL, enhanced the basal level of Beclin-1. Conversely Mcl-1 overexpression led to slight reduction in Beclin-1 level. These results suggest that there is a correlation between the level of Mcl-1 and Beclin-1 in the cell and that binding of Mcl-1 to Beclin-1 can possibly modulate Beclin-1 level. It is however unclear how exactly Mcl-1 exerts this effect on Beclin-1. Basal levels of Mcl-1 appear to be regulated by the HECT- and BH3-domain-containing E3-ligase Mule/ARF-BP1 (Zhong *et al.*, 2005). A highly speculative possibility thus, is that binding of Beclin-1 to Mcl-1 can enhance Beclin-1 degradation through introducing it, along with Mcl-1, to the proteasome. However this remains to be tested.

In the context of Ras, Mcl-1 knockdown further enhanced the upregulation of Beclin-1 in HOSE-Ras^{V12} cells expressing oncogenic H-Ras. The enhancement of Beclin-1 level by Mcl-1 knockdown correlated with sensitization to Ras-induced autophagy and cell death. Conversely, Noxa knockdown in H-Ras^{V12} expressing

cells, which as discussed earlier stabilizes Mcl-1 and diminishes Ras-induced autophagy, correlated with reduced induction of Beclin-1 upon H-Ras^{V12} induction further indicating a reciprocal relation between the levels of Noxa, Mcl-1 and Beclin-1.

These results suggest that Ras can modulate Beclin-1 by at least two ways: transcriptionally, through a pathway that involves ERK and ATF4, and by another unique mechanism that involves modulation of Noxa and Mcl-1 levels.

5.3.6 Melanoma cells harbouring Ras mutations express constitutively high levels of Noxa, Beclin-1 and Mcl-1

Consistent with what we observed upon oncogenic Ras overexpression in different cell lines, we have shown that two melanoma cell lines that harbour endogenous mutations in Ras express constitutively high levels of Noxa and Beclin-1 compared to normal melanocytes and two other cell lines HeLa and HOSE.Ras.V12 which die upon oncogenic Ras expression. The expression of Noxa and Beclin-1 in these melanoma cells lines was under the control of ERK as inhibition of MEK diminished their basal expression level. Strikingly, we found that these melanoma cells lines express comparably high level of Mcl-1, which we found is crucial for their survival. Ablation of Mcl-1 in these cells induced signs of autophagy that was followed by massive spontaneous cell death that resembled the cell death we observed in other cell lines upon forced expression of oncogenic Ras and was attenuated by simultaneous knockdown of Beclin-1 with Mcl-1.

These results clearly show the physiological/pathological relevance of our observations and suggest that Ras-induced autophagic cell death could be a caveat to target tumors that harbour Ras mutations. It however remains to be tested whether restoration of Ras-induced autophagy by targeting Mcl-1 in the types of cancer that are linked Ras mutations can prove useful for therapeutic intervention.

Chapter VI

General Discussion

6.1 Introduction

Many factors, such as environmental insults and failure in repairing DNA errors during cell division, continually expose our genomes to damage. Nevertheless, most people do not experience a single incidence of cancer in their entire life despite the ample risk of tumorigenesis they are exposed to every day. While "why and how cancer develops in certain individuals?" is an important question, an equally important question to ask is "why and how cancer does not occur in the vast majority of the population?" given the huge potential of its occurrence. The answer to the latter question lies in the many complex mechanisms that have evolved to sense and respond to any potentially tumorigenic cellular event. Those mechanisms represent a kind of innate tumor suppressor response and collectively make the incidence of tumours rather rare. For a cell to completely transform it has to overcome these transformation-limiting mechanisms. Therefore a single activating mutation is typically insufficient to induce carcinogenesis and cooperating transforming mutations of other proteins are required.

6.2 Ras-induced cell death as anti-proliferative response

Senescence, apoptosis and autophagic cell death are mechanisms that act as fail-safe responses in cells in response to potential tumorigenic events. Cellular senescence was shown to be triggered as an anti-proliferative response to oncogenic Ras expression in primary human cells (Serrano *et al.*, 1997). However, when oncogenic Ras is coupled with overexpression of c-myc, cells tend to bypass the senescence checkpoint and proceed to full transformation (Zhuang *et al.,* 2008). It has been shown that Ras expression alone is not sufficient to transform primary cells and other cooperating oncogenic alterations such as overexpression of c-myc, cyclin D1, Cdc25A and-B or the loss of p53, p16 or IRF-1 are required (Land *et al.*, 1983; Lovec *et al.*, 1994; Tanaka *et al.*, 1994; Galaktionov *et al.*, 1995; Serrano *et al.*, 1996)

Here we studied the consequences of mutational activation of Ras oncogene. We observed that mutational activation of Ras initially led to cell death in a big

percentage of the cells and was later followed by senescence induction in the cells that survived the initial elimination. Both responses may contribute to impeding cell growth and ultimately limiting the transformation of cells expressing oncogenic Ras.

6.3 Ras induces non-apoptotic cell death

Apoptosis is a process of programmed cell death that is distinguished by morphological and biochemical characteristic features. Deregulated expression of some oncogenes such as c-myc and E2F was shown to induce apoptosis (Prendergast, 1999, Thompson, 1998, Lowe *et al.*, 2004). Apoptosis is triggered under these conditions as a suicide program by which the cells that sense that they have become a threat commit themselves to an elimination program that aims to protect the overall well-being of the organism. As mentioned above, we have observed that the expression of oncogenic Ras triggers cell death. It was thus tempting to think that Ras, similar to some other oncogenes, triggers apoptosis. However, our initial morphological observations did not support this speculation as the cells dying upon expression of oncogenic Ras lacked the characteristic morphological features of apoptosis such as blebbing, loss of cell membrane asymmetry, nuclear fragmentation and chromatin condensation. Furthermore Ras-induced cell death did not correlate with the activation of caspases, another hallmark of apoptosis, suggesting that oncogenic Ras triggers another form of programmed cell death that is distinct from apoptosis. One possibility to explain why Ras triggers a non-apoptotic form of cell death is that activation of Ras evokes several anti-apoptotic signals downstream of its effector pathways PI3K and MEK/ERK as evading apoptosis is an essential perquisite for transformation.

Akt protects against apoptosis by phosphorylating a number of apoptosis regulators such as Bad leading to reducing its ability to interact with Bcl-2 and Bcl-xL (Datta *et al.*, 1997). Akt also phosphorylates caspase 9 leading to its inhibition (Fujita *et al.*, 1999). Akt has also been suggested to phosphorylate and

inactivate the AFX forkhead transcription factor leading to NF-kB release and the stimulation of expression of anti-apoptotic genes (Brownawell *et al.*, 2001).

The Raf/MEK/ERK pathway can also exert anti-apoptotic actions. Activation of the Raf/MEK/ERK pathway has also been shown to phosphorylate Bad on S112 which contributes to Bad inactivation and sequestration by 14-3-3 proteins (Fang *et al.*, 1999). The Raf/MEK/ERK pathway can also result in phosphorylation of Bim leading to its subsequent degradation by the proteasome (Sheridan *et al.*, 2008). In addition, Raf may contribute to the pro-survival response exerted by Ras by downregulating transcription of Par-4, a pro-apoptotic transcriptional repressor (Nalca *et al.*, 1999). Raf-1 was also shown to interact with mammalian sterile 20-like kinase (MST-2) (O'Neill *et al.*, 2004). Raf-1-MST-2 complex can also contribute to apoptosis inhibition by interacting and inhibiting with ASK1 (Du *et al*, 2004) ASK1 is a mediator of apoptosis in response to cytotoxic stress exerted by agents such as TNF, Fas and ROS (Allan *et al.*, 2003).

Collectively, such anti-apoptotic signals evoked by Ras can block apoptosis and lead the cell to activate another non-apoptotic program of cell death as an alternative cell death pathway.

6.4 Autophagy as a cell death program

The contribution of autophagy to cell death is controversial. A number of cell death pathways have been recognized. Cell death is classically classified according to morphological criteria into apoptosis and necrosis. Apoptosis is a programmed form of cell death with well-defined morphological traits such as cell retraction, membrane blabbing and chromatin condensation. These morphological features are often associated with activation of caspases. Necrosis is morphologically characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents (Duprez *et al.*, 2009). Autophagy is emerging a new form of

cell death that was coined the term "autophagic cell death". Accumulating evidences now suggest that autophagic cell death is an important physiological cell death process. This mode of cell death is characterized by massive degradation of cellular contents, including essential organelles such as mitochondria, by means of complicated intracellular membrane vesicle reorganization and lysosomal activity. Autophagic cell death has been suggested to be involved in development (Berry *et al.*, 2007) and has been observed in multiple neurodegenerative diseases (Ko *et al.*, 2005). Because the mechanism is not well defined, some autophagic cell death events might have been attributed to apoptosis. The link between apoptosis and autophagy is complicated and currently under intensive investigation. Autophagy has been proposed to protect from apoptosis (Pan *et al.*, 2009), act upstream of apoptosis to activate this cellular process (Laane *et al.*, 2009, Salazar *et al.*, 2009) or operate as an alternative cell death mechanism in cells that are defective in apoptosis (Rashmi *et al.*, 2008). Moreover, these two modes of cell death frequently occur in parallel (Mujumdar *et al.*, 2010). Both apoptosis and autophagic cell death are considered as programs of tightly regulated forms of cell death and was thus termed type I and type II programmed cell death respectively. However, necrosis has initially been suggested as an accidental form of cell death. More recent evidence has become available demonstrating that a form of programmed necrosis may also exist. This form of programmed necrosis is sometimes referred to by necroptosis to distinguish it from the mere accidental necrosis and to indicate a similarity with apoptosis in being an orchestrated death program (Duprez *et al.*, 2009). Interestingly, a link between programmed necrosis (necroptosis) and autophagy has recently been suggested. Bonapace and co-workers showed that induction of autophagy by Mcl-1 antagonist Obatoclax resensitized multidrug-resistant childhood ALL cells to glucocorticoids and other cytotoxic agents. The reversal of glucocorticoid resistance occurred through rapid activation of autophagy-dependent necroptosis, which bypassed the block in mitochondrial apoptosis. Execution of cell death, but not induction of autophagy, was strictly dependent on expression

of receptor-interacting protein (RIP-1) kinase and cylindromatosis (turban tumor syndrome) (CYLD), two key regulators of necroptosis (Vandenabeele *et al.*, 2010) as the inhibition of RIP-1 and interference with CYLD restored glucocorticoid resistance completely (Bonapace *et al.*, 2010).

These findings have changed our view of necrosis as a passive and essentially accidental form of cell death to that of an active, regulated and controllable process and presented a possible link between autophagy and necroptosis.
Thus, in conclusion cell death responses are extremely flexible despite being programmed and are possibly interconnected through common mediators and machineries.

6.5 Ras-induced cell death is mediated by autophagy

As we have observed that Ras-induced cell death was distinct from apoptosis, we sought to get further insight on the molecular mechanisms of Ras-induced cell death. Close observation of the cells dying in response to oncogenic Ras expression showed that cells exhibit massive cytoplasmic vacuolation before they round up, detach and eventually degenerate. Massive cytoplasmic vacuolation has been linked to the induction of autophagy in some contexts. Autophagy is the process of bulk degradation of cellular proteins through an autophagosomic-lysosomal pathway. During autophagy, autophagosomes (also known as autophagic vacuoles) form from the enclosure of cytoplasmic organelles and proteins by isolation membranes. These autophagic vacuoles then dock and fuse with lysosomes, allowing the lysosomal hydrolases to degrade the contents of the autophagic vacuoles. Autophagy is involved in the preservation of cellular nutrients under starvation conditions as well as the normal turnover of cytosolic components.

We set out to examine if Ras induces autophagy. A panel of typical markers of autophagy, including LC3I-to-LC3II conversion, p62 degradation, GFP-LC3 puncta formation, LC3 localization to the vacuoles membranes and positive

staining for the acidotropic agents, all confirmed that Ras induces strong autophagic activities. This raised an important question whether Ras-induced autophagy is linked to cell death or it is triggered to serve a pro-survival function in an attempt to maintain viability.

To test whether autophagy played a pro- or anti- cell death function in the context of Ras-induced cell death, we inhibited autophagy by separately knocking down three of the essential autophagy regulators Atg5, Atg7 or Beclin-1. Inhibition of autophagy diminished cell death suggesting that autophagy mediates Ras-induced cell death. Furthermore we found that Ras induces the expression of Beclin-1 through an ERK-dependent pathway. Ablation of Beclin-1 enhanced long term clonogenic survival of cells expressing oncogenic Ras suggesting that Beclin-1 may act to restrain Ras-induced transformation. Indeed, Beclin-1 has been shown to act as a tumor suppressor (Liang *et al.*, 1999) and a predictor of prognosis in patients with certain types of cancer. Higher expression levels of Beclin-1 correlated with positive prognosis in these types of cancer (Bao-Xiu *et al.*, 2009; Huang *et al.*, 2010).

6.6 Regulation of Ras-induced autophagy and cell death by the Bcl-2 family proteins

The essential role of the Bcl-2 family proteins in the regulation of apoptosis has been long established. Recently, Bcl-2 family proteins emerged as regulators of autophagy. Bcl-2 and Bcl-xL have been shown to bind and inhibit Beclin-1. Therefore we tested whether Bcl-2 family proteins play a role in Ras-induced autophagy and cell death. Screening for the modulation of protein levels of Bcl-2 family proteins showed that expression of Ras induces upregulation of the BH3-only protein Noxa. Noxa induction by Ras was found to be independent on p53 and to be regulated through an ERK-dependent pathway that involves the transcription factor ATF4.

Strikingly, we found that Noxa is essentially required for Ras-induced autophagy and autophagic cell death. Importantly, Noxa knockdown enhanced clonogenic survival of cells expressing oncogenic Ras. The observation that the pro-

apoptotic Noxa can promote another form of programmed cell death is intriguing but might be supported by the fact that Noxa exhibits only weak pro-apoptotic effect on its own.

The involvement of Noxa in Ras-induced autophagy and cell death led us to ask whether Ras-induced autophagic cell death can be modulated by the anti-apoptotic members of the Bcl-2 family. We found that forced expression of anti-apoptotic Bcl-2 proteins especially Mcl-1 and A1 inhibited Ras-induced autophagic cell death and enhanced long term clonogenic survival.

Concurrent with Noxa upregulation, Ras also induced Mcl-1 downregulation which we found to be important for the induction of autophagy and cell death as knockdown of Mcl-1 sensitized the cells to Ras-induced autophagy and autophagic cell death.

Immunoprecipitation of endogenous Beclin-1 or Mcl-1 before and after oncogenic Ras expression showed that Noxa competitively disrupts the interaction Beclin-1 and Mcl-1 after Ras expression liberating Beclin-1 from the inhibitory interaction with Mcl-1 to drive autophagy.

6.7 Regulation of Beclin-1 driven autophagy by Mcl-1

Previous reports showed that Bcl-2 and Bcl-xL bind and inhibit Beclin-1 (Pattingre *et al.*, 2005 and Maiuri *et al.*, 2007). While we found that forced expression of most anti-apoptotic Bcl-2 proteins inhibited Beclin1-driven autophagy, our results hinted to a particularly important role for endogenous Mcl-1 in regulating Beclin-1. Mcl-1 (along with A1) had the most efficient inhibitory effect on Ras-induced, Beclin-1 mediated autophagy and targeting Mcl-1 by Obatoclax induced autophagic cell death. In an attempt to understand the differential effect for Mcl-1 among the other anti-apoptotic Bcl-2 proteins on Beclin-1, we speculated that either endogenous Mcl-1 binds Beclin-1 more efficiently than other members of the family or the consequence of Mcl-1/Beclin-1 binding is different than when Beclin-1 binds Bcl-2 or Bcl-xL. Several proteins have been shown to be part of Beclin-1 complex that drives autophagy (reviewed in He *et al.*, 2010). Mcl-1 and A1 are unique among the anti-apoptotic proteins

for having bigger size and for almost exclusively binding Noxa. The stereochemical properties of Mcl-1 (and A1) might not favour the recruitment of some components of the Beclin-1 complex or by binding Noxa (which we demonstrated to be important for autophagy) Mcl-1 and A1 can inhibit autophagy. However these highly speculative possibilities remain to be tested. Surprisingly, while investigating this issue we found that Mcl-1 level in the cell impacted Beclin-1 in an inverse correlation. Ablation of Mcl-1 enhanced endogenous level of Beclin-1 and conversely overexpression of Mcl-1 diminished Beclin-1 level suggesting a link between Mcl-1 and Beclin-1 levels. Bcl-xL however did not seem to exert similar effect.

6.8 Mcl-1 is crucial for survival of melanoma cells harbouring Ras mutations

To test the observations we made in different cells upon overexpression of oncogenic Ras by another approach. We examined the expression levels of Beclin-1 and Noxa in two melanoma cells lines that harbour endogenous Ras mutations. We found that in agreement with our previous observations, endogenously active Ras mutations in these melanoma cell lines enhanced the constitutive expression levels of Beclin-1 and Noxa under the control of ERK in comparison to normal melanocytes or two other cell lines HeLa and HOSE. Strikingly, we found that these melanoma cell lines express constitutively high levels of Mcl-1 that we showed was crucial to keep Beclin-1/Noxa-mediated autophagy in check. Interestingly, silencing Mcl-1 induced spontaneous massive cell death that that resembled Ras-induced autophagic cell death in other cell lines. Cell death induced by silencing Mcl-1 in these melanoma cell lines was diminished when Beclin-1 was simultaneously knocked down together with Mcl-1.

6.9 Ras-induced autophagic cell death is linked to ER stress

ER stress has been linked to the induction of autophagy in some contexts. Our observation of the role of ATF4 in mediating Ras-induced autophagy through regulating the expression of Beclin-1 and Noxa and the established role for ATF4

in ER stress led us to ask whether ER stress is linked to Ras-induced autophagy. We found that oncogenic Ras expression induced markers of ER stress. Furthermore silencing ER stress regulators ATF4 or ATF6 diminished Ras-induced autophagy and cell death suggestive for a possible link between the two processes.

Appendix

Table 1: Plasmids used for work undertaken in this thesis

Plasmid name	Cloned by/Received from
pCDNA3	Invitrogen
pCDNA3-Ras^{V12G}	Laboratory of Prof. Julian Downward
pEF-Ras^{N17S}	Laboratory of Prof. Douglas Green
pEFmB-Raf^{WT}	Laboratory of Prof. Richard Marais
pEFmB-Raf^{V600E}	Laboratory of Prof. Richard Marais
pGSU6-scrambled shRNA	Laboratory of Dr Ameeta Kelekar
pGSU6-Beclin-1 shRNA	Laboratory of Dr Ameeta Kelekar
pBabe-N-Ras61K	Addgene
pBabe-K-RasV12	Addgene
pEGP-C3 p53	Laboratory of Dr Daniela Barcaroli
pcDNA3.HA p73 β	Laboratory of Prof Gerry Melino
pcDNA3.HA p73 α	Laboratory of Prof Gerry Melino
pRetroSuper-Mcl-1	Laboratory of Dr. Eric Eldering
pRetroSuper-p53	Oligoengine
pcDNA3-p53DD	Laboratory of Dr Carmen Marin
pRetroSuper-GFP	Laboratory of Dr. Eric Eldering
pRetroSuper-Noxa	Laboratory of Dr. Eric Eldering
pRetroSuper-Puma	Laboratory of Dr. Eric Eldering
pMKO1-Bad shRNA	Addgene
pMKO1-Bim shRNA	Addgene
pMKO1-Bmf shRNA	Addgene
pCDNA3-Bad	Dr Clare Sheridan (Martin Lab)
pCDNA3-Noxa	Dr Clare Sheridan (Martin Lab)
pCR3.1-Falg Beclin-1	Laboratory of Dr Beth Levine
pCDNA3-Flag Mcl-1	Dr Clare Sheridan (Martin Lab)

pCDNA3-Flag Bcl-xL	Dr Clare Sheridan (Martin Lab)
pCDNA3-Flag Bcl-2	Dr Clare Sheridan (Martin Lab)
pCDNA3-Flag Bcl-B	Dr Clare Sheridan (Martin Lab)
pCDNA3-Flag A1	Dr Clare Sheridan (Martin Lab)

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