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The p76^{Rb} and p100^{Rb} truncated forms of the Rb protein exert antagonistic roles on cell death regulation in human cell lines

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Abbreviations: eto, etoposide; E/TNF, emetine plus TNF-α; Rb, retinoblastoma protein; TNF, tumor necrosis factor; CMX-ros, chloromethyl-X-rosamine; ΔΨm: mitochondrial membrane potential.

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ABSTRACT

Several caspase-cleaved forms of the retinoblastoma protein have been described. Here, we compared the effect of full-length Rb versus the truncated $p76^{Rb}$ and $p100^{Rb}$ proteins on cell death regulation in five human cell lines. Interestingly, we observed that $p76^{Rb}$ triggers cell death in all tested cell lines and that $p100^{Rb}$ protects two cell lines against etoposide or TNF-α-induced cell death, whereas full-length Rb has no apoptotic effect. These results show that truncated forms of Rb can have specific activities in the regulation of cell death. They also suggest that caspase-cleavage of Rb should not be simply assimilated to a degradation process. Finally, we show that cell death induced by $p76^{Rb}$ is Bax-dependent and is diminished by Bcl-2 overexpression or by caspase inhibition and that $p100^{Rb}$ could inhibit cell death by decreasing both p53 stability and caspase activity.

Keywords: Retinoblastoma protein; Rb cleavage; caspase; apoptosis, transfection, GFP.

Introduction

The retinoblastoma protein (Rb) was the first identified tumour suppressor protein. Its loss of function is linked to the development of many human cancers. It has been shown that overexpression of Rb can induce growth arrest in a major G1 checkpoint blocking S-phase entry. The Rb protein governs cell cycle progression by controlling the expression of E2F-dependent genes in two ways: by directly binding and blocking the activation domain of E2F proteins or by active repression through chromatin remodelling.

Evidences for the importance of Rb in cellular differentiation and cell death come from studies of Rb knock-out mice, where the disruption of the Rb gene causes death by day 14 of gestation associated with defects in the development of the haematopoietic system, as well as in the central and peripheral nervous systems [1-3]. Interestingly, abolition of Rb function results in apoptosis involving both p53-dependent and -independent pathways [4, 5]. The transcription factor E2F-1 was proposed to be one of the possible links between Rb and p53. Indeed, free E2F-1 can transcriptionally activate $p19^{ARF}$, which stabilizes p53 by inhibiting the function of its regulator MDM2 [6]. Overexpression of Rb, which leads to a sequestration of E2F-1, can therefore inhibit apoptosis induced by p53.

A pro-apoptotic function of Rb has also been observed but is less well documented. Most of these studies show a direct role of Rb in the induction of apoptosis either by transcriptional activation or repression [7-9]. One model describes that Rb interacts with p53 and MDM2 in a trimeric complex that stabilizes both p53 and MDM2, enhancing p53-mediated transrepression which can trigger apoptosis [10]. More recently, two distinct Rb-E2F-1 complexes have been described, one of which activates transcription from pro-apoptotic genes to directly promote cell death [11]. These data suggest that the pro-apoptotic activities of Rb might be related to the complexes that it could form with its partners. Indeed,

our studies in drosophila have shown that the capacity of the drosophila homolog of Rb to be pro-apoptotic depends on the proliferation status of the cells in which it is expressed [12].

During apoptosis, Rb was shown to be cleaved by caspases, either at a C-terminal caspase-3-like consensus site generating a $p100^{Rb}$ form, lacking the last 42 amino-acids [13-15], or at an internal cleavage site producing two fragments of 68 and 48 kDa [16]. Transgenic mice containing a germline mutation (*RB-MI*) at the caspase cleavage site generating $p100^{Rb}$ were created. These mice exhibit resistance to apoptosis in selective tissues in response to specific death stimuli [17] and it was shown that this resistance involves a defect in Bid cleavage [18]. Recently, we identified the previously undescribed cleavage of Rb, at a LExD site, generating the $p76^{Rb}$ form, which antagonizes p53-induced apoptosis in rat embryo fibroblasts [19].

In this article, we compared the effect of full-length Rb versus $p76^{Rb}$ and $p100^{Rb}$, in several human cell lines. Interestingly, we report that although full length Rb does not have any apoptotic effect, $p76^{Rb}$ triggers cell death in the absence of any other apoptosis inducers in all the tested cell lines and $p100^{Rb}$ protects two cell lines against etoposide or TNF-α induced cell death. These results show that truncated forms of Rb can have specific activities in the regulation of cell death if compared to the full-length protein. Finally, we show on the one hand that $p76^{Rb}$-induced cell death is Bax-dependent and can be diminished by Bcl-2 overexpression or caspase inhibition and on the other hand that $p100^{Rb}$ could inhibit cell death by decreasing p53 protein level and caspase activity.

Materials and methods

Reagents. Emetine (E), mouse tumour necrosis factor-α (TNF-α) and etoposide (eto) were from Sigma. Chloromethyl-X-rosamine (CMX-ros) was from Molecular Probes. z-Val-Ala-DL-Asp-Fluoromethylketone (zVAD) was from Bachem. Stock solutions were prepared as follows: emetine and TNF-α in serum free medium, etoposide and CMX-ros in dimethyl sulfoxide (DMSO), zVAD in methanol. All stock solutions were stored at –20°C.

Cell lines, cell culture and induction of cell death. We used the following human cell lines: HT1080, HEK293T, SAOS-2, TC7 and HT29. All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and in DMEM/F12 medium supplemented with 10% fetal bovine serum together with penicillin (100 μg/ml), streptomycin (100 U/ml) and glutamax (1% v/v) from Invitrogen. Cell death was induced by the addition to exponentially growing adherent cells of etoposide or TNF-α plus emetine during 24h. The concentrations of apoptosis inducers were determined to induce between 30% and 60% cell death after a 24 h incubation. The following doses were used: 125 μg/ml etoposide for HEK293T, 30 μg/ml etoposide or 0.4 μg/ml emetine plus 2.5 ng/ml TNF-α for HT1080, 1.5 μg/ml emetine plus 12.5 ng/ml TNF-α for SAOS-2, HT29 and TC7. zVAD was used at 100 μM.

Plasmids. The GFP-Rb (pEGFPC1-Rb) construct was previously described [20] with Rb cDNA of human origin. cDNAs corresponding to truncated $p100^{Rb}$ and $p76^{Rb}$ were subcloned into pGEM-T vectors (Promega) after PCR amplification. PCR was performed on human wild type Rb coding sequence (NM_000321) contained in pEGFPC1-Rb plasmid. Primers used for PCR amplification are 5′-T*CTCGAG*CTATGCCGCCCAAAACCCCCCGAAAA-3′ and 5′-

G*AAGCTT***TCA**ATCTGCTTCATCTGATCCTTCA-3′ for p100Rb; 5′-T*CTCGAG*CT**ATG**ACAAGAATTATTGAAGTTC-3′ and 5′-G*AAGCTT*TCATTTCTCTTCCTTGTTTGAGGT-3′ for $p76^{Rb}$ (italic: XhoI or HindIII restriction sites, bold: start or stop codon introduced when necessary, underlined: specific Rb sequence). PCR was performed using standard methods with DyNAzymeTM EXT DNA polymerase (Finnzymes), performing 5 PCR cycles at 37°C (denaturation at 94°C for 30s, annealing at 37°C for 30 s and extension at 72°C for 3 min) followed by 25 PCR cycles at 55°C (denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 3 min), except for $p100^{Rb}$ amplification which required 30 cycles at 50°C instead of 25 cycles at 55°C. PCR products were extracted from agarose gel with JETsorb kit and ligated into pGEM-T vector using T4-DNA-ligase according to manufacturer's instructions (pGEM-T kit, Promega). pGEM-T-$p100^{Rb}$ and -$p76^{Rb}$ vectors were next digested using XhoI and HindIII enzymes and purified $p100^{Rb}$ and $p76^{Rb}$ fragments were ligated into XhoI/HindIII linearized pEGFPC1 vector (Invitrogen). Fusion cDNA sequences were checked by sequencing both strands using appropriate primers (MWG biotech). The use of pEGFPC1 vector conferred a GFP tag at the N-terminus of Rb, $p76^{Rb}$ and $p100^{Rb}$.

Transfection. The nucleofection technique was performed according to the manufacturers' instructions (Amaxa) and cells were transfected with the following combinations of nucleofection solutions and programs: HEK293T (L / A-020), SAOS-2 (V / D-024), TC7 (T / T-030), HT29 (R / W-017). The HT1080 cells were transfected with the lipofectamin 2000 technique according to the manufacturer's instructions (Invitrogen). Induction of cell death was performed 24 h after the beginning of transfection.

SiRNA treatments. Cells were seeded 24 h before transfection with control non silencing (QIAGEN) or Bax siRNA (Hs BAX 10 HP validated siRNA, QIAGEN). Lipofection was made using HiPerfect reagent (QIAGEN) and 25 nM siRNA according to the manufacturer's instructions. The cells were incubated with siRNA for 24h at 37°C before transfection with GFP-Rb or GFP-p76^{Rb} encoding vectors, as described above. Experiments (protein extraction for western-blot analysis and flow cytometry analysis) were carried out 24h to 48h later.

Flow cytometry. Flow cytometric measurements were performed using a XL3C flow cytometer (Beckman-Coulter). Cells expressing GFP-tagged proteins were identified by the emission of green fluorescence (525 nm) and mitochondrial membrane potential ($\Delta\Psi m$) was assessed by the retention of CMX-ros (620 nm). This fluorochrome, at low doses, specifically accumulates into the mitochondria depending on $\Delta\Psi m$. As a drop in $\Delta\Psi m$ characterises all types of cell death [21], cell viability was determined by measuring the percentage of cells with low CMX-ros fluorescence. Cell death was also checked by assessing the decrease in cell size / forward scatter that reflects cell shrinkage during cell death. These measurements were performed in GFP positive cells in order to characterize the effect of exogenous proteins on cell viability. The expression level of the fusion proteins was systematically assessed by measuring GFP fluorescence intensity .Briefly, the cells were trypsinized and pooled with their corresponding medium and centrifuged (200g, 5 min). Cells were then resuspended in complete medium with 150 μM CMX-ros and analyzed under FACS after 30 min incubation at 37°C.

Western blot analysis. Western blotting was performed as previously described [22]. The primary antibodies used were mouse monoclonal anti-p53 (Pab 122, gift from Dr. E.

May, IRSC, Villejuif, France), anti-MDM2 (SMP40, Santa Cruz), anti-enolase (gift from N. Lamande, College de France, Paris), anti-cleaved PARP (Asp214, BD Pharmingen), anti-Rb (G3-245, BD Pharmingen), rabbit polyclonal anti-p53-P (Ser-15, Santa Cruz), anti-caspase 9 (Cell Signaling) and anti-cleaved caspase-3 (Asp175, Cell Signaling) as well as goat polyclonal anti-PUMA-α (N-19, 1:200, Santa Cruz). Secondary antibodies (peroxidase-conjugated) were anti-mouse, anti-rabbit or anti-goat immunoglobulin (Biosystem). Immunoreactive spots were detected by ECL or ECLplus kits depending on signal intensity (Amersham).

Results

Our aim was to characterise potential roles of $p100^{Rb}$ and $p76^{Rb}$ in several human cell lines. To perform this study, we have used GFP-tagged proteins in order to assess cell viability only in cultured cells that were successfully transfected. We have also used cell lines with distinct p53 status (summarized in Fig. 1A) in order to test if $p100^{Rb}$ and $p76^{Rb}$ activities depended on p53. Each cell line was transfected with vectors encoding the three forms of Rb (full length Rb, $p100^{Rb}$ and $p76^{Rb}$) and 24 hours later, the cells were incubated or not with apoptosis inducers (emetine + TNF-α (E/TNF) or etoposide) during another 24 hours. The cells were then subjected to flow cytometric analysis to evaluate their viability (see Materials and Methods). Etoposide is a topoisomerase II inhibitor which can trigger the intrinsic pathway of apoptosis [23] whereas TNF-α is a cytokine which can trigger the extrinsic pathway of cell death [24]. TNF-α was used in combination with emetine, an inhibitor of protein synthesis which (at non toxic concentrations) potentiates the pro-apoptotic effect of this cytokine [25, 26]. HT29, TC7 and SAOS-2 were not sensitive to etoposide (probably because of their deficient p53 status) whereas HEK293T cells were insensitive to E/TNF (data not shown). The HT1080 cell line was the only one to be sensitive to inducers of both intrinsic and extrinsic apoptotic pathways. After drugs incubation, cellular viability was measured on GFP positive cells by characterizing the mitochondrial membrane potential ($\Delta\Psi m$) which has been shown to decrease during apoptosis, autophagy or necrosis [21]. Cell shrinkage was also assessed by flow cytometry to confirm the results (data not shown).

Fig. 1A shows that the full-length Rb protein has no significant effect (if compared to GFP alone) on cellular viability, in the presence or in the absence of apoptosis inducers, in all tested cell lines. This is also the case for $p100^{Rb}$ in the absence of apoptosis inducers. Interestingly, we show here that $p76^{Rb}$ significantly induces cell death on its own in the five

tested cell lines (compare GFP or GFP-Rb to GFP-p76^{Rb}). This suggests that the effect of p76^{Rb} is independent of the status of p53. p76^{Rb} also increases the level of cell death in the presence of apoptosis inducers, except in HT29 cells. We have also observed that p100^{Rb} inhibits apoptosis in HT1080 + etoposide and SAOS-2 + E/TNF (compare GFP or GFP-Rb to GFP-p100^{Rb}). The results obtained with the three forms of Rb are very interesting since they show that the truncated forms of Rb could have new and specific activities. Fig. 2B shows that the effects of these proteins are not related to a difference in their expression levels, indicated by the measurements of GFP fluorescence intensities. The fact that p76^{Rb} appears to be a little less expressed may be due to its specific toxicity.

In order to better understand how p76^{Rb} and p100^{Rb} regulate cell death, we tried to characterize several aspects of their mechanism of action. Fig. 2A shows that the broad spectrum caspase inhibitor zVAD significantly inhibits p76^{Rb} induced cell death in TC7 and HT29 cell lines, demonstrating that this cell death can be caspase-dependent. We have also used a variant of the SAOS-2 cell line conditionally overexpressing *bcl-2* (Tet off system from Clontech) and showed that Bcl-2 significantly inhibits p76^{Rb} induced cell death (Fig. 2B). Finally, we have tested the effect of Bax silencing on p76^{Rb} induced cell death in HT1080 cells. Fig. 2C (left panel) shows the efficiency of the siRNA treatment and points out that p76^{Rb} is able to trigger the accumulation of the pro-apoptotic Bax protein. Interestingly, it can also be observed from the corresponding analysis of cellular viability (right panel) that Bax siRNA decreases p76^{Rb} induced cell death, showing that it is Bax-dependent. Taken together, these results strongly suggest that GFP-p76^{Rb} triggers cell death by inducing the mitochondrial (intrinsic) pathway of apoptosis.

Next, we have investigated the mechanism underlying the anti-apoptotic role of p100^{Rb} in the HT1080 cells incubated with etoposide. We show in Fig. 3 that p100^{Rb} inhibits the accumulation of both total and the active p53 (phosphorylated on serine 15), triggered by

etoposide. Interestingly, this phenomenon occurs very early (between 5 and 8h of incubation) and is no more visible after 16h (coinciding with a decrease in $p100^{Rb}$ amount). Nevertheless, it seems to have consequences on the p53 transcriptional target and pro-apoptotic protein PUMA since its accumulation is specifically inhibited afterward. Interestingly, $p100^{Rb}$ also seems to inhibit processing of caspase-9 and caspase-3 as well as caspase-3 activity, visualised by PARP cleavage, after 16 h of incubation. It is thus tempting to hypothesize that the upstream events seen with p53 stability are responsible for the caspase inhibition and the anti-apoptotic activity of $p100^{Rb}$.

Discussion

Here, we have shown that $p76^{Rb}$ and $p100^{Rb}$ have specific roles on cell death regulation when compared to full-length Rb. In particular, we observed that $p76^{Rb}$ induces cell death in the five tested human cell lines, probably via the activation of a mitochondrial-dependent apoptotic pathway. We have also observed an anti-apoptotic activity of $p100^{Rb}$ in two cell lines. We have shown in the HT1080 cell line that $p100^{Rb}$ interferes with the stability and activity of the p53 protein whereas this mechanism appears impossible in SAOS-2 cells, since they are p53-deficient. This matter of fact strongly suggests that $p100^{Rb}$ could act at multiple levels to inhibit cell death. Indeed, if we consider that $p100^{Rb}$ inhibits both p53 and caspase activity, we can hypothesize that the inhibition of caspase activity could be partly independent from the effect on p53. Concerning the interplay between $p100^{Rb}$ and p53, two partners of Rb were already described to be involved in the regulation of p53 stability: MDM2 and E2F-1 [6, 27]. First, it has been reported that $p100^{Rb}$ exhibit an enhanced E2F binding [14], suggesting that $p100^{Rb}$ could inhibit E2F-1-dependent expression of $p19^{ARF}$ and consequently p53 stability [6]. Second, it has been shown that, in contrast to full-length Rb, $p100^{Rb}$ cannot interact with MDM2 [13]. Thus, the C-terminal cleavage of Rb could prevent the formation of the Rb-MDM2-p53 trimeric complex which is known to stabilize p53 [10]. In conclusion, implications of both E2F-1 and MDM2 proteins could be in agreement with the anti-apoptotic role of $p100^{Rb}$.

Finding that $p76^{Rb}$ significantly induces cell death in all tested human cell lines is interesting since it suggests that this effect is not cell type dependent. The effect of $p76^{Rb}$ is probably independent from p53 since it occurs in both wild type (HT1080) and p53-deficient (TC7, HT29, SAOS-2) cell lines (see Fig. 1A). However, we cannot exclude that several pathways could be triggered by $p76^{Rb}$, some pathways being p53-dependent or independent. Finally, by using GFP-tagged proteins, we have observed that full-length Rb, $p100^{Rb}$ and

$p76^{Rb}$ are located into the nucleus (data not shown). The fact that these three forms of Rb exhibit distinct activities suggests that they interact with different partners into this compartment to regulate the mitochondrial-dependent pathway of apoptosis. Thus a differential regulation of gene expression is probably central to the activity of the $p76^{Rb}$ and $p100^{Rb}$ truncated forms of the retinoblastoma protein. However the specific partners and the precise mechanism of this regulation remain to be determined.

The role of full length Rb in cell death regulation is complex since Rb was shown to exert opposite effects, depending on the cellular context. Our study suggests that such a complexity could exist for truncated forms of Rb since we have shown a few years ago that $p76^{Rb}$ can inhibit a p53-dependent cell death in immortalized or primary rodent fibroblasts [19]. The pro-apoptotic effect of $p76^{Rb}$ was first found in the human HEK293T cell line in which p53 may be inactivated by the presence of two viral oncoproteins: E1B and LT. Thus we have hypothesized that a difference in p53 activity could be responsible for the discrepancies between our two studies. However, we have tested the effect of $p76^{Rb}$ on human cell lines with distinct p53 status, and found that $p76^{Rb}$ was intrinsically and systematically pro-apoptotic. Since this new activity of $p76^{Rb}$ was a general observation in human cell lines, we now postulate that cell death regulations by the Rb pathway might be different between rodent and human cells, explaining the opposite roles of $p76^{Rb}$ in our two independent studies. Such an hypothesis would be in agreement with several of our previous studies, which showed that caspase inhibition can paradoxically accelerate cell death in rodent cells [19, 28-30] whereas we have never retrieved this effect in human cells. Finally, we are currently using *Drosophila melanogaster* in our laboratory as an *in vivo* model to study the role of truncated forms of the Rb homolog. Our results suggest that $p76^{Rb}$ accelerates cell death in specific tissues if compared to full length Rb (unpublished data), reinforcing the significance of the present study.

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Legends to figures

Fig. 1. Effect of GFP-p76^{Rb} and GFP-p100^{Rb} versus GFP-Rb and GFP on cellular viability in five human cell lines. (A) The four vectors encoding GFP, GFP-Rb, GFP-p76^{Rb} and GFP-p100^{Rb} were transfected in human cell lines with different p53 status (in HEK293T cells, two oncoproteins are present: E1B from the adenovirus and LT from SV40 which inhibit at least partially p53). The cells were incubated or not with apoptosis inducers (24h) and the cellular viability was determined by flow cytometric analysis (see Materials and Methods). *Statistically significant difference in the viability of cells transfected with vectors were indicated (GFP-Rb versus GFP; GFP-p76^{Rb} or GFP-p100^{Rb} versus GFP-Rb). Statistical analysis was determined using Student's t test. $P < 0.05$ was accepted as significant. (B) The relative GFP intensity was measured in each experimental condition to assess the expression level of the fusion proteins.

Fig. 2. Regulation of p76^{Rb}-induced cell death. All cell lines used here were transfected with the GFP and GFP-p76 encoding vectors. The TC7 and HT29 cells were incubated with the caspase inhibitor zVAD (100 μM) (A). The conditionally overexpressing *bcl-2* / SAOS-2 cell line (Tet off system, Clontech) was used to evaluate the effect of Bcl-2 (B). The HT1080 cells were treated or not with Bax siRNA and western blots of Bax and enolase were performed (C). In all cases, percentage of cell death was measured by flow cytometric analysis. *Statistically significant difference in the viability of cells transfected with vectors were indicated (-zVAD versus +zVAD, -Bcl-2 versus +Bcl-2). Statistical analysis was determined using Student's t test. $P < 0.05$ was accepted as significant.

Fig. 3. Effect of $p100^{Rb}$ on p53 stability and caspase activation. The HT1080 cells were transfected with the GFP and GFP-$p100^{Rb}$ encoding vectors and were incubated 24 hours later for different times with etoposide. Western blots were performed to assess the role of $p100^{Rb}$ on p53 accumulation and activity, as well as caspase activation and activity.

Figure 1

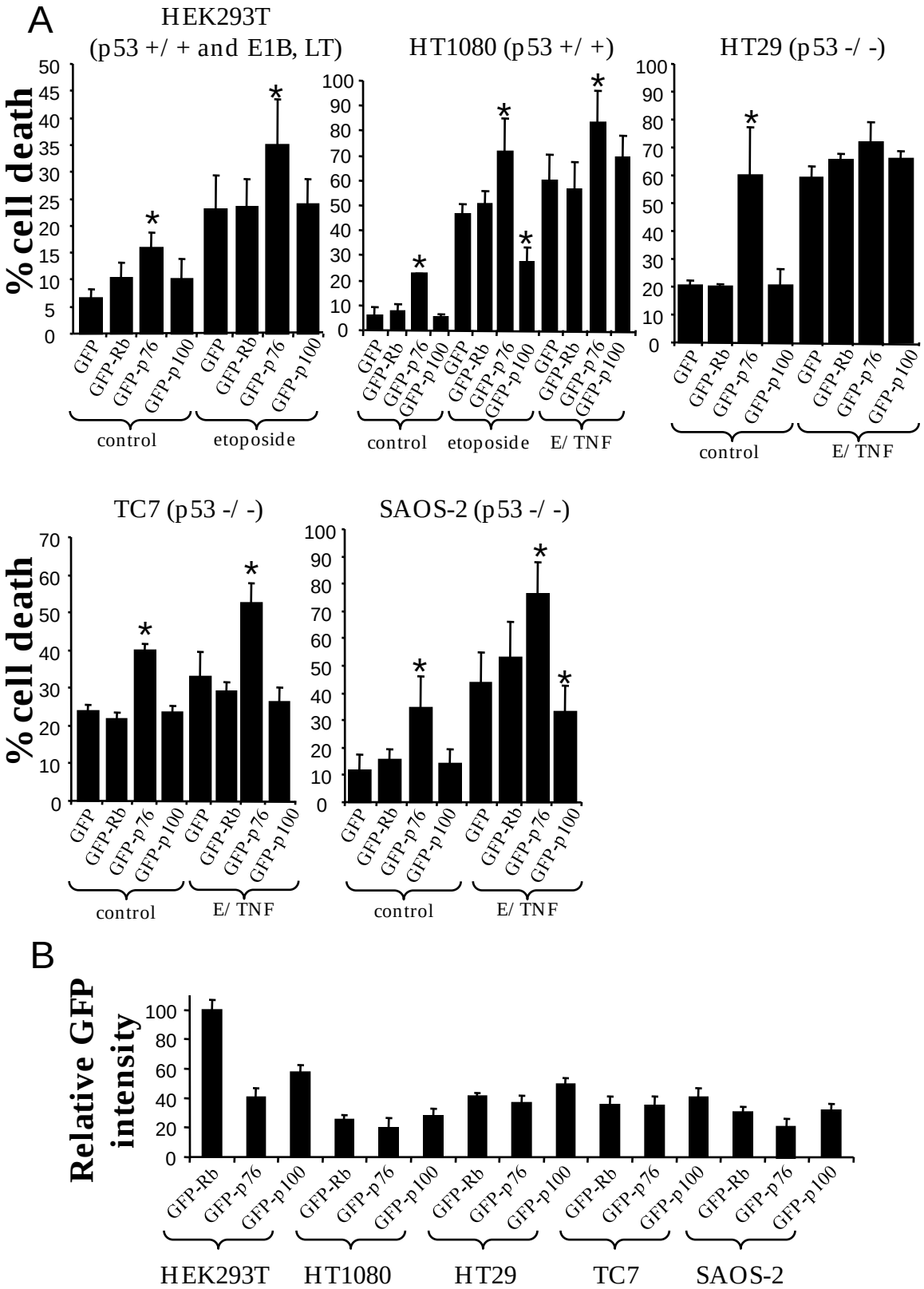

Figure 1

Figure 2

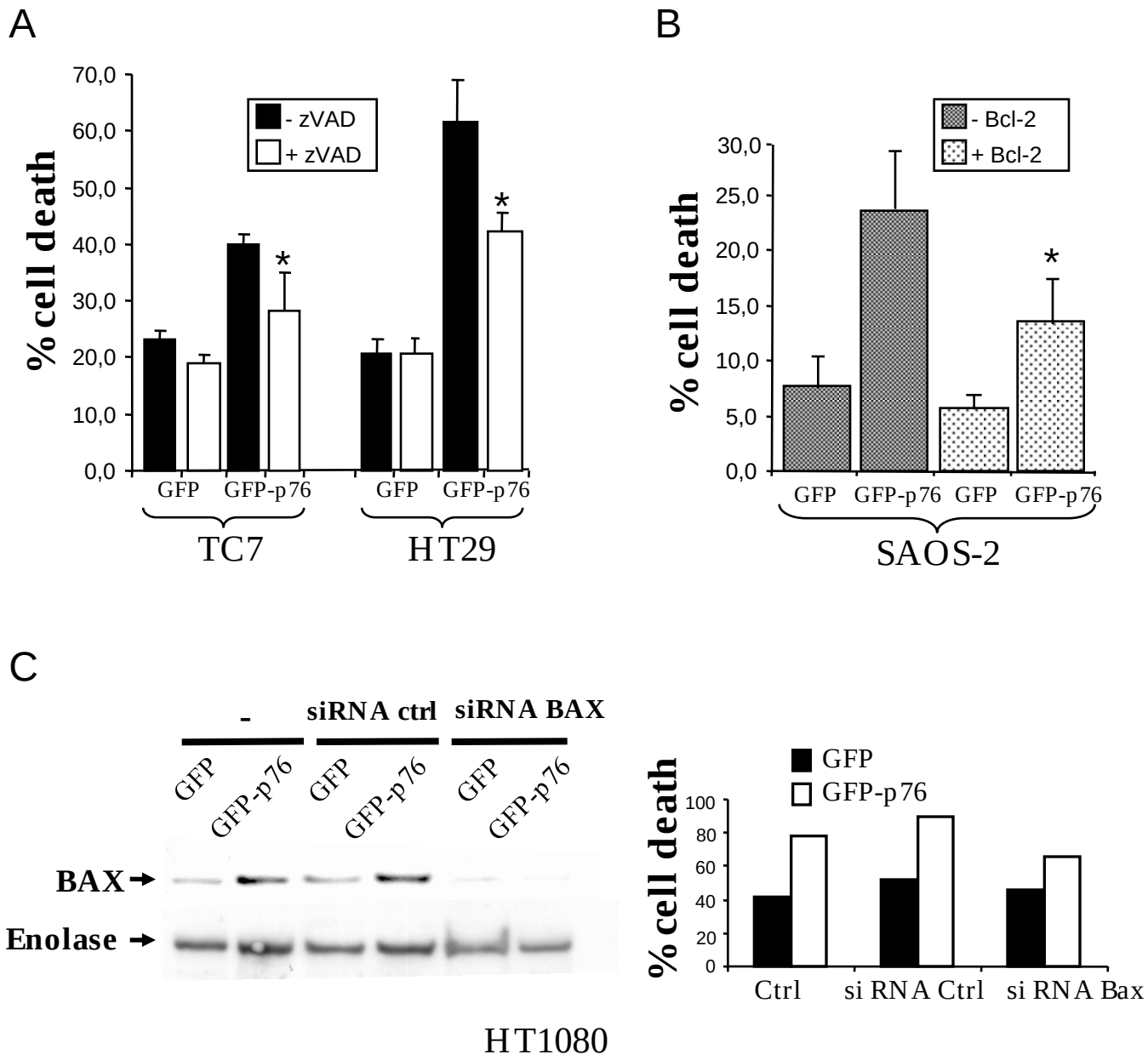

Figure 2

Figure 3

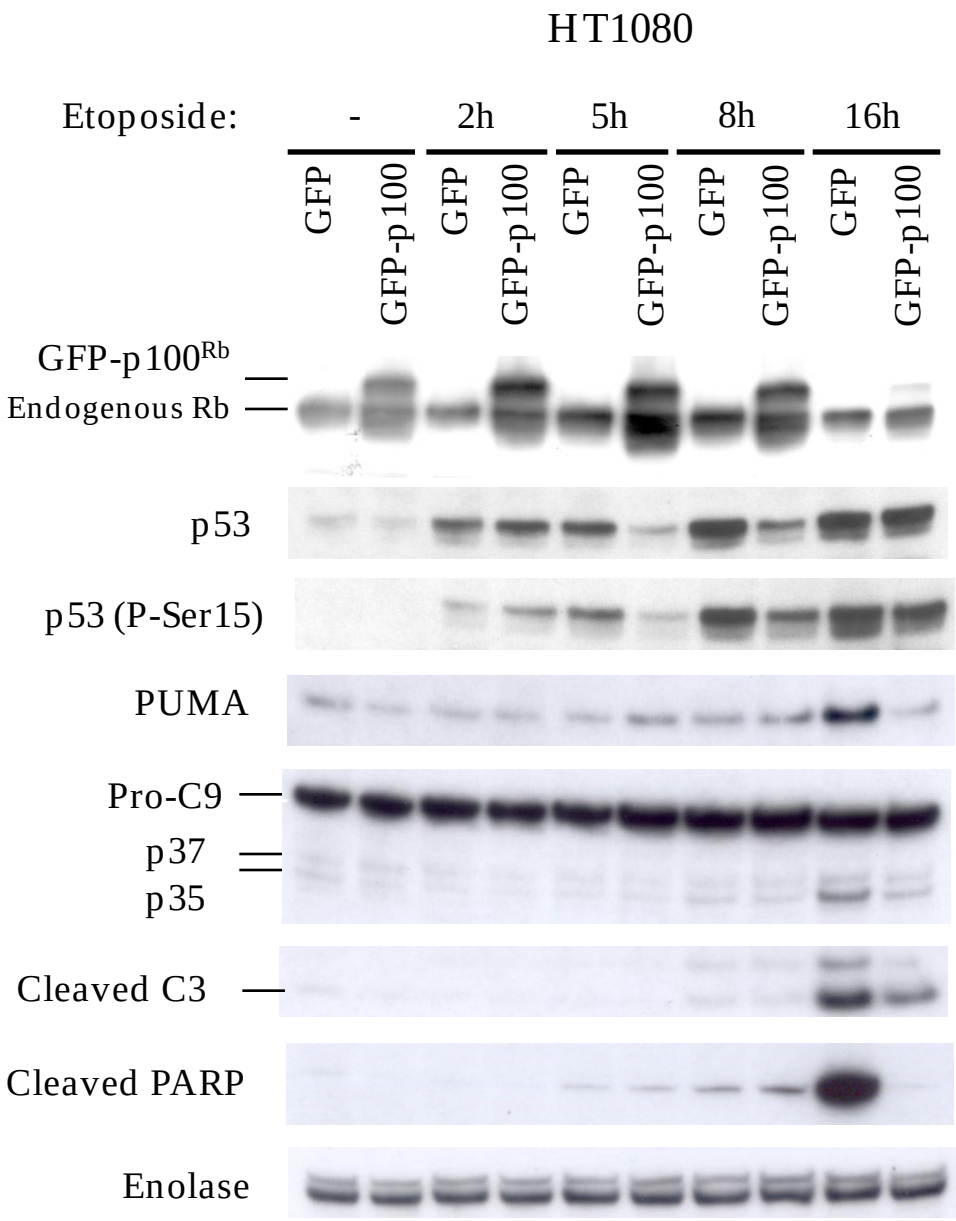

Figure 3