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Involvement of Noxa in cellular apoptotic responses to interferon, double-stranded RNA and virus infection

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Running Title: Viral and dsRNA induction of Noxa

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Double-stranded RNA (dsRNA) accumulates in virally infected cells, leading to induction of genes encoding proteins involved in signaling, apoptosis, protein synthesis/processing and cell metabolism. Noxa is a BH3-containing mitochondrial protein that contributes to apoptosis by disrupting mitochondrial outer membrane integrity. Here we demonstrate potent induction of Noxa expression by exposure of cells to dsRNA, interferon (IFN) and virus. Noxa induction was confirmed by using RT-PCR and immunoblot analyses in multiple human tumor cell lines. Importantly, Noxa regulation by IFN and dsRNA was independent of p53, thereby identifying a novel mechanism of Noxa induction. Ectopic expression of Noxa in HT1080 fibrosarcoma cells enhanced cellular sensitivity to viral or dsRNA/actinomycin D-induced apoptosis, typified by enhanced cytochrome C release from the mitochondrial to the cytosolic fraction and increased cleavage of caspases 3 and 9. Point and deletion mutations of Noxa confirmed that both the BH3 domain and the mitochondrial-targeting domain (MTD) were necessary for enhanced cellular apoptotic responses to dsRNA, IFN or virus. Treatment of cells with dsRNA or virus, but not etoposide, induced interaction between Noxa and Bax that required an intact Noxa BH3 domain. Interestingly, the Noxa MTD deletion mutant interacted with Bax in a dsRNA-dependent manner and redirected Bax away from the mitochondria, thus acting as a dominant negative protein. Together, these data suggest that Noxa is an important component of the innate immune response of cells to viral infection, leading to enhanced cellular apoptosis that may play a role in limiting viral dissemination.

To control viral infection, the host has developed an integrated defense network that comprises the innate and adaptive immune responses. Initial responses to viral infection involve primarily the innate arm of immunity and the killing of infected cells with cytotoxicity. Once the virus has invaded the cell, this host-mediated response is triggered that involves the induction of interferons (IFNs) (1,2). Both single-stranded and double-stranded RNA (dsRNA) accumulate in infected cells during virus replication where they induce IFNs and other proinflammatory cytokines (3). The IFNs are essential for initiating and coordinating a successful antiviral response by inducing a number of intracellular genes that directly prevent virus replication/cytolysis and stimulate the adaptive arm of the immune system (3). DsRNA can also induce directly a subset of innate immune genes that mediate antiviral/immunomodulatory responses (4).

Cellular mechanisms for preventing viral replication, dissemination or persistent infections include global inhibition of protein synthesis, blockage of protein transport and induction of apoptosis. A number of cellular proteins have been implicated as mediators of virus-induced apoptosis, including members of the interferon-regulatory factor (IRF) family, the dsRNA-dependent protein kinase R (PKR), ribonuclease L, TRAIL/Apo-2L, X-

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linked inhibitor of apoptosis-associated factor-1 and others (1,5-8). It is hypothesized that these proteins function individually and in concert to induce cellular apoptosis prior to completion of viral replication, thereby preventing viral spread. However, the exact mechanisms regulating IFN-induced or virus-induced cellular apoptosis are still under investigation.

Noxa is a Bcl-2 homology 3 (BH3) – only member of the Bcl-2 family of proteins (9). Known also as ATL-derived PMA-responsive gene (APR) (10), expression of Noxa is induced by phorbol ester, p53, ultraviolet (UV) radiation and other DNA damaging agents such as etoposide or adriamycin (9-13). Noxa is a critical mediator of the p53-dependent apoptosis (9) and has recently been implicated in hypoxia-induced apoptosis (14). The human Noxa gene is located on chromosome 18q21 and encodes a 54 a.a. protein with a single BH3 motif, which is in contrast to the murine Noxa protein that has two putative BH3 domains (9). The human and murine proteins also contain mitochondrial targeting domains (MTD) (9, 15). As a BH3-only member of Bcl-2 family, Noxa is proposed to mediate apoptosis by interacting with other pro- or anti-apoptotic Bcl-2 family members (such as Bax or Bak) directly or indirectly to promote mitochondrial membrane changes that lead to membrane permeabilization and efflux of apoptogenic proteins (16-18). The exact mechanisms of Noxa induced apoptosis remain undefined, including whether Noxa secondary modification is required for activation.

Recent gene profiling experiments have implicated human Noxa as an IFN- and dsRNA-stimulated gene (19-22). Here we confirm that human Noxa mRNA and protein are induced by IFN, dsRNA and viral stimulation, and that induction correlates with sensitization of cells to apoptotic signals. We show also that induction is independent of p53, but does require prototypical IFN- and dsRNA-response pathways. Our studies suggest that the apoptotic function of Noxa contributes to viral-induced cell death and IFN-dependent sensitization of cells to other pro-apoptotic agents, and further highlight the association between the innate immune system and cellular apoptotic machinery.

Materials and Methods

Cell lines, plasmids, transfection and retrovirus production: HT1080 fibrosarcoma, HT1080/GSE56, HT1080/LXSN (23), A375 and WM9 melanoma cell lines were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 5% bovine growth serum (Hyclone). PC-3 and LNCaP prostate tumor cell lines were maintained in Ham's F12K medium and RPMI 1640 medium, respectively, both supplemented with 5% bovine growth serum. A full-length human Noxa cDNA was cloned from dsRNA-treated HT1080 fibrosarcoma cell total cellular RNA (tcRNA) by using RT-PCR, and subsequently sequence confirmed as wild type Noxa. The cloned Noxa cDNA was inserted into expression vector pcDNA3.1-myc-HIS (-) (Invitrogen) under the control of a CMV promoter, yielding plasmid pcD-Noxa. Mutant Noxa constructs were generated from the pcD-Noxa parental plasmid by using the Transformer™ site directed mutagenesis kit (BD Biosciences). Noxa BH3-D contained a deletion of the BH3 domain (a.a. 29-37), while BH3-PM contained a leucine to alanine mutation at position 29 within the BH3 domain. Noxa MTD-D had the entire MTD deleted (a.a. 41-50). Transient and stable transfections were performed using Lipofectamine reagent (Invitrogen). To generate stable transfectants, transfected cells were cultured in G418 for 3 weeks, after which individual clones were isolated and Noxa expression confirmed by using Western blotting with a Noxa monoclonal antibody (Alexis Biochemicals).

For retroviral expression of Noxa antisense, the Noxa cDNA was cloned in an antisense orientation into the pBabepuro vector and infectious retrovirus particles produced by using GP+ENV AM12 amphotropic packaging cells. HT1080 fibrosarcoma cells were infected at an m.o.i. = 0.5 and then placed into puromycin selection medium (1 μg/ml) for one week. Individual clones were expanded and screened for reduced basal and dsRNA-induced Noxa expression by using RT-PCR and Western blot analysis.

Cell treatments: Stocks of dsRNA (2 mg/ml) were prepared by dissolving poly(I) poly(C) (Amersham Pharmacia Biotech) in PBS at 50˚C for 1 h. For dsRNA treatment, cells were washed with PBS and serum-free DMEM and then dsRNA added to the medium at the desired final concentrations for the

indicated times. For apoptosis induction, cells were co-treated with actinomycin D (Act. D; 6 ng/ml final concentration). Encephalomyocarditis virus (EMCV) or vesicular stomatitis virus (VSV) were adsorbed to cells at various m.o.i. for 1 h in serum free medium, followed by addition of complete medium for the indicated times. IFN-β (Rebif; Serono SA) was used at a final concentration of 500 U/ml for the indicated times. Etoposide was dissolved in DMSO and used at a final concentration of 4 μg/ml in serum-free medium for 6 h. Phorbol-12-myristate-13-acetate (Calbiochem, EMD Biosciences) was dissolved in DMSO and was used at a final concentration of 300 nM in serum free medium for 3 h.

RNA isolation and reverse transcriptase-coupled polymerase chain reaction (RT-PCR): Total cellular RNA was isolated by using TRIzol reagent (Invitrogen), reverse transcribed (2 μg) using Moloney murine leukemia virus RT (Promega) and random hexamer primers in a total volume of 20 μl at 42˚C for 60 min. One tenth of the RT reaction was subjected to PCR analysis using primer pairs specific for Noxa (5'-CGAGAATTCGAGATGCC TGGGAAG-3' and 5'-CTTGGTACCGGTTCCTGA GCAG-3') and GAPDH (5'-AAATCC CATCACCAT CTTCC-3" and 5'-GTCCACCACCCTGTTGCTGC-3'). PCR products were resolved on 1% agarose ethidium bromide gels, bands visualized on a digital camera (Eastman Kodak) and images inverted for presentation to give dark bands on a light background.

Immunoblotting, cell fractionation and cytochrome c release: For immunoblot analysis of protein expression, cell lysates were prepared as described (22). One hundred µg of protein was resolved on 12.5% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. Noxa detection was carried out with monoclonal antibodies against Noxa (Alexis Biochemicals), p53 (Santa Cruz Biotechnology), cytochrome oxidative complex 4 (COX4) (Molecular Probes), cytochrome c (BD PharMingen), Bax (BD PharMingen), c-myc (Invitrogen) or actin (Sigma) respectively. Bands were visualized with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody (Santa Cruz Biotechnology) and detected by using chemiluminescence (Pierce) followed by autoradiography.

To obtain mitochondria and cytosolic fractions, cells left untreated or treated with dsRNA plus Act. D were washed once in cold phosphate buffered saline (PBS), resuspended in 6 volumes of mitochondria isolation buffer (320 mM sucrose, 1mM K-EDTA, 10 mM Tris-HCl pH 7.4, 1 mM PMSF, 1 μg/ml leupeptin, 21 μg/ml pepstatin, 31 μg/ml aprotinin) and homogenized in an ice cold tissue grinder with 10 strokes. The homogenate was centrifuged at 500 g for 15 min at 4˚C. The resulting supernatant was then twice centrifuged at 17,000 g for 15 min at 4 ˚C and the supernatant collected as the cytosolic fraction. The pellet was incubated in 50 μl lysis buffer for 15 min on ice, and then clarified by centrifugation at 13,000g for 4 min. Protein concentration was measured and equal amounts of protein were analyzed by using SDS-PAGE. Immunoblotting was used to determine the relative expression of cytochrome c, COX 4, Noxa and actin in the different fractions.

Proliferation, cell viability and apoptosis assays: For growth assays, cells were seeded at a density of 2,000 per well in 96-well plates. After 12 h, cells were treated with dsRNA at the indicated concentration with or without 6 ng/ml Act. D. co-treatment for 48 h. Alternatively, cells were plated at 10,000 cells/well and infected with EMCV at the indicated m.o.i. for 24 h. Surviving cells were gently washed with PBS and fixed with 10% trichloroacetic acid (Sigma) for 1 h at 4˚C. Cells were then stained with 0.4% SRB (sulforhodamine B, ICN Biomedicals) at room temperature for 1 h, the plates washed with 1% acetic acid and air-dried. Dye was eluted in 10 mM unbuffered Tris (Fisher) and the absorbance read at 490 nm on a V-max microplate reader (Molecular Devices). Absorbance data were used to calculate relative cell growth as described previously (8). Treatments were conducted in triplicate and the mean values ($\pm$ SD) of a typical experiment are presented. All experiments were conducted a minimum of three times.

Trypan blue exclusion studies were carried out as described above, with the exception that cells were plated at 20,000 cells/ml in 12-well dishes and viable cells counted at the end of the treatment period. Cells were trypsinized, stained with 0.4 %

trypan blue solution for 5 min at room temperature and unstained cells counted on a hemacytometer. Data were again presented as relative cell numbers (compared to untreated controls).

To assess apoptosis, cells were left untreated or were treated with dsRNA (50 μg/ml) combined with actinomycin D (6 ng/ml) or IFN-β (500 U/ml),. or treated with EMCV (M.O.I = 0.3) for the indicated times. After treatment, cell lysates were prepared and poly ADP-ribose polymerase (PARP; BD PharMingen), caspase 3 (Cell Signaling) or caspase 9 (BD PharMingen) cleavage were assessed by using Western blot analysis.

Noxa/Bax interaction studies: Cells expressing Noxa protein and the various Noxa mutant proteins were left untreated or treated with dsRNA (50 μg/ml, ±6 ng/ml Act. D), EMCV (M.O.I.= 0.3) or etoposide (4 μg/ml) for 6 or 12 h. After that time cells were lysed (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM Imidazole and 20 mM Tris pH 7.9) and lysates cleared by centrifugation at 13,000g for 4 min. PBS equilibrated 50% Ni-NTA slurry (Qiagen) was added to the cleared lysates (50 μl/ml) and mixed gently by shaking at 4°C for 2 h. After two washes (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM Imidazole) bound proteins were resolved by SDS-PAGE and Bax, Noxa and Noxa mutant proteins detected by immunoblotting with specific antisera.

RESULTS

Several recent oligonucleotide gene array profiling studies had implicated human Noxa as an IFN-regulated gene in HT1080 fibrosarcoma (19), WM9 and WM35 melanoma cell lines (20) and in isolated peripheral blood mononuclear cells (21). Noxa was also implicated as a dsRNA-induced gene in HT1080 fibrosarcoma cells (22) and as an IRF-3 regulated gene in Jurkat cells (24). To confirm Noxa induction by these stimuli, HT1080 cells were treated for the indicated times with 500 U/ml IFN-β and/or 50 μg/ml dsRNA. As a positive control, cells were treated in parallel with the phorbol ester TPA, a known regulator of Noxa expression (10). Noxa mRNA induction was observed with all treatments, but was maximal with dsRNA treatment (Fig. 1A). Similar Noxa mRNA induction was observed following treatment of A375 melanoma cells with these same stimuli (Fig. 1A). Combined IFN and dsRNA treatments enhanced stimulation only slightly as compared to dsRNA treatment alone (Fig. 1A and data not shown). Noxa protein induction by dsRNA and IFN-β was confirmed in HT1080, A375 and WM9 cells by using immunoblot analysis with a Noxa monoclonal antibody. In all three cell types, Noxa protein was strongly up-regulated with 3 h (dsRNA) or 8 h (IFN-β) stimulation (Fig. 1B). Induction of Noxa by two RNA viruses, EMCV and VSV, was also confirmed in HT1080 cells (Fig. 1C), where expression persisted until complete cell lysis was observed (data not shown).

Induction of genes by dsRNA or virus can occur directly or via a feedback loop involving production of an IFN intermediate. Although our previous gene array studies had implicated direct Noxa mRNA induction by dsRNA in cells unresponsive to IFNs (22), we sought to confirm that Noxa protein expression was induced by dsRNA in an IFN-independent manner. DsRNA treatment of IFN-unresponsive U4C cells, a Jak1-deficient cell line derived from HT1080 cells through chemical mutagenesis (25), resulted in Noxa protein induction that was similar in magnitude and duration to the induction observed in IFN-responsive parental HT1080 cells (Fig. 1D). These data confirmed that Noxa was regulated directly by dsRNA and did not require IFN feedback. Noxa induction by IFN likely involved the typical Jak/Stat pathway since induction by IFN was not observed in the U4C cells (data not shown).

Since Noxa was previously identified as a p53-regulated gene, we sought to determine whether p53 was required for IFN and/or dsRNA-dependent induction of Noxa. Our first strategy employed HT1080 cells expressing a dominant-negative p53 genetic suppressor element (HT1080 GSE56) or empty vector (HT1080 LXSN) (23). The GSE56 cells expressed a fragment of the p53 protein (amino acids 275-368) derived from the oligomerization domain that inhibits the activity of the endogenous p53 protein (23). Although p53 protein in the GSE cells accumulates to high levels, it is largely nonfunctional (23). When treated with dsRNA (Fig. 2A) or IFN (supplemental data), HT1080 LXSN and HT1080 GSE56 cells both exhibited a time-

dependent induction of Noxa. In contrast, the LSXN controls, but not the GSE56 HT1080 cells, exhibited enhanced Noxa expression in response to etoposide (Fig. 2A and data not shown). To extend these data, we compared Noxa induction by dsRNA or etoposide in p53-null PC3 prostate tumor cells to p53 expressing LNCaP prostate tumor cells (26,27). Induction of Noxa by dsRNA was identical in both cell lines, but induction by etoposide was observed only in the p53-positive LNCaP cells (Fig. 2B). Together these data suggest that Noxa can be induced by IFN and dsRNA in a p53-independent manner.

To assess the effects of Noxa protein on cellular growth responses to dsRNA, IFN and virus, HT1080 and A375 cells were stably transfected with pcD-Noxa, which conferred constitutive Noxa expression from a CMV promoter. Both cell lines tolerated constitutive Noxa protein expression, consistent with a previous report on Noxa where normal fibroblasts infected with adenoviral Noxa were unaffected unless stimulated with an additional apoptotic signal (13). A Noxa-expressing HT1080 clone with high level ectopic Noxa protein expression, but normal induction of the endogenous Noxa protein by dsRNA, was selected for further analysis (see Fig. 3A). Endogenous and ectopic Noxa proteins were both localized to the mitochondrial fraction, the integrity of which was confirmed by reprobing the blot with antibodies against the mitochondrial specific proteins COX4 and cytochrome c (Fig. 3A). In untreated cells most of the cytochrome c was associated with the mitochondrial fraction, but in dsRNA/Act. D treated cells redistribution to the cytoplasm was observed, a feature that was enhanced in the pcD-Noxa stable transfectants (Fig. 3A). To assist in studies on the biological consequence of IFN- or dsRNA-induced Noxa, a Noxa antisense retroviral construct (pBabepuro Noxa-AS) was used to infect HT1080 cells. Over 60 puromycin-resistant clones stably infected with this antisense construct were assessed for Noxa induction by dsRNA. From these, a single clone was identified that exhibited approximately 90% reduction in Noxa protein expression as compared to vector controls (Fig. 3B). Importantly, this clone did not exhibit constitutive IFN production that can sometimes plague antisense experiments (28), as demonstrated by lack of constitutive IFN-stimulated gene expression or constitutive viral resistance (data not shown).

Cells constitutively expressing Noxa had growth characteristics comparable to empty vector-expressing cells, and were morphologically similar (data not shown). However, upon treatment with dsRNA/Act. D, but not Act. D alone, pcD-Noxa HT1080 cells underwent morphological changes consistent with apoptosis, including cell rounding and eventual detachment and overall loss in cell numbers. When cell numbers were assessed in 48 h growth assays, Noxa-expressing cells were roughly 60% more sensitive to the cytotoxic effects of dsRNA/Act. D as compared to vector controls, but were not more sensitive to either stimulus when used as single agents (Fig. 3C). In contrast, the Noxa antisense expressing cells were less sensitive to dsRNA/Act. D treatment as compared to either vector control line (Fig. 3C). To determine whether increased apoptosis contributed to the enhanced cytotoxicity observed in the Noxa-expressing cells, apoptosis was evaluated using a variety of methods, including assessment of cytochrome C redistribution (Fig. 3A), caspase 3, caspase 9 and poly ADP ribose polymerase (PARP) cleavage (Fig. 3D, 3E and data not shown). In each case, enhanced substrate cleavage was observed in the Noxa-expressing cells, and decreased activation was observed in the antisense expressing cells (Fig. 3D and data not shown). Similar results were obtained following infection of cells with EMCV (Fig. 3E). These data suggested that Noxa contributed to the cellular apoptotic response to virus or the viral mimetic dsRNA. It should be noted that the doses of Act. D (6 ng/ml) used in these and subsequent studies were insufficient to block Noxa protein induction by dsRNA (Fig. 3F), but were sufficient to synergize with dsRNA to promote apoptosis.

Noxa mutational studies were conducted to identify Noxa protein structural motifs that contributed to the enhanced apoptotic response to dsRNA/Act. D or EMCV infection. Three mutant Noxa variants were generated by using site-directed mutagenesis, including a leucine to alanine point mutation in the BH3 domain (Noxa BH3-PM), deletion of the entire BH3 domain (Noxa BH3-D) and deletion of the mitochondrial targeting domain (Noxa MTD-D) (Fig. 4A). All three mutant proteins

were expressed constitutively in HT1080 cells and clones with similar protein expression levels selected for further analysis (Fig. 4B). In trypan blue dye exclusion viability studies, cells expressing wild type Noxa or Noxa BH3-PM exhibited enhanced sensitivity to dsRNA/Act. D treatment as compared to untransfected cells or vector controls (Fig. 4C). The BH3-D mutant had no effect on cell viability as compared to controls, while the MTD-D appeared to provide slight protection against cytotoxic effects of dsRNA/Act. D (Fig. 4C). In biochemical assays of apoptosis, wild type Noxa once again enhanced caspase 3 cleavage, as did Noxa BH3-PM, albeit slightly less than the wild type protein (Fig. 4D). Cells expressing the Noxa BH3-D mutant did not exhibit enhanced apoptosis, while the Noxa MTD-D mutant exhibited less caspase cleavage as compared to control cells (Fig. 4D).

To extend these studies to virus infected cells, HT1080 clones expressing wild type Noxa or the various mutant proteins were infected with increasing titers of EMCV and assessed for apoptosis after 24 h. Cells expressing wild type Noxa or Noxa BH3-PM were more sensitive to virus-induced cytolysis and apoptosis, as shown by trypan blue exclusion (Fig. 5A) and caspase 3 cleavage (Fig. 5B). Cells expressing the Noxa BH3-D mutant behaved similarly to the vector control cells, while the Noxa MTD-D cells again exhibited less apoptosis as compared to the controls (Fig. 5).

IFNs are known to sensitize cells to other apoptotic signals, including members of the TNF superfamily (TRAIL/Apo-2L, FasL, TNFα) (8, 29), DNA damaging agents (etoposide, camptothecin) (30), dsRNA or certain viruses (31). To determine whether Noxa contributes to the ability of IFN to sensitize cells to dsRNA-induced apoptosis, empty vector or Noxa transfected clones were treated with dsRNA alone, in the presence of Act. D, or following 24 h pretreatment with IFN-β. IFN pretreatment effectively sensitized the pcD-Noxa, but not the control cells to dsRNA-dependent apoptosis as measured by caspase 9 cleavage (Fig. 6A). These results were extended to cell viability assays, where it was observed that pcD-Noxa cells were 50-60% more sensitive to IFN/dsRNA cotreatment as compared to vector controls (Fig. 6B). Noxa MTD-D transfected cells were less sensitive to the combined treatment (Fig. 6B).

Noxa/Bax interaction was assessed in cells expressing wild type and Noxa mutant proteins. Treatment with dsRNA/Act. D induced association between wild type Noxa and Bax (Fig. 7A). Perturbation of the BH3 domain resulted in either reduction (BH3-PM) or elimination (BH3-D) of Noxa/Bax association (Fig. 7A). Deletion of the MTD, however, did not prevent Noxa/Bax interaction (Fig. 7A). To determine whether dsRNA-induced association between Noxa and Bax was reflective of events that occur in cells following virus infection, $\mathrm{Noxa}_{myc\mathrm{His}}$ expressing cells were left untreated or treated with dsRNA, etoposide or virus for 12 h. After that time cell lysates were prepared and Noxa/Bax association assessed as above. Virus infection and dsRNA treatment induced Noxa/Bax association, but etoposide treatment did not (Fig. 7B), suggesting that the mechanism of Noxa induction but DNA damage may be different than it is following virus or dsRNA stimulation. To assess Bax subcellular distribution, HT1080 cells stably transfected with wild type Noxa or Noxa MTD-D were left untreated or treated with dsRNA for 12 h, cell lysates separated into cytosolic and mitochondrial fractions and Noxa and Bax proteins detected by immunoblotting. These cell fractionation studies confirmed that wild type Noxa (either ectopic or dsRNA-induced endogenous protein) was largely mitochondrial, but that the Noxa MTD-D mutant localized predominantly to the cytosolic fraction (Fig. 7C). Treatment of pcD-Noxa cells with dsRNA led to a redistribution of Bax from the cytosolic to the mitochondrial fraction (Fig. 7C). However, in the Noxa MTD-D cells Bax remained largely cytosolic even after dsRNA treatment, although some did move to the mitochondrial fraction (Fig. 7C).

DISCUSSION

Cellular apoptotic responses to virus infection constitute an important component of the innate immune system (1, 32). Genes contributing to this response have been identified, but it is clear that the coordinated efforts of numerous gene products ultimately determine cellular sensitivity to apoptotic stimuli. Proteins constitutively present in cells such as caspases and their regulators are involved in the

apoptotic response to virus or dsRNA (1). Other pro-apoptotic proteins are induced in response to the initial viral infection or to the subsequent IFN feedback loop (33). The data presented herein suggest that Noxa constitutes a novel component of this response and is involved in dsRNA and/or viral apoptotic responses that ultimately dictate cellular survival during innate immune responses to viral challenge.

Human Noxa was first identified as a phorbol ester-responsive gene in T-cells (10), but studies aimed at assessing the biological role of Noxa were not initiated until the murine gene was shown to be regulated by DNA damaging agents in a p53-dependent manner (9). Subsequent cell and animal-based studies have revealed important roles for murine Noxa in p53-dependent apoptotic responses to UV, etoposide or adriamycin (11, 12, 13). Noxa has also recently been implicated in cellular responses to hypoxia (14). Noxa overexpression enhanced apoptotic responses to these and other stimuli (9, 15), and apoptotic responses to DNA damaging agents were decreased in Noxa knockout animals as compared to wild type controls (13, 34). Our data suggest a direct role of Noxa in viral responsiveness based upon the specific transcriptional regulation of the Noxa gene by both IFNs and dsRNA and enhanced cellular cytotoxic response to these stimuli in cells expressing ectopic Noxa.

Signal transduction pathways activated by IFNs and dsRNA converge in the nucleus where both activate transcription factors that bind to common DNA elements. Type I IFNs interact with a specific cell surface receptor complex (the Type I IFN receptor) to activate formation of IFN-stimulated gene factor 3 (ISGF3), a heterotrimeric transcription factor comprised of STAT1, STAT2 and IFN regulatory factor 9 (IRF9) (35,36). In the nucleus, ISGF3 binds to IFN-stimulated response elements (ISRE) found in the regulatory regions of most IFN-regulated genes (37). DsRNA activates several signaling pathways, the best known initiated following interaction with Toll-like receptor 3 (TLR3) in endosomal vessicles (38). TLR3 engagement eventually leads to activation of NF-κB and IRF3 that are required for transcriptional induction of downstream responsive genes, including IFNs (38). IRF3 binds to a specific subset of ISRE sequences and activates transcription in conjunction with p300/CBP (5, 39). The regulatory region of the human Noxa gene contains a putative ISRE sequence and a consensus NF-κB binding element, and we have found that both are required for full induction by dsRNA (Y. Sun, D. Leaman, unpublished observation).

Although p53 has been implicated in aspects of cellular responses to virus (40), p53 defective tumor cells retain antiviral responsiveness and in many cases pro-apoptotic responsiveness (41). Nevertheless, several studies were conducted to assess the role of p53 in the observed induction of Noxa by dsRNA and virus. By using cells either p53-null prostate tumor cells or HT1080 fibrosarcoma cells expressing a dominant-negative p53, we were able to demonstrate the dsRNA-induced expression of Noxa was unaffected by p53 status. In contrast, Noxa induction by the DNA damaging agent etoposide was blocked under p53-null conditions. These data, combined with the above results implicating traditional dsRNA and IFN regulated pathways, suggest that induction of Noxa by virus, dsRNA and IFN is direct and involves signaling cascades commonly associated with these stimuli independent of a p53 intermediate.

Bax and Bak are necessary downstream effectors of BH3-only protein mediated apoptosis. Bim, Bid, Bad and Noxa were unable to kill embryonic fibroblast lacking both Bax and Bak, but could efficiently kill cells having either one of these two genes (17, 18). For some BH3-only family members, association with Bax/Bak oligomers and induction of apoptosis involved post-translational modification, such as proteolytic cleavage (Bid) (16), phosphorylation (Bim, Bmf and Bik) (42,43) or dephosphorylation (Bad) (44). Bid/Bak, Bid/Bax (16, 45) and Bim/Bax (46) interactions have been observed, although direct Noxa/Bax interaction has not been reported. Murine Noxa has been proposed not to bind Bax directly, but rather to mediate displacement of pro-apoptotic Bax activators (such as Bid) from Bcl-2 (47,48). Our data offer a modified view of this mechanism. While the data support the hypothesis that Noxa/Bax interaction does not occur constitutively, they suggest an activating event such as dsRNA stimulation can induce association (Fig. 7). Upon dsRNA stimulation we observed Bax translocation from the cytoplasm to the mitochondia where interaction with

wild type Noxa occurred. It is possible that Bcl-2/Noxa or Bcl-xL/Noxa disassociation in response to dsRNA triggers Noxa/Bax interaction. However, another signal is apparently also needed since Noxa MTD-D/Bax interaction was also observed in the cytoplasm after dsRNA treatment (Fig. 7), suggesting that Noxa/Bcl-2 dissociation, if it occurs, is not the sole determinant of Noxa/Bax interaction. Thus, we propose that dsRNA treatment induces post-translational modification of either Noxa or Bax that promotes association between the two molecules. Since dsRNA activates a variety of kinases, including PKR, TANK-binding kinase 1, IκB kinase-ε, c-jun N-terminal kinase and others (1, 49, 50), one possibility is that Noxa phosphorylation status is altered by dsRNA treatment. Additional work is necessary to address this possibility, but the fact that etoposide treatment failed to induce a similar association suggests that this activity is unique to the dsRNA/virus response pathway.

The ability of Noxa MTD-D to act as a dominant negative inhibitor of dsRNA/Act D-induced apoptosis was interesting and unexpected observation. Our data suggested that Noxa MTD-D acted by sequestering Bax in the cytoplasm, thereby reducing mitochondrial destabilization after dsRNA treatment (Fig. 7). Regardless of the mechanism, the observed dominant negative effect of the Noxa MTD-D protein provided important corroboration of the potential importance of Noxa in viral-mediated apoptosis that was implicated by using the Noxa antisense construct. Both reduced dsRNA/Act.D and virus-induced apoptosis, although neither eliminated it completely. This suggests that Noxa contributes to the magnitude of response to these stimuli, but does not play an obligatory role. Similar results have been observed in Noxa knockout mice, where responses to DNA damaging agents were muted but not eliminated the Noxa-null background (13, 34).

Noxa is not the only BH3-containing protein implicated in mediating IFN responses. An IFN-regulated BH3-containing splice variant of 2'-5' oligoadenylate synthetase was shown previously to contribute to cellular apoptotic responses (51). IFN-α treatment promoted downregulation of Bcl-XL protein in multiple myeloma cells, which was proposed to contribute to IFN-induced apoptosis in these cells (52). Overexpression of Bcl-2 blocked IFN-induced apoptosis (53) while Bcl-2 downregulation enhanced IFN-α-induced apoptosis (54). Consistent with our dsRNA observations, Panaretakis et al. observed IFN-dependent activation of Bax/Bak (55). Thus it appears that a variety of members of the Bcl-2 superfamily may contribute the overall apoptotic response of cells to IFN. Viruses also target the Bcl-2 family (56), and roles for apoptosis in both the propagation and suppression of virus replication have been described (1). With few exceptions (57), however, the in vivo functions of these proteins are largely unknown. Lytic viruses may utilize host apoptotic cascades to aid in cell destruction and virus dissemination, and cells counter with sensitive detection systems to attempt to short-circuit replication by undergoing an "altruistic" apoptosis before the virus has completed the entire replication process (53). Whether Noxa functions primarily to inhibit viral replication (via the latter mechanism) or contributes to enhanced replication of specific virus types remains to be determined.

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FOOTNOTES

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ABBREVIATIONS: APR: ATL-derived PMA-responsive gene. BH3: Bcl-2-homology 3. dsRNA: Double-stranded RNA. IFN: Interferon. IRF: Interferon regulatory factor. ISG: Interferon-stimulated Gene. JAK: Janus kinase. MTD: mitochondrial targeting domain. NF-κB: Nuclear factor-kappa-B. PKR: Protein kinase R. STAT: Signal transducer and activator of transcription.

FIGURE LEGENDS

Fig. 1 - Noxa induction by dsRNA, IFN-β, TPA and virus. A). HT1080 fibrosarcoma or A375 melanoma cells were treated with dsRNA (50 μg/ml), IFN-β (500 U/ml) or TPA (300 nM) for the indicated times. Total cellular RNA was isolated and carried through RT-PCR analysis using specific primers for Noxa or GAPDH. **B).** HT1080, A375 and WM9 melanoma cells were treated with dsRNA (50 μg/ml) or IFN-β (500 U/ml) for the indicated time. Cell lysates were isolated for immunoblotting with the specific antibodies against Noxa and α-actin. **C).** HT1080 cells were left uninfected or infected with EMCV or VSV at m.o.i. = 0.3 for the indicated times. Cell lysates were then prepared and immunoblotted for Noxa induction. Blots were subsequently stripped and reprobed with α-actin antibody as a loading control. **D).** WT HT1080 and U4C (JAK1-minus) cells were treated with dsRNA (50 μg/ml) for 3, 6 and 12 h, after which time cell lysates were isolated and Noxa or α-actin protein expression examined as above.

Fig. 2 – Noxa induction by dsRNA is independent of p53. A). HT1080 LXSN (empty vector) and HT1080 GSE56 (p53 dominant negative) cells were treated with dsRNA (50 μg/ml) or etoposide (4 μg/ml) for the indicated times. Cell lysates were isolated for immunoblotting with specific antibodies against p53, Noxa and α-actin. **B).** Noxa induction by etoposide and dsRNA was assessed in p53-null PC3 prostate tumor cells and in p53-positive LNCaP prostate tumor cells. Cells were left untreated or treated with each stimulus for 6 h, after which time cell lysates were prepared and immunoblotted for Noxa induction. The blot was subsequently reprobed for α-actin to serve as a loading control.

Fig. 3 - Ectopic Noxa expression, subcellular localization and antisense inhibition. A). HT1080 cells were transfected with pcD-Noxa and cultured in 800 μg/ml G418 for three weeks, after which individual clones were subcultured, expanded and assessed for constitutive Noxa expression using a myc mAb. HT1080-pcD vector or pcD-Noxa expressing cells were treated with dsRNA (50 μg/ml) and Act D (6 ng/ml) for 12 h. Following treatment, adherent and floating cells were harvested for fractionation. Cytosolic supernatants and mitochondrial-containing pellets were obtained by double centrifugation. The resulting samples were used for SDS-PAGE and immunoblotting with the specific antibodies against Noxa, Cytochrome C, COX4 and α-actin. Mitochondrial fraction purity was demonstrated with COX4 mAband the α-actin antibody served as a loading control. **B).** WT HT1080, HT1080-pBabe-puro or HT1080-Noxa-antisense transfected clones were left untreated or treated with dsRNA (50 μg/ml) for 6 h. **C).** HT1080-pcDNA Vector, pBabe-vector, pcD-Noxa (Noxa sense expression) and pBabe-Noxa-antisense (constitutive Noxa antisense expression) were treated with dsRNA/Act D at the indicated doses for 48 h. Following treatment, attached cells were fixed with 10% TCA, stained by SRB and absorbance read at 490 nm on a plate reader. The data are presented as relative cell numbers ± SD from triplicate samples. **D).** HT1080 pBabepuro, pcD-Noxa and pBabe-Noxa-AS cells were treated with dsRNA (50 μg/ml)/Act D (6 ng/ml) for the indicated times. Cell lysates were used to detect pro-caspase 3 and its cleaved fragments by immunoblotting. **E).** The same cells as in C were infected with EMCV (m.o.i. = 0.3) for the indicated times and then cell lysates were used to detect caspase 3 as above. **F).** HT1080 cells were treated with Act. D (6 ng/ml) with or without dsRNA (25 μg/ml) for the indicated times. Cell lysates were then used for immunoblot analysis using a Noxa mAb. The blot was subsequently stripped and reprobed for α-actin.

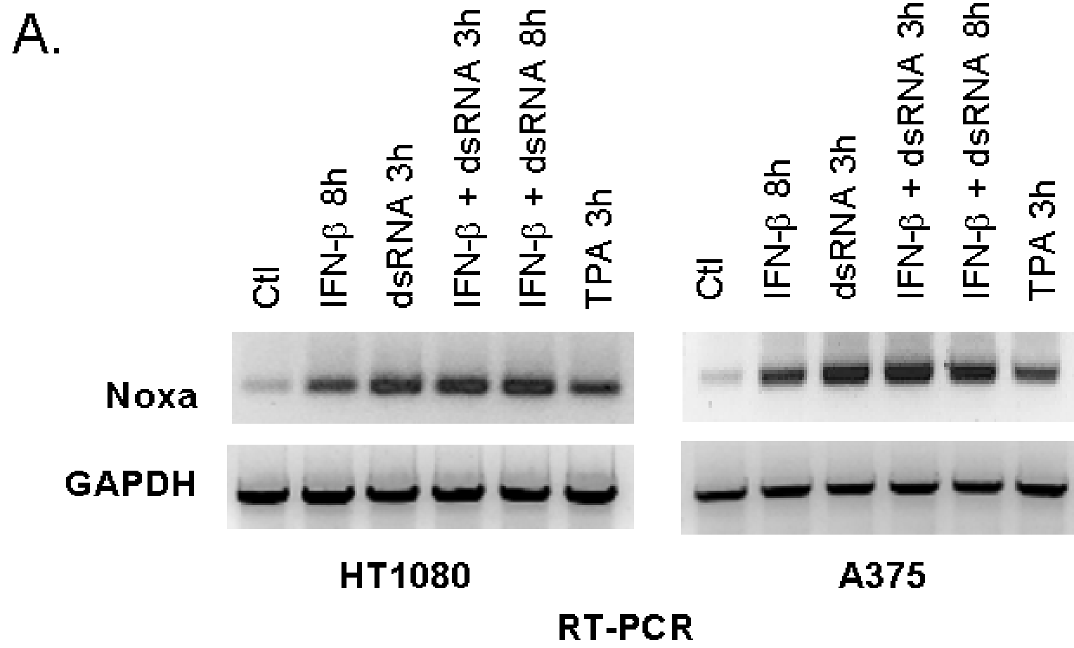
Fig. 4 – Targeted mutations of the Noxa protein. A). Schematic representation of Noxa point and deletion mutations. The Noxa BH3-PM harbored a single leucine to alanine mutation at a.a. position 29 within the BH3 domain. The BH3-D and MTD-D had deletions of the entire BH3 and MTD domains, respectively. **B).** Lysates from HT1080 cell clones stably expressing each of the Noxa proteins diagrammed in A were subjected to immunoblot analysis using a Noxa mAb. The blot was stripped and

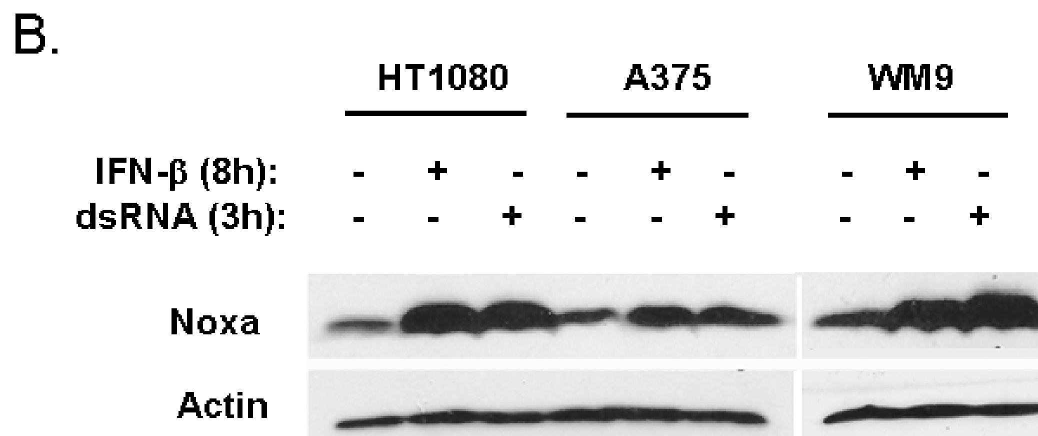
reprobed with α-actin to serve as a loading control. **C).** HT1080 cells stably transfected with pcDNA3.1 (control), pcD-Noxa, Noxa BH3-PM, Noxa BH3-D or Noxa MTD-D were treated with dsRNA/Act D at the indicated doses for 48 h. Following treatment, viable cells numbers were determined by staining with trypan blue and counting unstained cells on a hemacytometer. The data are presented as relative cell numbers ± SD from triplicate samples. **D).** The same cell lines described in C were left untreated or treated with dsRNA (50 μg/ml)/Act. D (6 ng/ml) for 6 or 12 h, and then cell lysates subjected to immunoblotting to detect pro-caspase 3 and its cleaved fragments. Stripping the blot and reprobing for α-actin illustrated equal gel loading.

Fig. 5 – Effect of Noxa mutations on EMCV-induced cytopathicity and apoptosis. A). HT1080 cells stably transfected with pcDNA3.1 (control), pcD-Noxa, Noxa BH3-PM, Noxa BH3-D or Noxa MTD-D were infected with increasing titers of EMCV (m.o.i. =0~0.3) for 24 h. Following treatment, viable cells numbers were determined by staining with trypan blue and counting unstained cells on a hemacytometer. The data are presented as relative cell numbers ± SD from triplicate samples. **B).** The same cell lines described in A were left uninfected or were infected with EMCV (m.o.i. = 0.3) for the indicated times, and then cell lysates subjected to immunoblotting to detect pro-caspase 3 and its cleaved fragments. Stripping the blot and reprobing for α-actin illustrated equal gel loading.

Fig. 6 – Effect of Noxa on IFN sensitization of cells to apoptosis. A). HT1080 cells stably transfected with pcDNA3.1 (control) or pcD-Noxa were left untreated or treated with dsRNA (50 μg/ml), Act. D (6 ng/ml) or IFN-β (500 U/ml) alone or in combination as indicated. Act. D and dsRNA treatments were for 12 h (either alone or when combined). IFN-β treatments were for 36 h, and combined IFN-β/dsRNA treatments included 24 h of IFN-β alone, followed by 12 h IFN-β/dsRNA. After treatment, cell lysates were subjected to immunoblotting to detect pro-caspase 9 and its cleaved fragments. Stripping the blot and reprobing for α-actin illustrated equal gel loading. **B).** The indicated cell lines were left untreated or treated with dsRNA or IFN-β alone or in combination as described above. After the appropriate time, cells were fixed with 10% TCA, stained by SRB and absorbance read at 490 nm on a plate reader. The data from a representative experiment are presented as relative cell numbers ± SD from triplicate samples.

Fig. 7 – dsRNA- or virus-induced association between Noxa and Bax. A). HT1080 cells stably transfected with pcDNA3.1 (control), pcD-Noxa, Noxa BH3-PM, Noxa BH3-D or Noxa MTD-D were left untreated or were treated with dsRNA (50 μg/ml)/Act D (6 ng/ml) for 12 h, after which time cell lysates were prepared. The myc-HIS tagged wild type or mutant Noxa proteins were captured on nickel beads and the bound proteins subjected to immunoblotting using a mAb to Bax. The blot was subsequently stripped and reprobed with Noxa mAb to illustrate relative levels of the various Noxa mutant proteins. **B).** Noxa/Bax association was assessed as in A following 12 h treatment of cells with dsRNA (50 μg/ml), etoposide (4 μg/ml) or EMCV (M.O.I. = 0.3). Bax was detected in nickel bead-associated lysates by immunoblotting, and the stripped blot was subsequently probed for Noxa to demonstrate efficient pull-down in all samples. **C).** HT1080 pcD-Noxa or Noxa MTD-D stable cells were left untreated or treated with dsRNA (50μg/ml) for 12 h. Cell lysates were then fractionated into cytosolic and mitochondrial fractions by using differential centrifugation. Bax and Noxa proteins were identified in the respective fractions by immunoblotting with specific antibodies. The locations of the ectopic and endogenous Noxa proteins are indicated. Probing with a mAb against COX4 confirmed that the cytosolic fraction was free of contaminating mitochondria. The blot was subsequently stripped and reprobed with an α-actin mAb. Whole cell extract (WCE) from untreated pcD-Noxa cells was used as a positive control.





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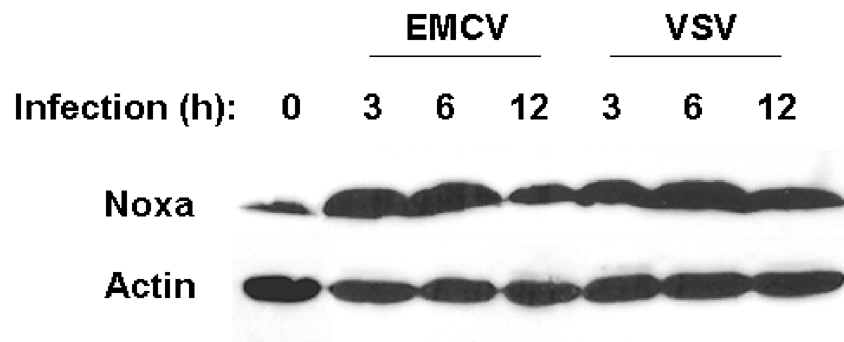

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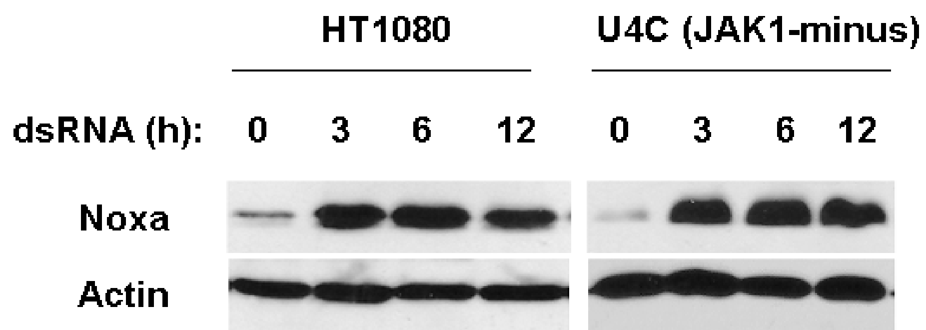

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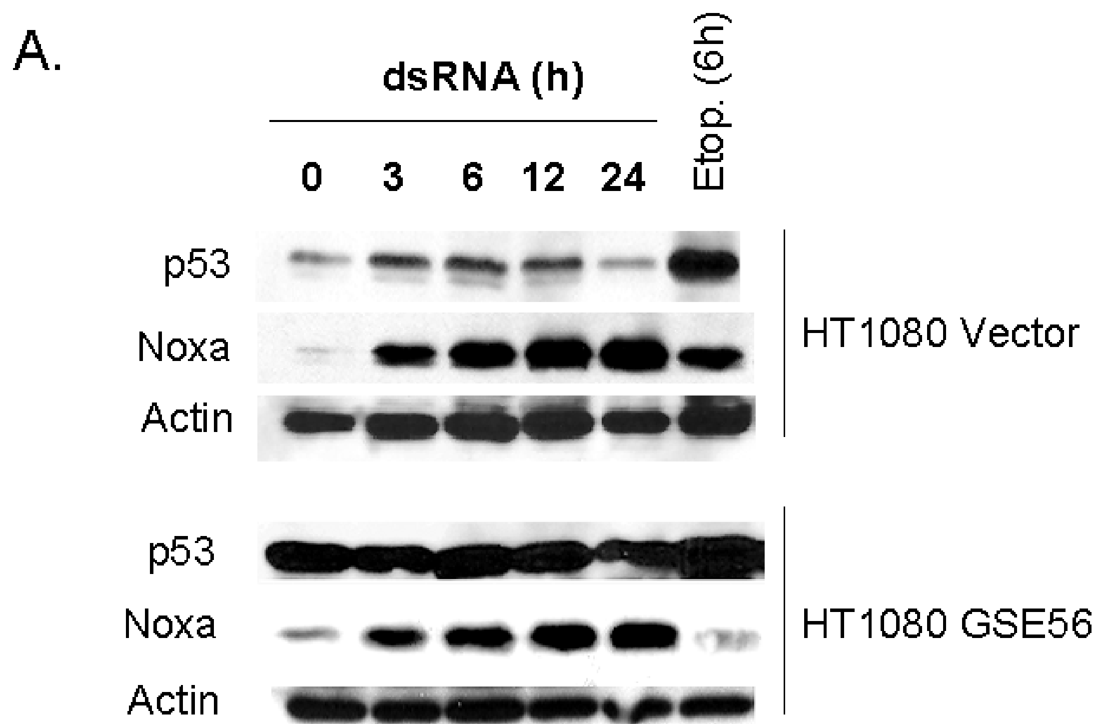

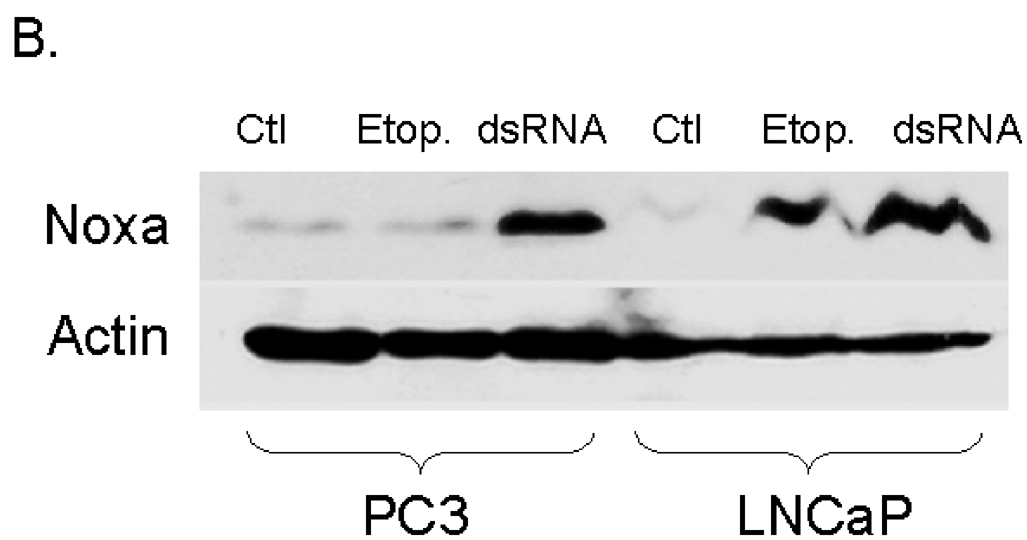

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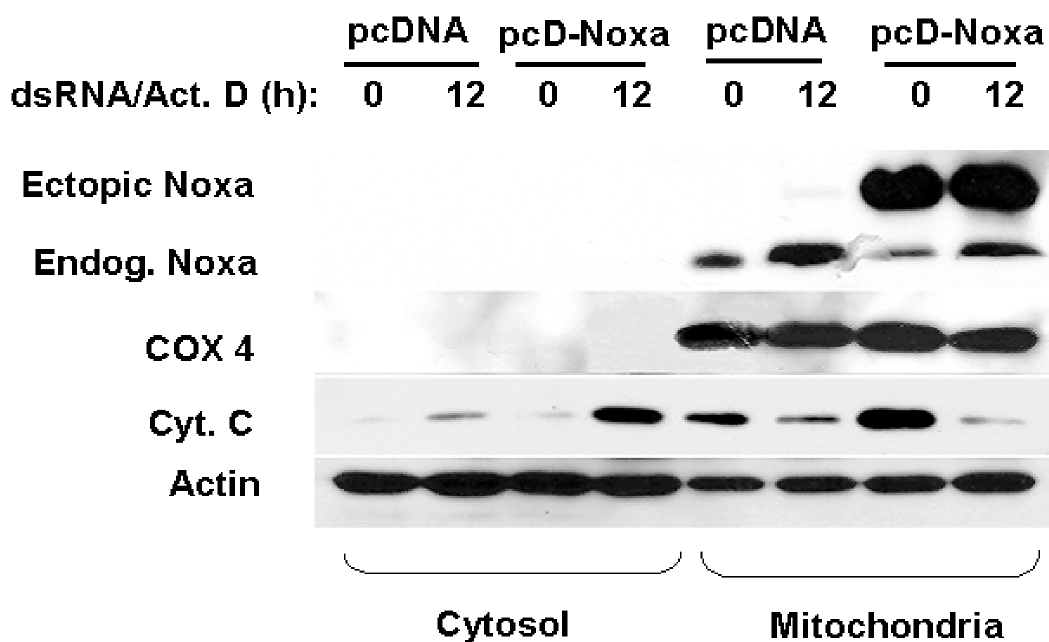

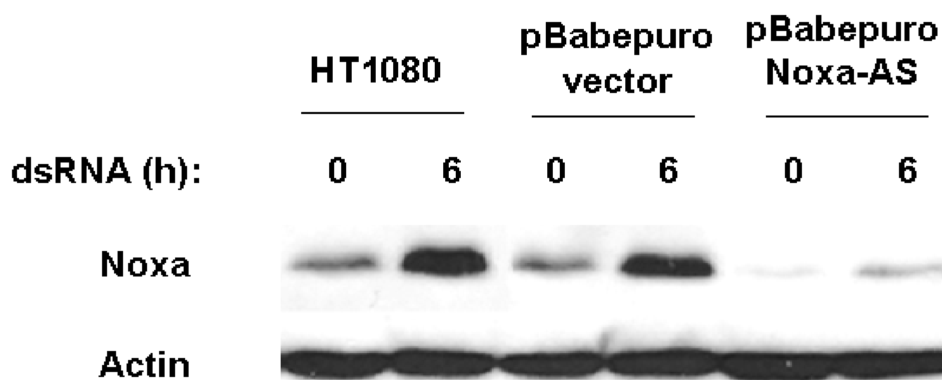

Fig. 3

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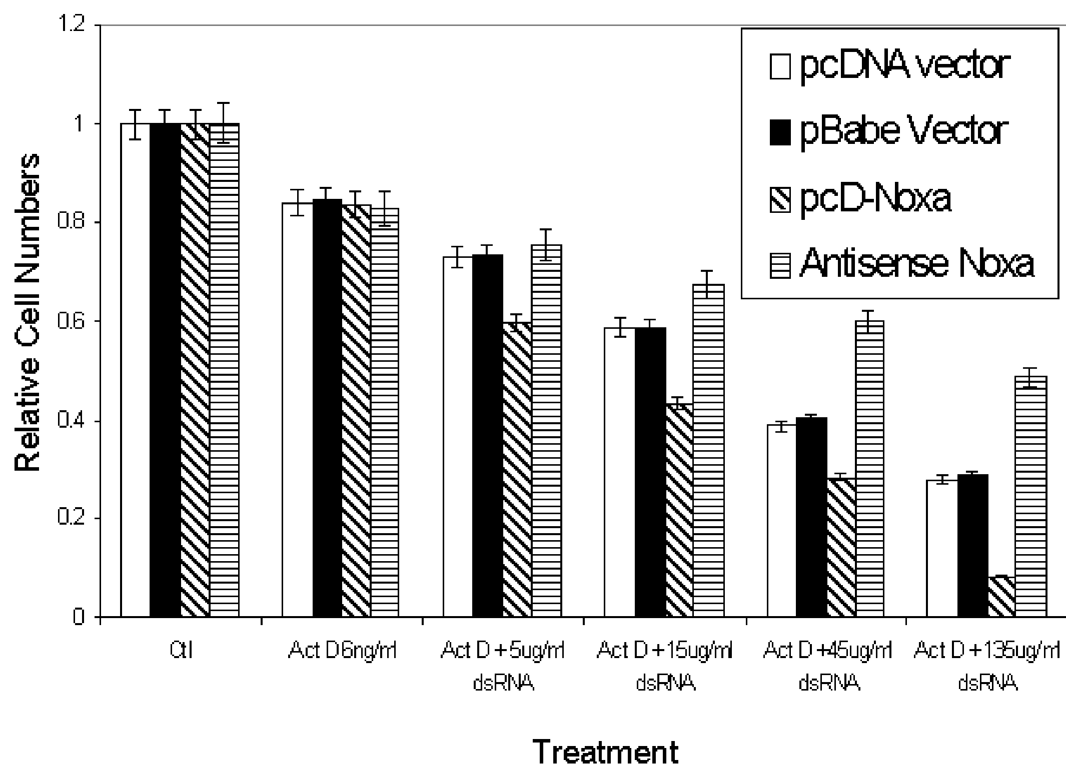

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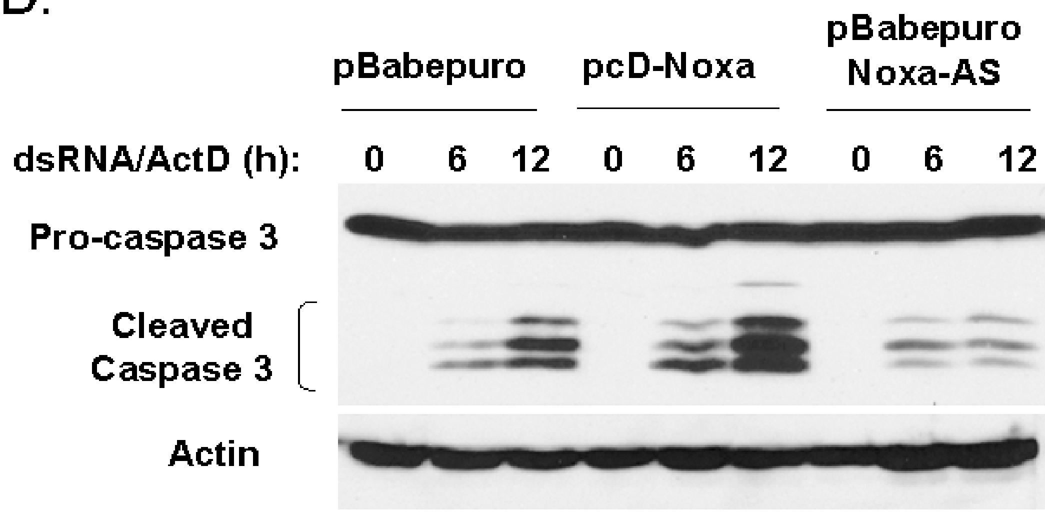

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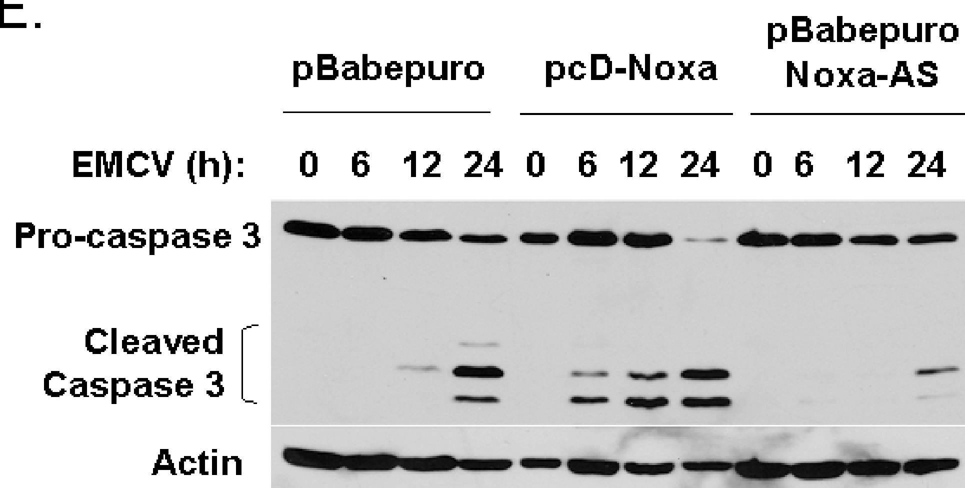

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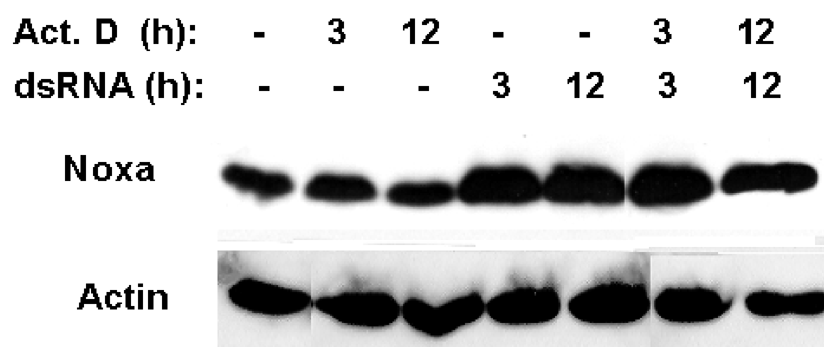

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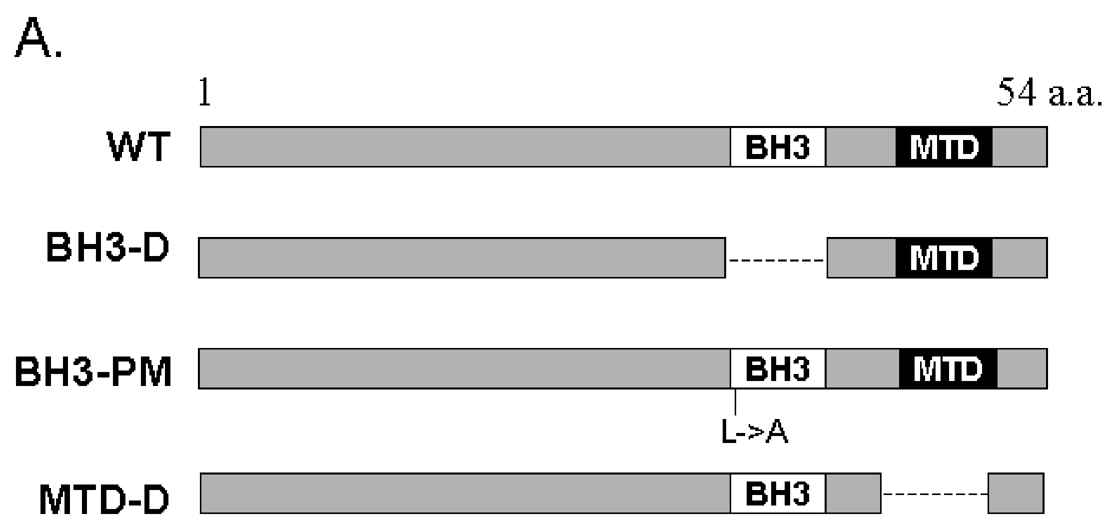

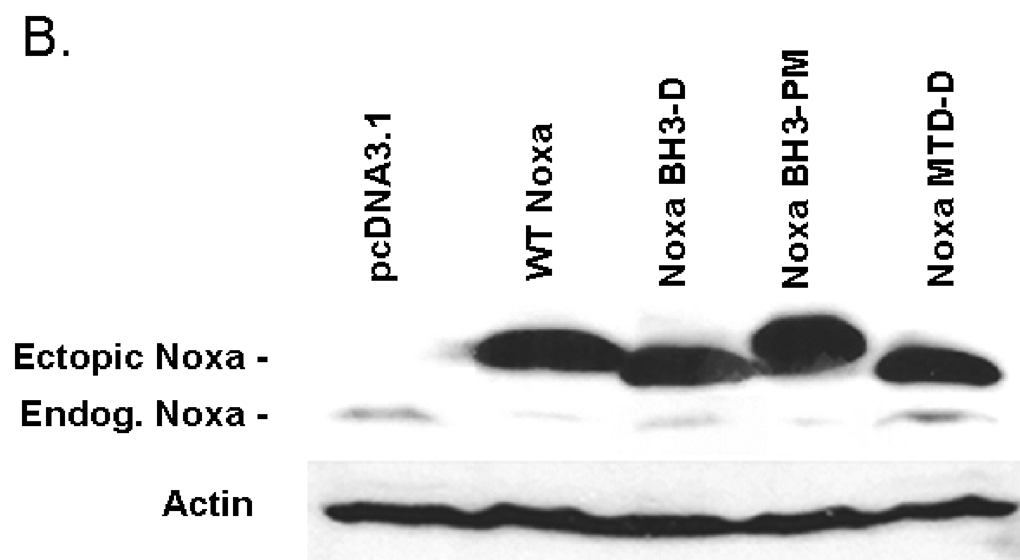

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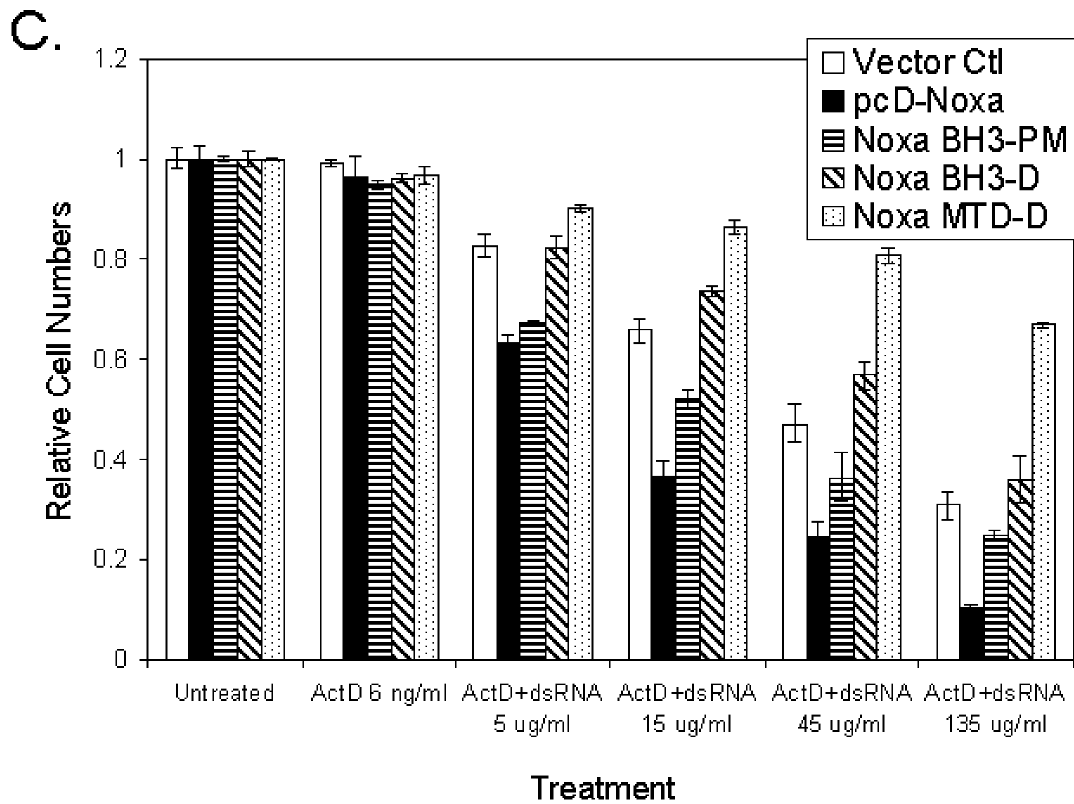

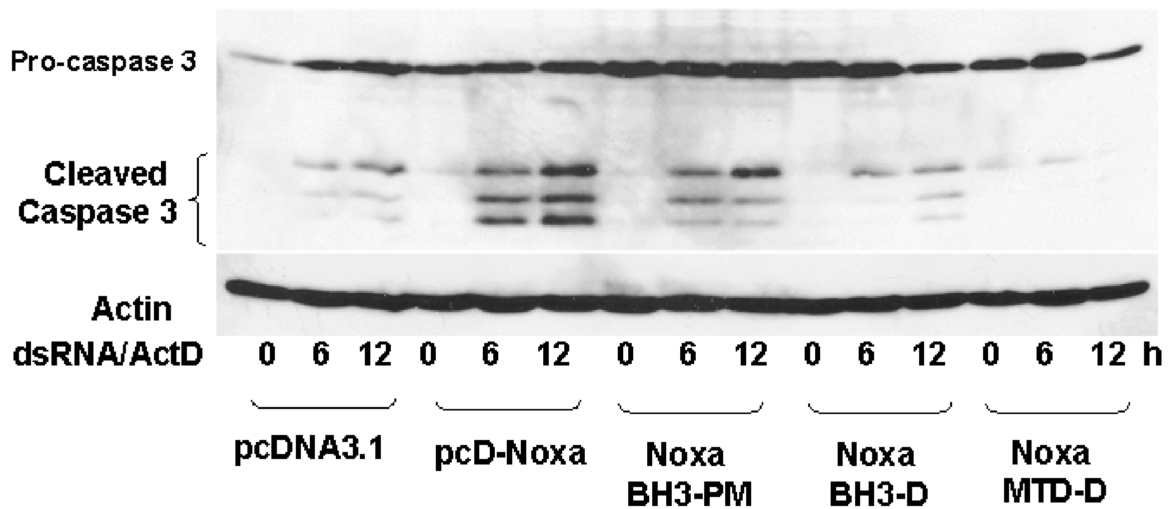

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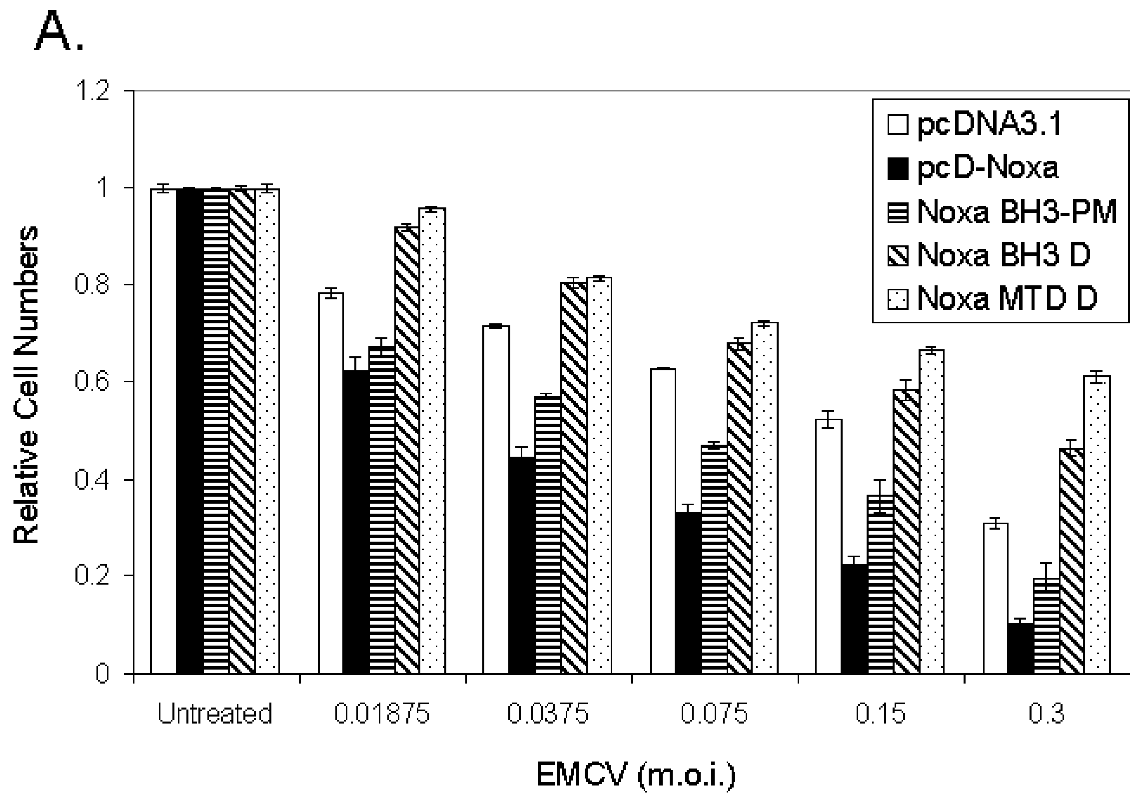

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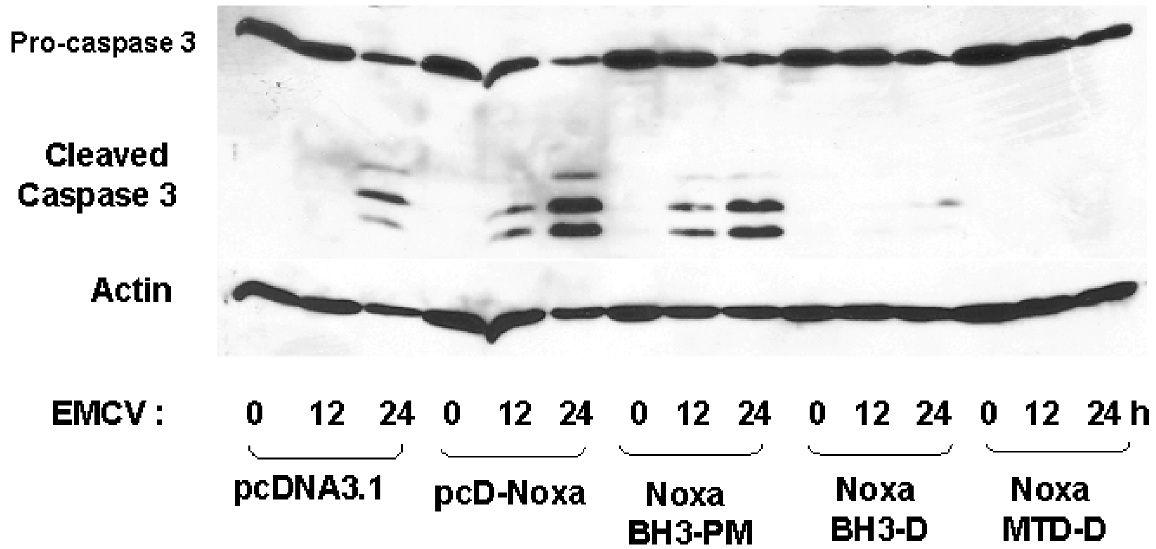

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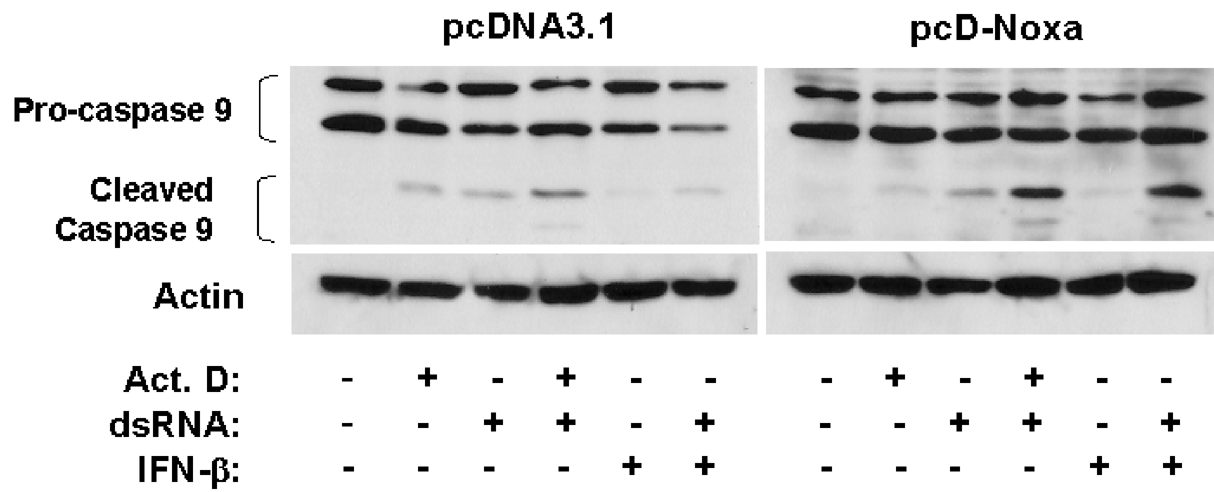

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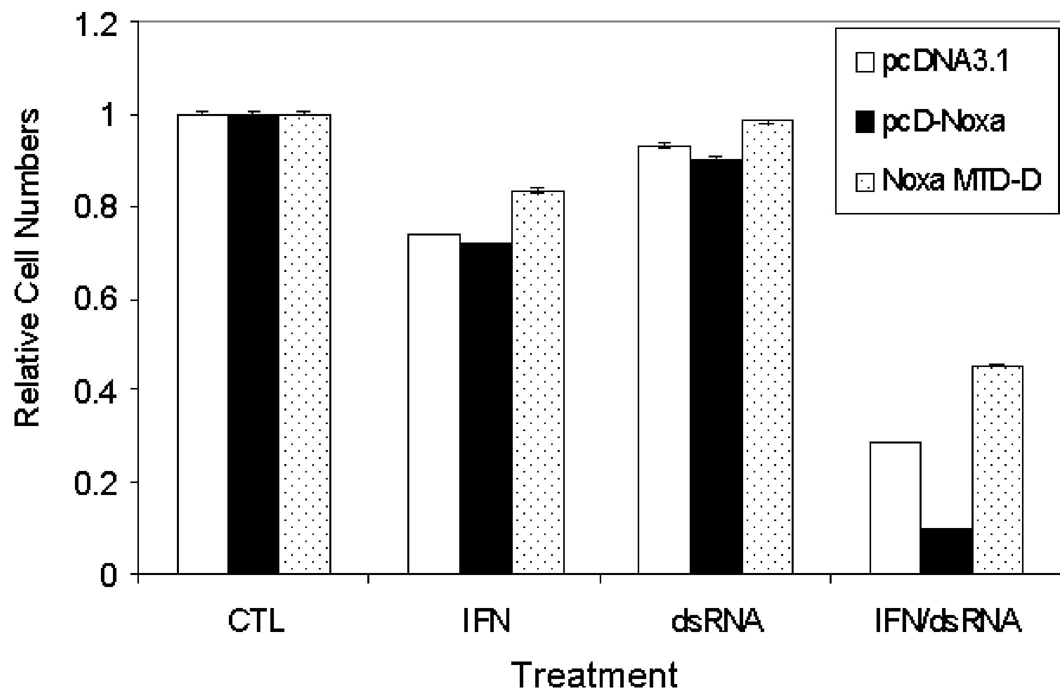

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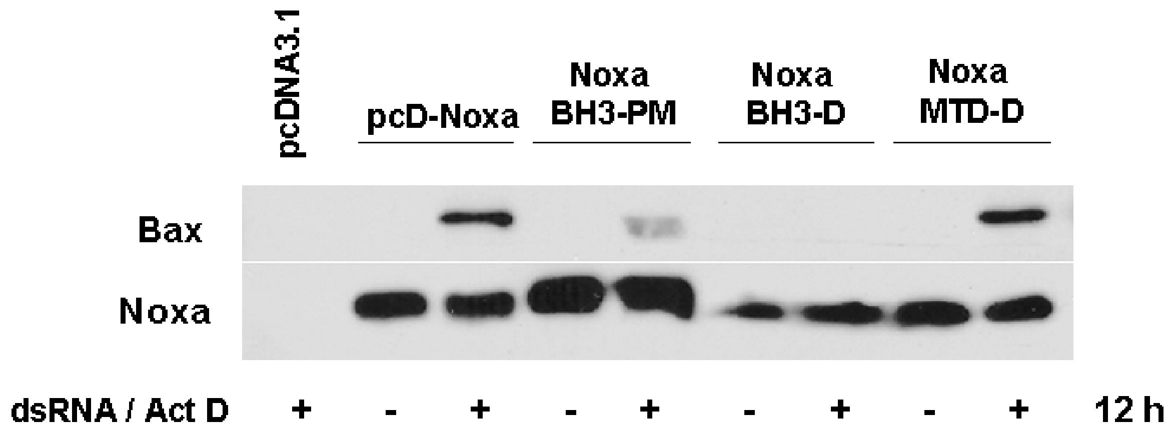

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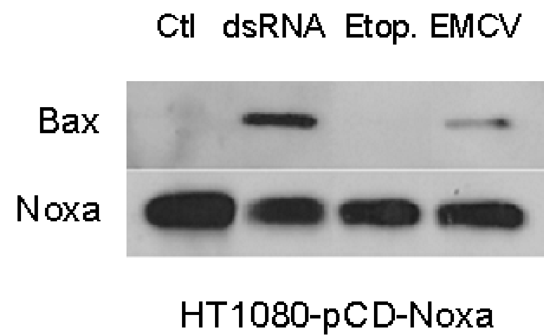


Fig. 7

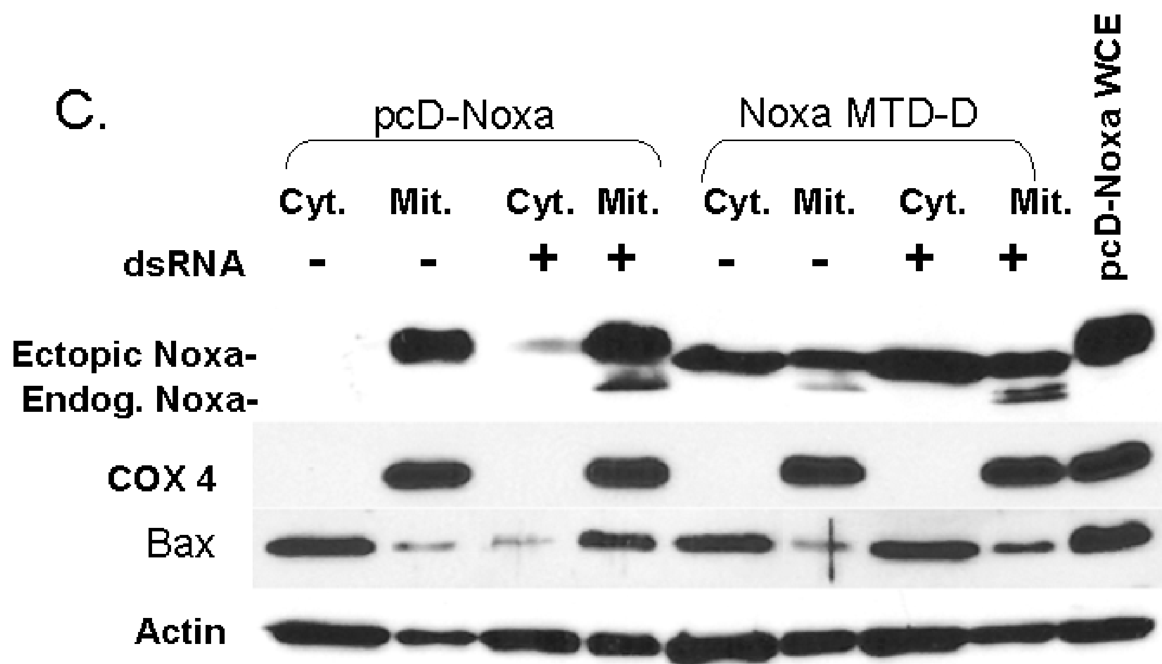
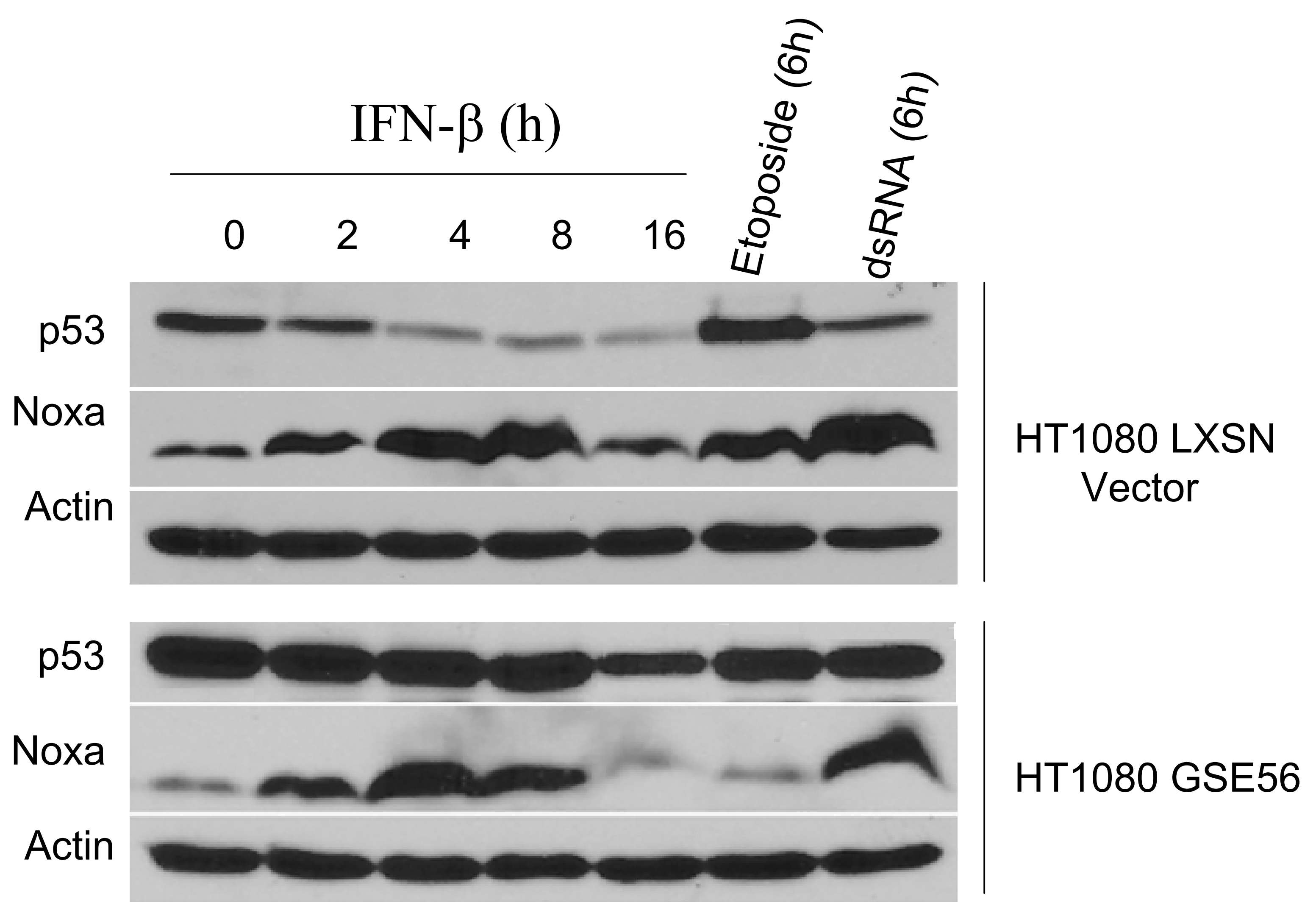
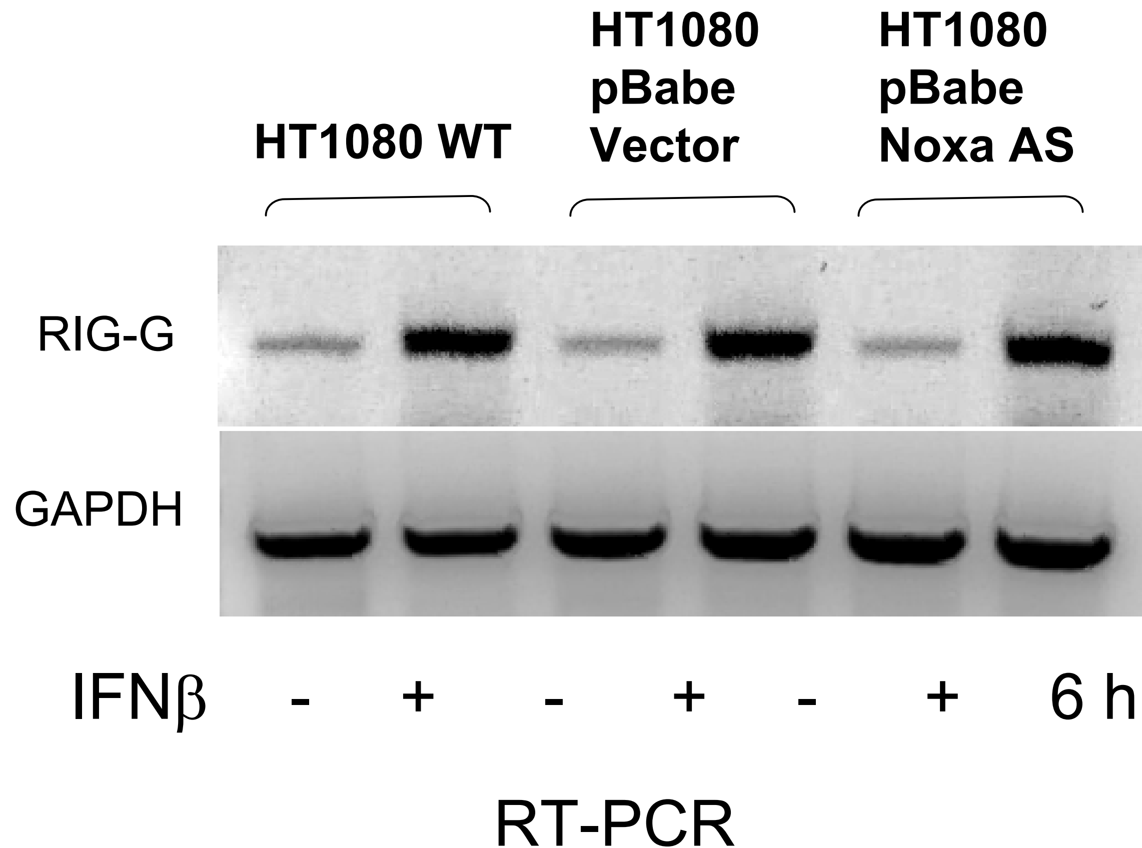


Fig. 7



Supplemental Fig. 1 - Noxa induction by IFN is independent of p53. A). HT1080 LXSN (empty vector) and HT1080 GSE56 (p53 dominant negative) cells were treated with IFN-β (500 U/ml), dsRNA (50 μg/ml) or etoposide (4 μg/ml) for the indicated times. Cell lysates were isolated for immunoblotting with specific antibodies against p53, Noxa and α-actin. Noxa induction by IFN was not affected by p53 inhibition, but etoposide-dependent induction was eliminated.



Supplemental Fig. 2 – Laack of IFN feedback in Noxa AS expressing cells. Wild type (WT) HT1080, HT1080 cells stably infected with empty retroviral vector pBabepuro or HT1080 cells stably expressing Noxa AS were left untreated or treated with IFN-b (500 U/ml) for 6 h. After that time total cellular RNA was extracted and ISG expression assessed by using RT PCR. The IFN-regulated gene RIG-G was evaluated since it exhibits low constitutive expression but potent upregulation by IFN. As shown above, basal and IFN-induced RIG-G expression was nearly identical in all three cell lines.