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The Tumor Suppressor RASSF1A and MAP-1 Link Death Receptor Signaling to Bax Conformational Change and Cell Death

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Summary

Tumor cells typically resist programmed cell death (apoptosis) induced by death receptors. Activated death receptors evoke Bax conformational change, cytochrome c release, and cell death. We report that the tumor suppressor gene RASSF1A is required for death receptor-induced Bax conformational change and apoptosis. TNFα or TRAIL stimulation induced recruitment of RASSF1A and MAP-1 to receptor complexes and promoted complex formation between RASSF1A and the BH3-like protein MAP-1. Normally, MAP-1 is inhibited by an intramolecular interaction. RASSF1A/MAP-1 binding relieved this inhibitory interaction, resulting in MAP-1 association with Bax. Deletion of the RASSF1A gene or short hairpin silencing of either RASSF1A or MAP-1 expression blocked MAP-1/Bax interaction, Bax conformational change and mitochondrial membrane insertion, cytochrome c release, and apoptosis in response to death receptors. Our findings identify RASSF1A and MAP-1 as important components between death receptors and the apoptotic machinery and reveal a potential link between tumor suppression and death receptor signaling.

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Introduction

Apoptosis is critical for multiple physiological processes, including organ formation, neuronal connectivity, immune cell selection, and the killing of certain pathogens (Chao and Korsmeyer, 1998; Cox and Der, 2003; Evan et al., 1995). Two types of signaling pathways promote apoptosis. The "intrinsic" pathway is activated by noxious factors, such as DNA damage, unbalanced proliferative stimuli, and nutrient or energy depletion. In contrast, specific death receptors (e.g., tumor necrosis factor α [TNFα] receptor, TNFα apoptosis-inducing related ligand [TRAIL], or Fas [CD95]) stimulate the "extrinsic" pathway (Danial and Korsmeyer, 2004; Thorburn, 2004). Mitochondria play a central role in nearly all apoptotic pathways, serving to integrate upstream apoptosis-inducing (proapoptotic) signals and promote the release of small apoptogenic molecules (Kroemer, 2003).

Activated death receptors trigger a series of events that result in the formation of trimeric receptor complexes and the death-inducing signaling complex (DISC). DISC assembly and subsequent activation of initiator caspases (such as caspase-8) convey signals to the mitochondria ultimately resulting in cytochrome c release into the cytosol. Released cytochrome c (together with caspase-9 and Apaf-1) promotes the assembly of a multiprotein complex (the apoptosome) that activates downstream effector caspases (Adams and Cory, 2002; Cain et al., 2002; Ho and Zacksenhaus, 2004). Intrinsic pathway stimulation also leads to cytochrome c release from the mitochondria. Activated effector caspases, generated by either pathway, cleave important nuclear proteins (such as lamin B and poly [ADP-ribose] polymerase [PARP]) and activate specific DNA endonucleases. These events result in many of the biochemical and morphological changes observed during apoptosis, including nuclear and cytoplasmic breakdown (Degterev et al., 2003; Denecker et al., 2001; Riedl and Shi, 2004).

Apoptosis is regulated by Bcl-2 family proteins, which are defined by the presence of one or more "Bcl-2 homology" (BH) domains. Multi-BH-domain proapoptotic proteins (such as Bax and Bak) are required for most, if not all, forms of cell death (Bouillet and Strasser, 2002; Scorrano and Korsmeyer, 2003). These molecules normally exist as monomers, but upon activation by upstream signals, they are thought to oligomerize and either themselves form a pore that permits release of inner mitochondrial membrane proteins (such as cytochrome c) or to interact with intrinsic mitochondrial proteins to form such a pore (Bouillet and

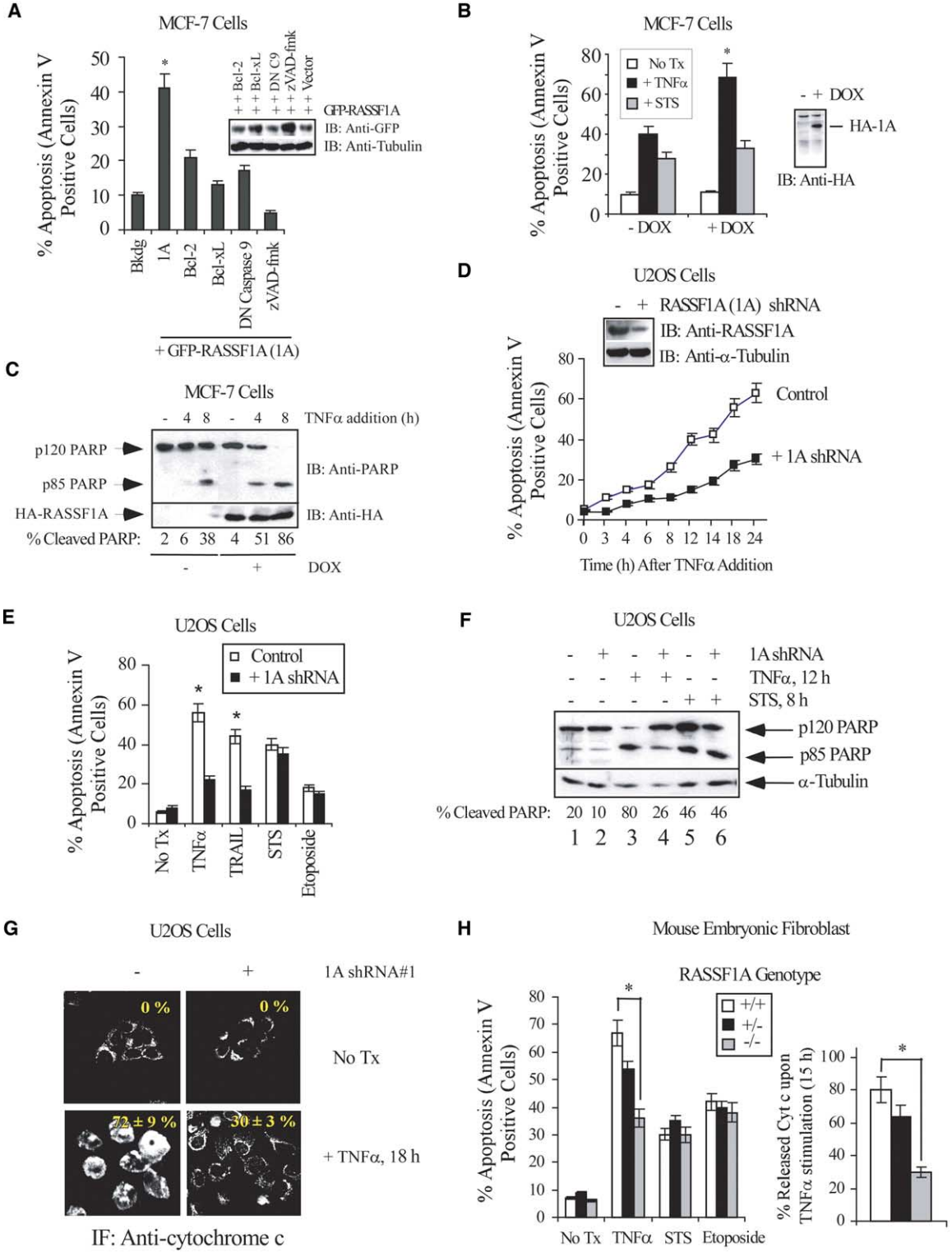

Figure 1. RASSF1A Promotes Death Receptor-Dependent Apoptosis

(A) Annexin V staining in MCF-7 cells 48 hr after transient expression of GFP-RASSF1A and antiapoptotic proteins or in the presence of zVAD-FMK as indicated. Significance was evaluated by ANOVA; p value < 0.0001.

(B) Annexin V staining of doxycycline (DOX)-inducible HA-RASSF1A MCF-7 cells (side panel) left untreated (No Tx) or stimulated with TNFα or staurosporine (STS) for 12 hr. *p value < 0.001 for TNFα treatment –/+ DOX.

(C) Immunoblot (IB) showing precocious TNFα-evoked PARP cleavage (see IB: anti-PARP) in MCF-7 cells inducibly expressing HA-RASSF1A (see IB: anti-HA). Percent-cleaved PARP was quantified by densitometry (intensity of p85 band/intensities of p85 + p120 bands).

Strasser, 2002; Kuwana et al., 2002; Mikhailov et al., 2003). BH3-only proteins (e.g., Bid, Bad, and Bim) promote Bak/Bax oligomerization and pore formation, with different BH3-only proteins acting as sentinels that respond to various insults and promote Bax activation (Bouillet and Strasser, 2002; Danial and Korsmeyer, 2004; Letai et al., 2002). DISC assembly and subsequent caspase-8 activation result in Bid cleavage and translocation from the cytosol to the mitochondrial membrane (Chao and Korsmeyer, 1998; Kuwana et al., 2002). How Bax activation occurs has remained unclear, although it is known that Bax undergoes a conformational change prior to or coincident with its insertion into the mitochondrial membrane. Apoptosis-inhibiting (antiapoptotic) multidomain Bcl-2 proteins, such as Bcl-2 and Bcl-xL, function, at least in part, to sequester BH3-only proteins, thereby preventing Bax/Bak activation (Kuwana et al., 2002).

Neoplastic cells typically resist apoptosis, because many oncogene and/or tumor suppressor gene mutations impair proapoptotic signaling (Ivanov et al., 2003; Vousden, 2002). *RASSF1A* is a tumor suppressor gene on chromosome 3p21 that is inactivated in many carcinomas (Dammann et al., 2003). The *RASSF1* locus encodes three isoforms (A, B, and C), which arise by multiple promoter usage and alternative splicing. In tumor cells, exon 1α, encoding the amino terminus of the 1A isoform, is epigenetically silenced by promoter-specific methylation, resulting in the loss of *RASSF1A* expression. Restoring *RASSF1A* to nonexpressing cancer cells reduces colony formation, suppresses anchorage-independent growth, and inhibits tumor formation in nude mice (Dammann et al., 2000). Mice with heterozygous or homozygous deletion of RASSF1A have a significant, although small, increase in spontaneous tumors; notably, these mice retain expression of the RASSF1C isoform (Tommasi et al., 2005). These data suggest a tumor suppressor function specific for the RASSF1A isoform and the importance of the unique exon 1α encoding for the amino terminus of RASSF1A. However, the mechanism of action of RASSF1A has remained unclear.

Here, we report a mechanism by which RASSF1A functions to regulate cell survival. Ectopic expression of RASSF1A in MCF-7 cells enhanced death receptor-evoked apoptosis, acting upstream of the mitochondria. Conversely, reducing RASSF1A levels by RNA interference (RNAi) (in U2OS or MCF10A cells) or gene deletion (RASSF1A$^{-/-}$ mouse embryonic fibroblasts) specifically impaired death receptor-dependent apoptosis. Death receptor stimulation resulted in the formation of a complex between RASSF1A and the BH3-like protein modulator of apoptosis-1 (MAP-1), which is normally held in an inactive conformation by an intramolecular interaction. RASSF1A binding relieves this inhibitory interaction, allowing MAP-1 to bind Bax. RASSF1A/MAP-1 interaction is required for Bax conformation change, mitochondrial membrane insertion, and maximal apoptosis in response to death receptor stimulation. Furthermore, RASSF1A and MAP-1 are recruited to both the TNFα and TRAIL receptor complexes in response to their respective cognate ligands. Our findings identify RASSF1A and MAP-1 as important components in death receptor signaling and reveal a mechanism by which tumor cells resist death receptor-dependent apoptosis.

Results

RASSF1A Expression Promotes Apoptosis

Previous studies suggested roles for RASSF1A in control of G1/S (Shivakumar et al., 2002) or mitotic progression (Liu et al., 2003; Song and Lim, 2004). However, we observed no effect of RASSF1A expression on these processes (Figure S1A available in the Supplemental Data with this article online and data not shown). Instead, transient expression of RASSF1A in MCF-7 breast cancer cells, which lack endogenous *RASSF1A*, resulted in morphological and biochemical effects suggestive of apoptosis, including cell rounding and nuclear condensation (data not shown), increased Annexin V staining (Figure 1A), and the appearance of a sub-G1 population (Figure S1A). RASSF1A-induced apoptosis was inhibited by the nonspecific caspase inhibitor zVAD-FMK or by transient overexpression of dominant-negative caspase-9, suggesting a role for caspase activity and apoptosome formation in RASSF1A-induced apoptosis (Figure 1A). Furthermore, transient overexpression of the antiapoptotic proteins Bcl-2 or Bcl-xL inhibited RASSF1A-evoked apoptosis (Figure 1A). Taken together, these data suggest that RASSF1A acts upstream or at the level of mitochondria to promote apoptosis.

To further characterize its proapoptotic mechanism, we generated MCF-7 cells (MCF7-1A) in which expression of RASSF1A (containing an amino terminal HA epitope tag) was induced upon doxycycline addition (+DOX) (Figure 1B, side panel). Nonexpressing (–DOX) or RASSF1A-expressing MCF-7 cells (+DOX) were subjected to various stimuli, and apoptosis was quantified by Annexin V staining. At this level of expression, RASSF1A alone did not induce cell death, but addition of an extrinsic pathway stimulus (TNFα) resulted in signifi-

(D) Pooled U20S cells expressing shRNA#1 (+ 1A shRNA) exhibit reduced TNFα-evoked apoptosis when compared to pooled U2OS cells expressing shRNA vector only (control). Apoptosis was quantified by Annexin V staining. Immunoblot shows RASSF1A knockdown, with α-tubulin as a loading control. Error bars correspond to an error of ± 8% for control cells and ± 10% for 1A shRNA cells.

(E) Annexin V staining of control or 1A shRNA U20S cells; TNFα or TRAIL at 18 hr; and STS or Etoposide at 12 hr. *p value < 0.001 for significance between control and 1A shRNA cells under the indicated treatments.

(F) Defective TNFα-evoked PARP cleavage (quantified as in [C]) in 1A shRNA cells.

(G) Reduced TNFα-evoked cytochrome c release (monitored by immunofluorescence [IF]) in 1A shRNA cells. Note punctate mitochondrial staining in the absence of TNFα and diffuse pan-cellular cytoplasmic staining in the presence of TNFα. Numbers represent the percentage of cells containing diffuse cytochrome c staining (released cytochrome c).

(H) Reduced TNFα-evoked apoptosis and cytochrome c release in primary RASSF1A$^{-/-}$ MEFs when compared to wild-type (wt) MEFs (+/+) (TNFα stimulation for 15 hr). STS was added for 9 hr, and etoposide (100 μM) was added for 20 hr. Annexin V staining was carried out as in (B) and cytochrome c release as in (G). See Figure S2C for representative fields; *p value < 0.005.

cantly increased apoptosis compared to nonexpressing MCF-7 cells (Figure 1B). Cleaved PARP, a marker of active effector caspase activity, appeared earlier and at elevated levels in MCF7-1A cells when compared to parental MCF-7 cells (Figure 1C). RASSF1A expression also promoted precocious cytochrome c release, as determined by immunofluorescence (Figure S1B). In contrast, stimulation of the intrinsic pathway by staurosporine (Figure 1B) or etoposide (data not shown) did not enhance MCF7-1A cell death. Thus, ectopically expressed RASSF1A preferentially sensitizes MCF-7 cells to death receptor-evoked apoptosis. The apparent discrepancy between the effects of transient (Figure 1A), and stable, inducible (Figure 1B) expression of RASSF1A probably reflects much higher RASSF1A expression in transiently transfected cells, which may bypass the requirement for membrane-proximal signals.

RASSF1A Depletion Inhibits Death Receptor-Dependent Apoptosis

To determine whether RASSF1A is required for death receptor-induced apoptosis, we utilized RNA interference to decrease endogenous RASSF1A levels in U2OS osteosarcoma cells (Brummelkamp et al., 2002). Stable expression of a RASSF1A short hairpin RNA (shRNA1) resulted in >80% decrease in RASSF1A expression compared to "control" cell pools containing the expression vector alone (Figure 1D, inset). Consistent with the effects of RASSF1A overexpression, cell cycle distribution was unaltered in U2OS cells lacking RASSF1A (Figure S1C). However, RASSF1A-depleted U2OS cells (+1A shRNA) had a markedly reduced apoptotic response to TNFα (Figure 1D) but retained normal responsiveness to intrinsic proapoptotic stimuli (Figure 1E, STS and Etoposide). TRAIL- and, to a small extent, Fas-induced apoptosis also were impaired in 1A shRNA cells (Figure 1E and data not shown). Furthermore, TNFα-induced apoptosis was defective in the nontransformed, immortalized MCF10A mammary cells rendered RASSF1A deficient by shRNA treatment (Figure S1D). These data suggest a general requirement for RASSF1A in death receptor-dependent apoptosis in transformed and nontransformed cells.

We next assessed how RASSF1A acts within the death receptor signaling pathway. Reducing RASSF1A expression did not alter surface expression of the TNFα receptor nor the ability of TNFα to promote IκBα degradation (Figures S2A and S2B, respectively). However, compared to controls, 1A shRNA cells exhibited reduced PARP cleavage (Figure 1F, compare lanes 3 and 4), impaired cytochrome c release (Figure 1G), decreased caspase-3 activity, and retained mitochondrial membrane potential (data not shown) after TNFα stimulation. Consistent with the lack of effect of RASSF1A on apoptosis via the intrinsic pathway (Figure 1E), staurosporine-evoked PARP cleavage (Figure 1F, lanes 5 and 6) and caspase-3 activation (data not shown) were comparable in control and 1A shRNA cells.

Similar to the effects of loss of expression of RASSF1A by RNA interference, RASSF1A$^{-/-}$ mouse embryonic fibroblasts (MEFs) had significantly reduced TNFα-evoked apoptosis when compared to wild-type (wt) cells (Figure 1H, left). However, apoptosis in response to intrinsic pathway activators, such as staurosporine and etoposide, was unimpaired in RASSFF1A$^{-/-}$ MEFs (Figure 1H, left). Cytochrome c release in response to death receptor stimulation also was reduced in RASSF1A$^{-/-}$ MEFs (Figure 1H, right and see Figure S2C for representative fields).

RASSF1A Is Required for Bax Conformational Change and Translocation to Membranes

Our data suggested a role for RASSF1A upstream or at the level of the mitochondria. Bid and Bax play important roles in promoting the release of mitochondrial inner membrane proteins in response to death receptor stimuli (Bouillet and Strasser, 2002; Kuwana et al., 2002; Mikhailov et al., 2003). Bid is thought to aid in Bax insertion into the outer mitochondrial membrane. To carry out this function, Bid must be cleaved into an active p15 fragment by caspase-8 (Kuwana et al., 2002). Antiapoptotic proteins, such as Bcl-xL, prevent the generation of effector caspase activity via the mitochondrial pathway as well as the potential generation of additional caspase-8 activity by a proposed amplification loop (Boatright et al., 2003; Degterev et al., 2003). Thus, cells overexpressing Bcl-xL can be used to monitor initiator caspase activity (mainly caspase-8). TNFα-evoked caspase-8 activation was comparable in control and 1A shRNA cells transfected with Bcl-xL (Figure 2A). Consistent with these findings, endogenous Bid cleavage (Figure 2B) and translocation of GFP-Bid to mitochondria (Figure 2C) were similar in these cells. Therefore, RASSF1A appears to influence a pathway parallel to Bid cleavage/translocation and is required for death receptor-evoked cytochrome c release.

Although Bax is important for activating the mitochondrial pathway of apoptosis (Esposti and Dive, 2003; Kandasamy et al., 2003), the precise mechanism by which death receptor signaling activates Bax has remained elusive (Degenhardt et al., 2002; Esposti and Dive, 2003; Sundararajan et al., 2001). RASSF1A-depleted U20S cells showed impaired Bax translocation to the P100 fraction, comprising of mainly mitochondria and membrane components, in response to TNFα stimulation (Figure 2D). Furthermore, the ability of death receptor stimuli (TNFα or TRAIL) to promote Bax conformational change (as monitored by the Bax conformation-specific antibody 6A7) was reduced, whereas staurosporine-evoked Bax conformational change was unimpaired (Figure 3A). RASSF1A depletion also inhibited Bax conformational change in MCF10A cells (Figure 3B) and in RASSF1A$^{-/-}$ MEFs (Figure 3C and Figure S2D) in response to TNFα stimulation, again consistent with a general requirement for RASSF1A in death receptor-dependent apoptosis in primary, transformed, and nontransformed cells. ShRNA#1 did not affect the expression of another RASSF1 isoform, RASSF1C, indicating a selective proapoptotic function for the 1A isoform (data not shown). As an additional control, shRNA#2, directed to a different region of RASSF1A, also resulted in impaired Bax conformational change and apoptosis in response to TNFα stimulation, whereas a variant hairpin containing two base pair differences from the shRNA1 sequence (1A shRNA mismatch) failed to suppress RASSF1A expression and

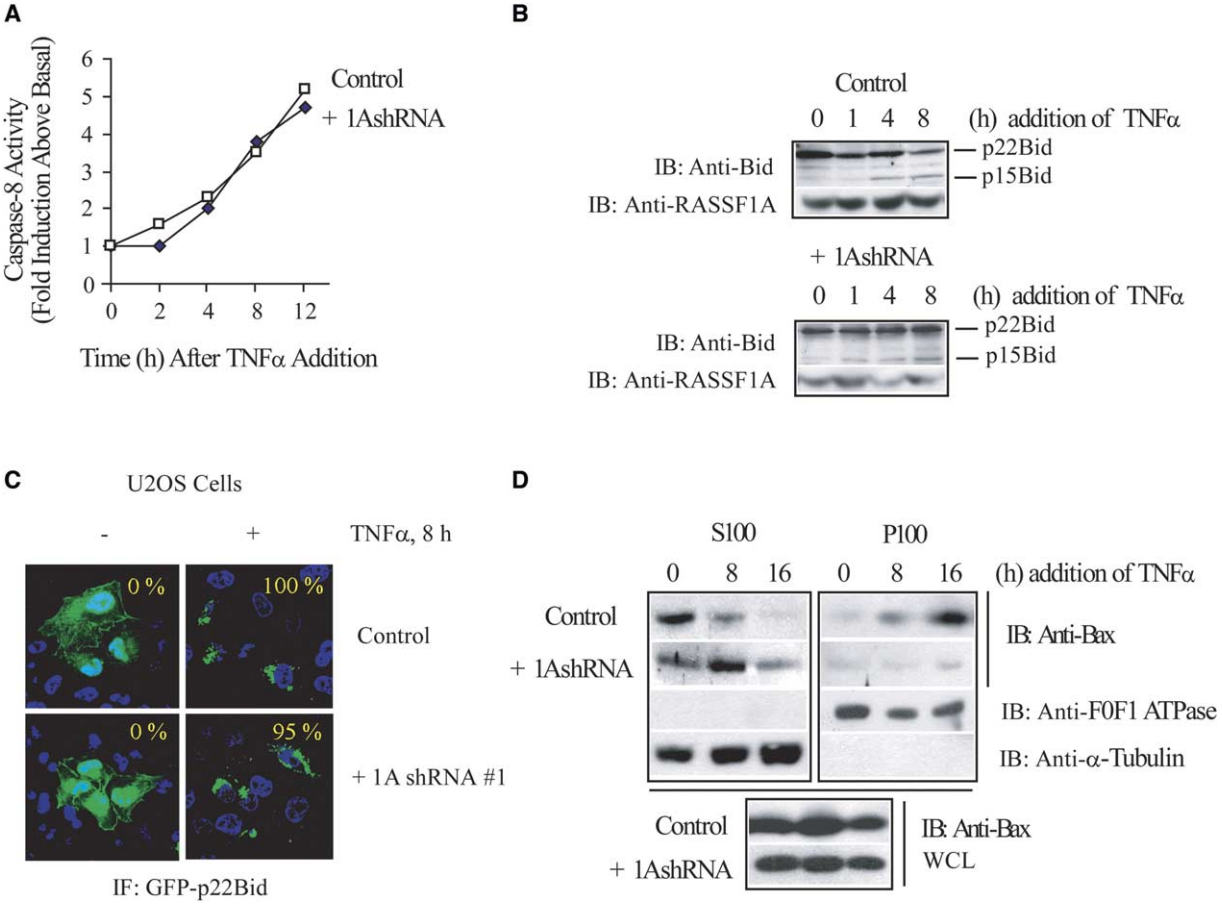

Figure 2. RASSF1A Depletion in U2OS Cells Perturbs Bax, but Not Bid, Translocation

(A) Comparable caspase-8 activation in control and 1A shRNA cells transfected with Bcl-xL. Expression of Bcl-xL was similar in control and 1A shRNA cells (data not shown).

(B) Endogenous Bid is cleaved with similar kinetics in control and 1A shRNA cells. Total cell lysate was prepared and immunoblotted with the indicated antibodies. Immunoblot for Bid was visualized by using an ECL-Supersignal reagent from Pierce.

(C) Loss of RASSF1A by RNA interference does not affect Bid translocation. Immunfluorescence was carried out on cells transiently transfected with a p22Bid expression vector containing a carboxy-terminal GFP tag. Numbers indicate the percentage of GFP-positive cells with a punctate mitochondrial staining pattern.

(D) Reduced Bax translocation in control and 1A shRNA cells. Purity and recovery of fractions were assessed by anti-F0F1 (P100, membrane fraction) or anti-α-tubulin (S100, cytosolic fraction) immunoblotting. WCL, whole-cell lysate.

had no effect on either Bax conformational change or apoptosis (Figures S2E and S2F). Furthermore, RASSF1A-induced apoptosis was comparable in immortalized fibroblasts from wt, Bid$^{-/-}$, and Bak$^{-/-}$ mice with or without the addition of TNFα. However, fibroblasts lacking Bax and Bak (Bax$^{-/-}$/Bak$^{-/-}$) were completely resistant to the proapoptotic effects of RASSF1A (Figure 3D), suggesting an importance of Bax in RASSF1A-mediated apoptosis.

MAP-1 Associates with RASSF1A and Is Required for Death Receptor-Dependent Apoptosis

We could not detect coimmunoprecipitation of RASSF1A with Bax either basally or in response to death receptor stimulation (data not shown). However, in a yeast two-hybrid screen for RASSF1A-interacting partners, we observed specific association with the BH3-like protein MAP-1 (F.L. and S.F., unpublished data). A previous study showed that MAP-1 associates with Bax, but not with Bak, Bid, BimL, or Bcl-w. Association of MAP-1 with Bax required the BH3-like domain of MAP-1 (Tan et al., 2001), suggesting that MAP-1 may regulate Bax activity and the possibility that RASSF1A promotes Bax conformational change via MAP-1. Consistent with this hypothesis, coimmunoprecipitation of endogenous RASSF1A and MAP-1 was observed after TNFα stimulation by using antibodies to MAP-1 (Figure 4A, top) or RASSF1A (Figure 4A, bottom).

These findings suggested that RASSF1A might function via MAP-1, in which case MAP-1 depletion should have effects similar to RASSF1A deficiency. Indeed, U2OS cells stably expressing a MAP-1 shRNA had markedly reduced MAP-1 protein levels (Figure 4B, inset) and decreased TNFα- and TRAIL-evoked apoptosis, as shown by decreased Annexin V staining (Figures 4B and 4C) and nuclear condensation (Figure S3A). Fur-

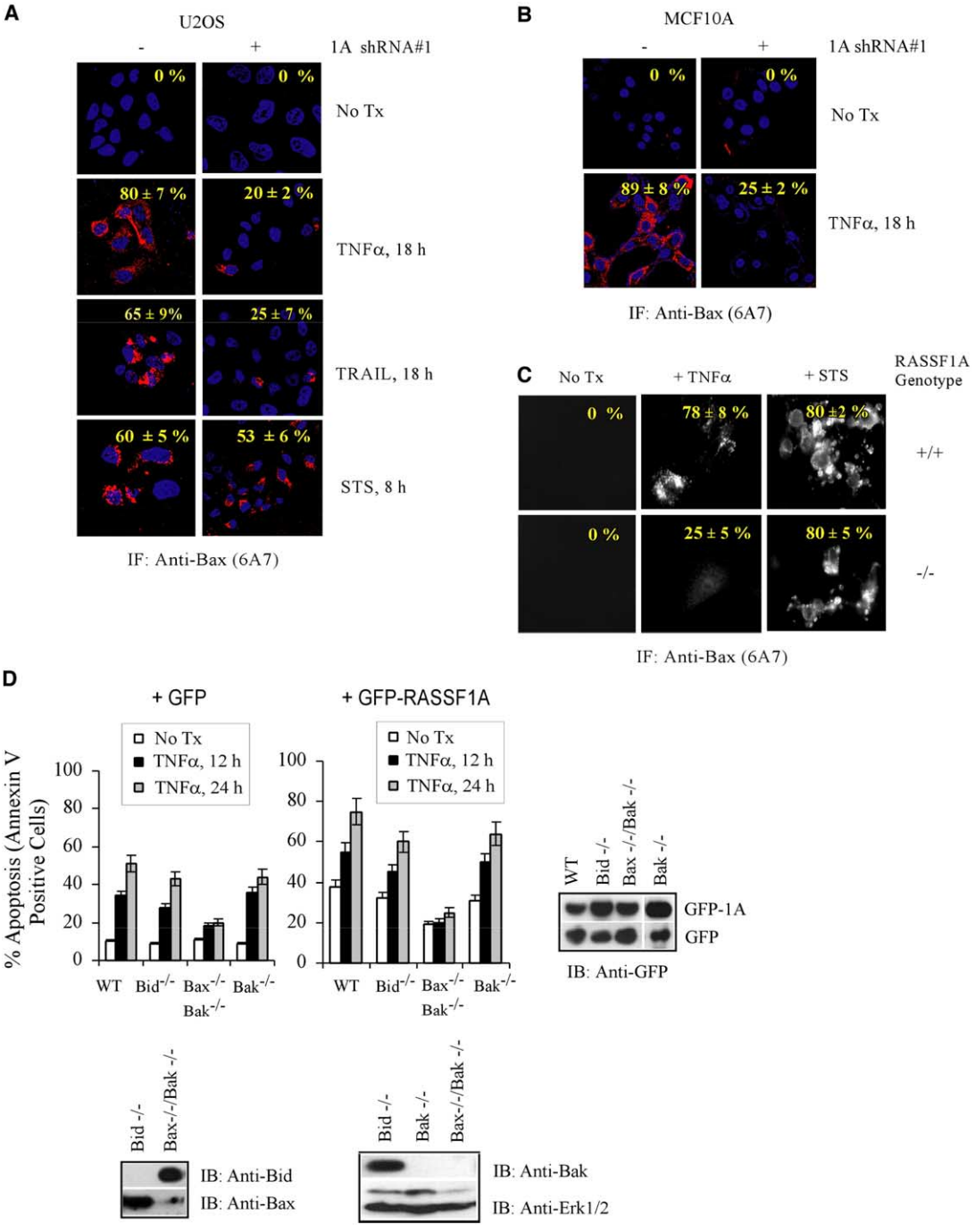

Figure 3. Depletion of RASSF1A (by RNAi) or by Genetic Deletion Perturbs Bax Conformational Change, and RASSF1A-Induced Apoptosis Requires the Presence of Bax

(A) Reduced Bax conformational change in 1A shRNA cells treated with TNFα or TRAIL, but not STS (detected by immunostaining with the conformation-specific monoclonal antibody 6A7 [in red]). Cell nuclei were stained with TOPRO-3 (blue). Numbers indicate the percentage of cells with conformationally altered Bax.

(B) Knockdown of RASSF1A by RNA interference impedes Bax conformational change (as monitored by Bax 6A7 antibody) in MCF10A cells stimulated with TNFα (red); nuclei were stained with TOPRO-3 (in blue). Numbers indicate the percentage of cells with conformationally altered Bax.

(C) Reduced TNFα-stimulated Bax conformational change (as monitored by Bax 6A7 antibody) in primary RASSF1A$^{-/-}$ MEFs when compared to wt MEFs (+/+). However, staurosporine-stimulated Bax conformational change was unaltered. Numbers indicate the percentage of cells with conformationally altered Bax.

(D) Transient expression of GFP-RASSF1A induces apoptosis in wt, Bid$^{-/-}$, and Bak$^{-/-}$ (p values < 0.001 and < 0.02, respectively), but not Bax$^{-/-}$/Bak$^{-/-}$ fibroblasts. Top, Annexin V staining of GFP-positive cells at the indicated times post mouse TNFα stimulation; bottom and side panels, expression of the indicated proteins. Experiments in (A) and (B) used pools of 1A shRNA#1-expressing cells; similar results were obtained with single clones of shRNA#1-expressing cells or using 1A shRNA#2 (see Figures S2C and S2D).

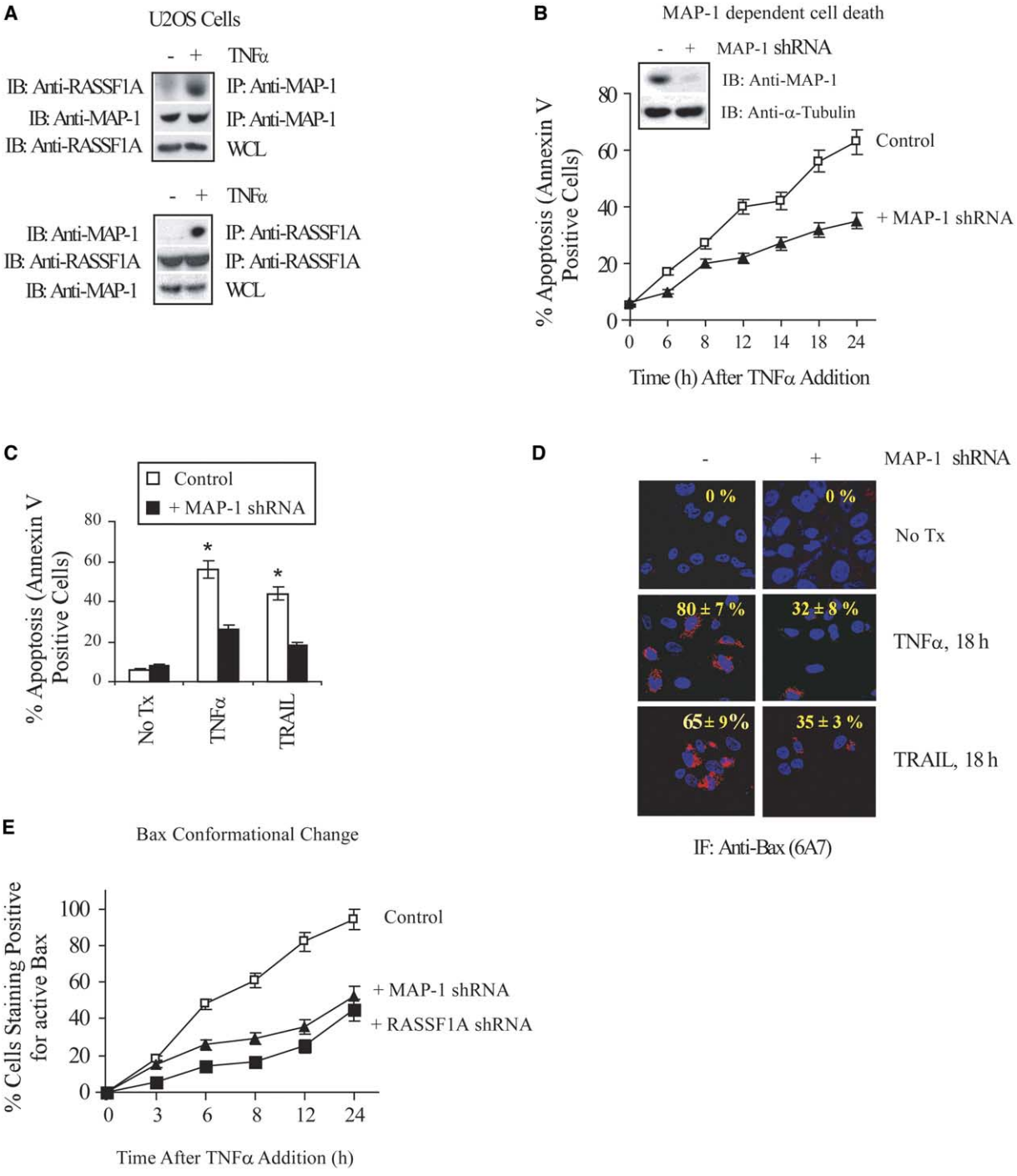

Figure 4. MAP-1 Associates with RASSF1A in Response to TNFα Stimulation and Is Required for Death Receptor-Dependent Apoptosis

(A) Coimmunoprecipitation of endogenous MAP-1 and RASSF1A after TNFα stimulation (3 hr) with the indicated antibodies.

(B) Stable pools of U20S cells expressing MAP-1 shRNA (+ MAP-1 shRNA) exhibit reduced TNFα-evoked apoptosis when compared to stable pools of U2OS cells expressing only the shRNA vector (control). Apoptosis was quantified by Annexin V staining. Inset, immunoblot shows MAP-1 knockdown with α-tubulin as a loading control. Error bars correspond to an error of ± 8% for control cells and ± 6% for MAP-1 shRNA cells.

(C) Decreased apoptosis in stable pools of MAP-1 shRNA cells (+ MAP-1 shRNA) 18 hr post-TNFα or TRAIL stimulation. *p value < 0.003 between control and MAP-1 shRNA cells under the indicated treatments.

(D) Reduced Bax conformational change (detected by immunostaining with the conformation-specific monoclonal antibody 6A7 [in red]) in response to death receptor stimulation in MAP-1 shRNA cells. Nuclei were stained with TOPRO-3 (blue). Numbers indicate the percentage of cells with conformationally altered Bax.

(E) Stable pools of U2OS cells containing the indicated shRNA vectors were stimulated with TNFα and active Bax determined by immunofluorescence using the Bax 6A7 antibody. Numbers indicate the percentage of cells staining positive for the 6A7 antibody. Error bars correspond to an error of ± 6% for control cells, ± 13% for 1A shRNA cells, and ± 11% for MAP-1 shRNA cells.

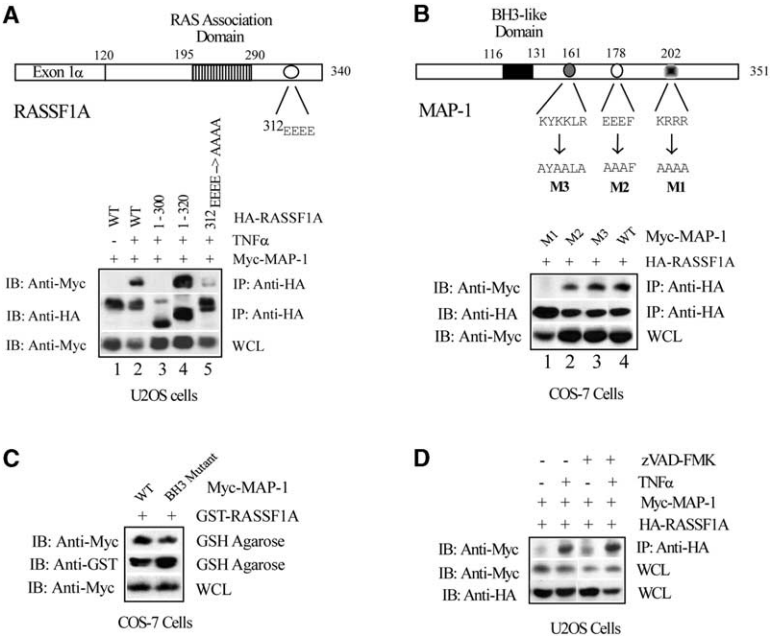

Figure 5. Determinants of RASSF1A/MAP-1 Association

RASSF1A and MAP-1 were ectopically expressed, and proteins associated with RASSF1A were recovered by IP with anti-HA antibodies (A, B, and D) or binding to glutathione agarose (C) followed by immunoblotting with the indicated antibodies. Schematics in (A) and (B) indicate the locations of critical residues and domains (numbers indicate amino acid location).

(A) Association of RASSF1A with MAP-1 requires an acidic region within the carboxyl terminus of RASSF1A (^{312}EEEE), a sequence missing within the deletional mutant 1–300 (lane 3). Lane 5, amino acid changes (within the context of the wt sequence) are indicated.

(B) Association of MAP-1 with RASSF1A requires a basic region within the carboxyl terminus of MAP-1 (^{202}KRRR, mutant M1, lane1). MAP-1 mutants M1, M2, and M3 contain the indicated amino acid changes within the context of wt MAP-1.

(C) Association of MAP-1 with RASSF1A does not require the BH3-like domain of MAP-1.

(D) RASSF1A/MAP-1 association does not require caspase activity. The caspase inhibitor zVAD-FMK was added at 50 μM for 1 hr prior to TNFα stimulation. For (A) and (D), TNFα stimulation was carried out for 3 hr.

thermore, TNFα- and TRAIL-evoked Bax conformational change was impaired in MAP-1-depleted cells (Figure 4D), similar to what was observed for RASSF1A-depleted cells (Figure 4E).

Determinants of RASSF1A/MAP-1 Association

We further characterized how RASSF1A associates with MAP-1. Transiently expressed RASSF1A and MAP-1 coimmunoprecipitated upon TNFα-stimulation (Figure 5A, lanes 1 and 2). Deletion of the carboxy-terminal 40 amino acids of RASSF1A (1–300) abolished association with MAP-1 (Figure 5A, lane 3). Adding back 20 amino acids (1–320) restored MAP-1 interaction (Figure 5A, lane 4), and analysis of additional mutants identified the sequence ^{312}EEEE as essential for MAP-1 binding (Figure 5A, lane 5). The acidic nature of the RASSF1A sequence required for MAP-1 binding (^{312}EEEE; Figure 5A) suggested that a basic motif in MAP-1 might mediate an electrostatic interaction with RASSF1A. Indeed, conversion of a specific basic stretch in MAP-1 to alanines (mutant M1, ^{202}KRRR→^{202}AAAA) abolished MAP-1/RASSF1A association (Figure 5B, lane 1), whereas mutating another stretch of basic residues upstream from ^{202}KRRR (mutant M3, ^{161}KYKKLR→^{161}AYAALA) or an acidic region (mutant M2, ^{178}EEEF→^{178}AAAF) in MAP-1 had no effect (Figure 5B, lanes 3 and 2, respectively). The BH3-like domain of MAP-1 also was dispensable for MAP-1/RASSF1A interaction (Figure 5C) but is required for association with Bax (Tan et al., 2001). Furthermore, the association of RASSF1A with MAP-1 was insensitive to zVAD-FMK treatment and thus is independent of caspase activity (Figure 5D).

RASSF1A Regulates MAP-1 Association with Bax

Next, we characterized the association of MAP-1 with Bax. Coimmunoprecipitation of endogenous MAP-1 and Bax was observed after TNFα stimulation of U2OS cells (Figure 6A). Similarly, transiently expressed MAP-1 coimmunoprecipitated with endogenous Bax upon TNFα-stimulation (Figure 6B, lane 1, row 2). However, in RASSF1A "knockdown" cells, association with Bax was lost (Figure 6B, lane 1, row 3). These data suggested that in the absence of RASSF1A, MAP-1 exists in an inactive ("closed") conformation in which its BH3-like domain is unavailable for interaction with Bax. Association with RASSF1A would then expose the MAP-1 BH3-like domain ("open" conformation), permitting its association with Bax (see Figure 7D).

Such a model predicts that a MAP-1 mutant that is already open should associate with Bax in the absence of RASSF1A. Indeed, MAP-1 mutants M1 (Figure 6B, lane 2, row 3) and M2 (^{178}EEEF→^{178}AAAF) (Figure 6B, lane 4, row 3) had this property, in contrast to wt MAP-1 (Figure 6B, lane 1, row 3) and mutant M3 (Figure 6B, lane 3, row 3). Thus, the M1 and M2 motifs may interact to maintain the closed conformation of MAP-1. The association of mutants M1 and M2 with Bax in the absence of TNFα stimulation was comparable to the association of wt and mutant M3 with Bax in TNFα-stimulated cells (compare lanes 2 and 4 in row 1 with lanes 1 and 3 in row 2), suggesting that once MAP-1 is in the open conformation, its association with Bax is no longer dependent upon TNFα.

Taken together, our observations suggested that RASSF1A is necessary and sufficient to promote MAP-1 conformational change and Bax association, resulting in Bax conformational change and ultimately, cell death. To test this hypothesis, we examined the effects of TNFα on control and 1A shRNA cells stably expressing the aforementioned MAP-1 mutants (Figure 6C). Remarkably, either M1 or M2 sensitized U2OS cells to death receptor-evoked apoptosis (compared to wt

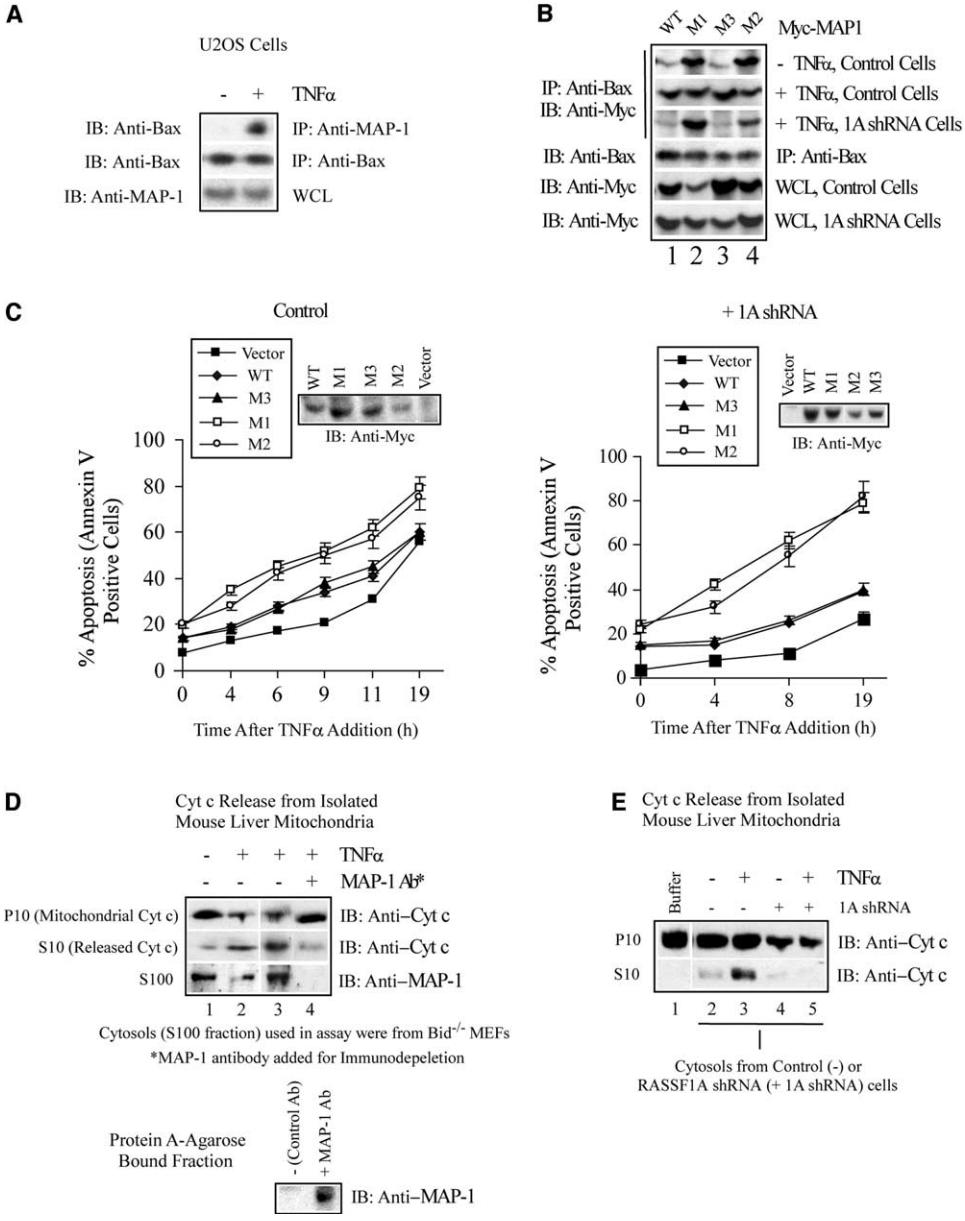

Figure 6. RASSF1A Regulates MAP-1 Association with Bax and Cytochrome C Release

(A) Association of endogenous MAP-1 with Bax upon TNFα stimulation (3 hr) in U2OS cells.

(B) MAP-1/Bax association requires RASSF1A to promote the open conformation of MAP-1. Control or 1A shRNA cells were transiently transfected with the indicated Myc-MAP-1 expression constructs. After TNFα stimulation (3 hr) and immunoprecipitation with anti-Bax antibody 5B7, recovered proteins were detected by immunoblotting with the indicated antibodies.

(C) Open mutants of MAP-1 enhance TNFα-evoked apoptosis. Wt, open mutants of Myc-tagged MAP-1 (M1 and M2), or control mutants (M3) were expressed stably in control (left) or 1A shRNA cells (right) at the indicated levels (inset). Response to TNFα stimulation was carried out for the indicated times, and apoptosis was quantified by Annexin V staining. Significance was evaluated by ANOVA; p value < 0.003.

(D and E) An in vitro cytochrome c release assay was carried out by using isolated mouse liver mitochondria (prepared as described in Gogvadze et al., [2001]). Bid$^{-/-}$ MEFs (D) or control or 1A shRNA cells (F) were stimulated with TNFα using either mouse (D) or human (E) TNFα 2 hr prior to subcellular fractionation. The cytosolic (S100) fraction was isolated and mixed with isolated mouse liver mitochondria for 60 min at 30°C, followed by centrifugation at 10,000 × g at 4°C for 5 min to obtain pellet (P10, containing intact mitochondria) and supernatant (S10, containing released cytochrome c) fractions. In (D), the S100 fraction was incubated with control ([D], lane 3) or MAP-1 antibodies ([D], lane 4) overnight, and immune complexes were removed with protein A agarose (see bottom). The supernatant after protein A-agarose precipitation was mixed with isolated mouse liver mitochondria as described above. MAP-1-depleted supernatant (note MAP-1 immunoblot, [D], lane 4) or supernatant from 1A shRNA cells ([E], lanes 4 and 5) cannot promote cytochrome c release.

MAP-1 and the M3 mutant; Figure 6C). MAP-1 mutants M1 and M2 also sensitized RASSF1A knockdown cells to death receptor-evoked apoptosis (Figure 6C, right), and significantly enhanced Bax conformational change in the absence of RASSF1A (Figure S3B). In contrast, wt MAP-1 (or mutant M3) sensitized U2OS cells to death

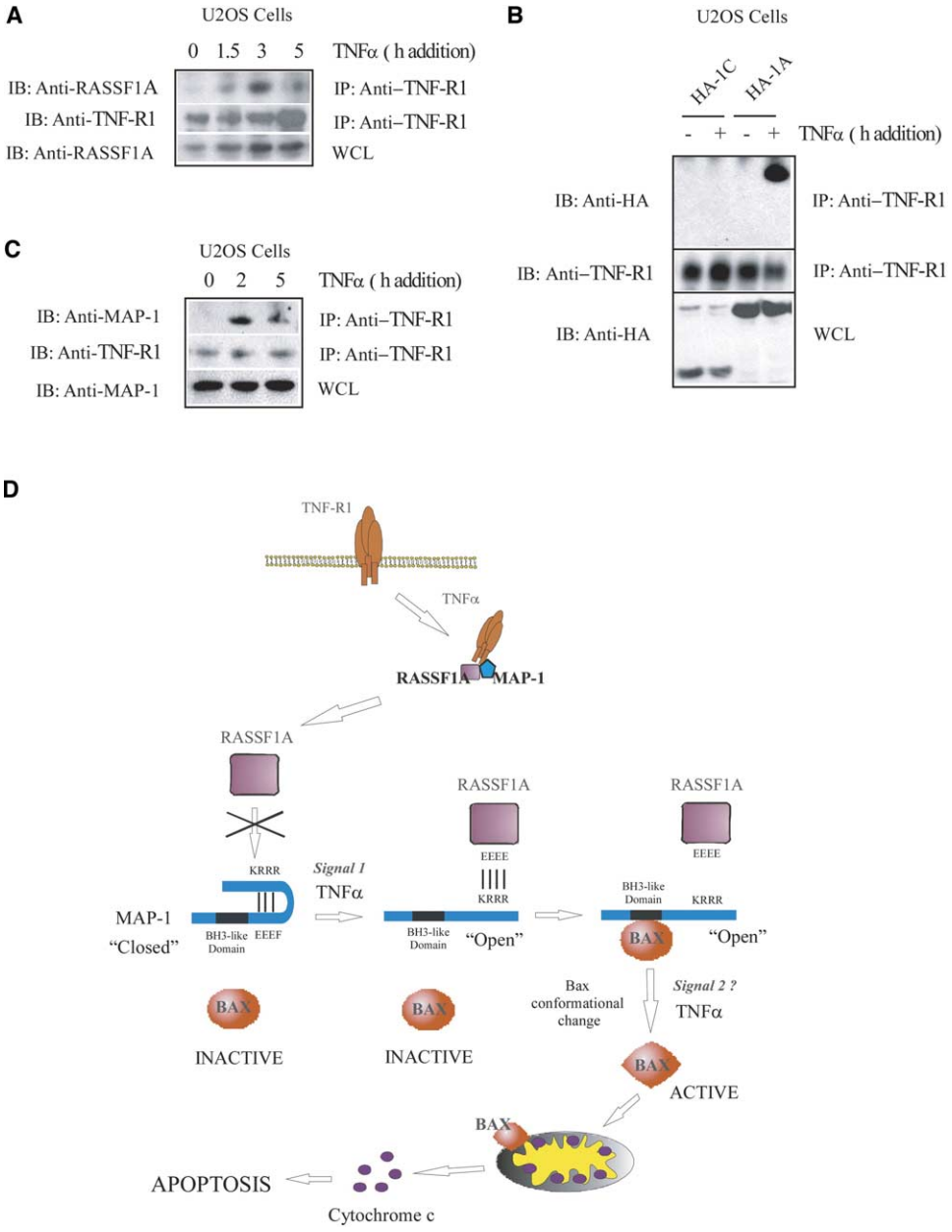

Figure 7. TNFα Stimulation Recruits RASSF1A and MAP-1 to Death Receptors Complexes

(A) Coimmunoprecipitation of endogenous RASSF1A with TNF-R1 after TNFα stimulation in U2OS cells.

(B) RASSF1A, not RASSF1C, associates with TNF-R1 after TNFα stimulation in U2OS cells.

(C) Coimmunoprecipitation of endogenous MAP-1 with TNF-R1 after TNFα stimulation in U2OS cells.

(D) Model of the proposed RASSF1A/MAP-1 pathway. TNFα stimulation recruits MAP-1 and RASSF1A to death receptor complexes. This results in the association of RASSF1A and MAP-1, and subsequently, RASSF1A regulates Bax conformational change via the BH3-like protein MAP-1. MAP-1, normally kept inactive via an intramolecular electrostatic interaction, associates with RASSF1A upon death receptor activation and acquires an open conformation. Together with as yet unidentified additional death receptor-evoked signal(s) (signal 2), RASSF1A promotes Bax conformational change, mitochondrial membrane insertion of Bax, and ultimately, cytochrome c release and apoptosis. See text for further details.

receptor apoptosis only when RASSF1A was expressed (Figure 6C, compare right and left panels for wt MAP-1 and mutant M3). Although the open mutants of MAP-1 resulted in an increase in basal apoptosis, they further enhanced death receptor-evoked apoptosis upon TNFα stimulation (Figure 6C, right), suggesting that at least one additional death receptor signal collaborates with the RASSF1A/MAP-1/Bax pathway after MAP-1 has acquired the open conformation (see Discussion). Consistent with this notion, RASSF1A/MAP-1 association occurs independently of caspase activity (Figure 5D), but caspase activation was still required for TNFα-evoked apoptosis in cells expressing the MAP-1 open mutants (S.B. and B.G.N., unpublished data). The identity of this

additional caspase-dependent signal (signal 2) is currently under investigation.

Loss of MAP-1 or RASSF1A Fails to Promote Cytochrome C Release

We further evaluated the role of RASSF1A and MAP-1 in TNFα-dependent cytochrome c release in the both Bid$^{-/-}$ fibroblasts and U2OS-RASSF1A knockdown cells. Cytosol (S100 fraction) from TNFα-stimulated Bid$^{-/-}$ cells promoted cytochrome c release in an in vitro cytochrome c release assay by using isolated mouse liver mitochondria (Figure 6D, lane 2). Immunodepletion using MAP-1 antibodies (Figure 6D, lane 4), but not control antibodies (Figure 6D, lane 3), rendered this fraction unable to promote cytochrome c release. Cytosol from RASSF1A-depleted cells (1A shRNA) also was unable to promote cytochrome c release in this assay (Figure 6E, compare lanes 3 and 5). These results suggest that both RASSF1A and MAP-1 are required to promote Bax activity (in releasing cytochrome c), consistent with the ex vivo studies above.

Recruitment of RASSF1A and MAP-1 to Death Receptor Complexes

Finally, we evaluated the ability of RASSF1A and/or MAP-1 to be recruited to activated death receptor complexes. Endogenous RASSF1A was recruited to the TNFα receptor (TNF-R1) in response to TNFα stimulation (Figure 7A) and to the TRAIL-R1 receptor (TRAIL-R1) in response to TRAIL addition (Figure S3C) in U2OS cells. Interestingly, association with TNF-R1 was specific for RASSF1A and was not observed with RASSF1C (Figure 7B), suggesting specificity for the unique amino terminus of RASSF1A encoded by exon 1α. MAP-1 also was recruited to TNF-R1 (Figure 7C) and TRAIL-R1 receptor complexes (Figure S3D) in response to their respective cognate ligand. Preliminary analysis suggests that RASSF1A and MAP-1 associate with internalized death receptors (S.B. and B.N.G, unpublished data), a situation that is commonly observed for the recruitment of proapoptotic components to TNF-R1 or TRAIL-R1 complexes (Micheau and Tschopp, 2003; Schneider-Brachert et al., 2004; Thorburn, 2004). These data suggest that death receptor activation functions to bring together RASSF1A and MAP-1 in death receptor complexes in order for RASSF1A to influence MAP-1 conformational change and promote MAP-1/Bax association (Figures 6A and 6B), Bax activity (Figures 2D and 3A–3C), stimulation of cytochrome c release (Figures 1G and 1H), activation of effector caspases (data not shown), and promotion of condensed nuclei formation (Figure S3A) and apoptosis (Figures 1D and 1E–1H).

Discussion

Although *RASSF1A* is epigenetically silenced in many carcinomas, its mechanism of action has remained unclear. Here, we have uncovered an important role for this protein in regulating death receptor-induced apoptosis. Inducible expression of RASSF1A in MCF-7 cells resulted in sensitization to death receptor-dependent apoptosis with no augmentation of the intrinsic pathway of apoptosis (Figures 1A and 1B). Furthermore, knockdown of RASSF1A expression in U2OS cells resulted in the specific loss of death receptor-dependent apoptosis (Figures 1D and 1E). Confirming the results of our RNA interference studies, RASSF1A$^{-/-}$ MEFs have defective death receptor-dependent apoptosis with no change in intrinsic pathway responses (Figure 1H). We identify the BH3-like protein MAP-1 as a downstream mediator of RASSF1A-dependent apoptosis in response to death receptor stimulation. RASSF1A/MAP-1 association is essential for Bax conformational change, mitochondrial membrane insertion, and cytochrome c release both in vitro and ex vivo (Figures 3–5).

Our results lead to a model for RASSF1A and MAP-1 in death receptor-evoked apoptosis (Figure 7D). In the absence of extrinsic apoptotic stimuli, RASSF1A is not associated with MAP-1, and MAP-1 exists in a closed conformation with its BH3-like domain inaccessible. Death receptor stimulation (e.g., by the addition of TNFα and TRAIL) promotes the recruitment of both RASSF1A and MAP-1 to receptor complexes within 2–3 hr of ligand stimulation. The majority of DISC components, such as TRADD, FADD, and caspase-8, are recruited to death receptor complexes within an hour of stimulation and associate mainly with the internalized receptor (Harper et al., 2003; Micheau and Tschopp, 2003; Schneider-Brachert et al., 2004). Shortly after internalization, death receptors (such as TNF-R1) encounter an "apoptotic checkpoint," whereby complexes are brought together to continue to promote survival or stimulate apoptosis. We propose that RASSF1A and MAP-1 associate with TNF-R1 during this apoptotic checkpoint and may influence the direction of TNF-R1 signaling. We do not have an explanation for why the association of RASSF1A and MAP-1 with death receptors is later than the recruitment of most proapoptotic components to TNF-R1, although conceivably RASSF1A and/or MAP-1 may influence a final step in the decision to promote apoptosis. The precise mechanism and dynamics by which these two proteins are recruited to internalized death receptors also awaits future experimentation.

As mentioned above, death receptor signaling brings RASSF1A and MAP-1 into close proximity. RASSF1A and MAP-1 association occurs via an intermolecular electrostatic interaction involving the C terminus of RASSF1A and a basic stretch within MAP-1 (Figure 7D, signal I). This association promotes the open form of MAP-1, with its BH3-like domain exposed. The open form of MAP-1 can now associate with Bax and induce Bax conformational change (Figures 6B and Figure S3B). Consistent with our model, stable cell lines containing open mutants of MAP-1 are sensitized to death receptor-dependent apoptosis in the absence of RASSF1A, suggesting that they no longer require the presence of RASSF1A in order to initiate apoptosis (Figure 6C). However, maximal or sustained Bax activity and subsequent apoptosis require an additional, as yet unidentified, death receptor signal (Figure 7B, signal 2), as further cell death occurs when cells expressing the open mutants of MAP-1 are stimulated with TNFα (Figure 6C, right and Figure S3B). RASSF1A/MAP-1 interaction, together with this second TNFα-dependent signal, is required to promote Bax conformational change and

its insertion into the mitochondrial membrane (Figure 7D). The nature of this second signal is currently being investigated, but preliminary results suggest that it may involve proteolytic cleavage of MAP-1 (S.B and B.G.N., unpublished data).

In our model, Bid (acting in a parallel pathway) plays a potentiating rather than an essential role in promoting cytochrome c release and apoptosis. Consistent with this suggestion, Bid$^{-/-}$ cells retain TNFα- and RASSF1A-evoked apoptosis (Yin et al. [1999] and Figure 3D), and the level of caspase-8 activation (Figure 2A) and Bid cleavage (Figure 2B) was comparable in control and RASSF1A knockdown cells. Furthermore, because MAP-1 associates with Bax, but not Bak (Tan et al., 2001), and Bak$^{-/-}$ cells retained TNFα- and RASSF1A-evoked apoptosis, we propose that RASSF1A selectively influences how MAP-1 regulates the activity of Bax and subsequent Bax-modulated release of cytochrome c from the mitochondria.

Our results establish that RASSF1A is required for death receptor-evoked proapoptotic signaling, but the relationship between this function and its role as a tumor suppressor gene remains to be clarified. Consistent with a causal link between the proapoptotic and tumor suppressor actions of RASSF1A, most tumor cells fail to undergo apoptosis in response to death receptor activation (Ivanov et al., 2003; Thorburn, 2004). Although genetic ablation of single death receptor pathways typically does not result in increased tumor incidence, RASSF1A and MAP-1 are required for apoptosis in response to multiple death receptors. Conceivably, pan-deficiency in death receptor signaling, together with other tumor-associated mutations, could be prooncogenic; this issue can now be clarified with the availability of the RASSF1A$^{-/-}$ mice (Tommasi et al., 2005).

Of the RASSF1 isoforms, only *RASSF1A* is frequently inactivated in tumors by methylation of the promoter for exon 1α (Dammann et al., 2005). RASSF1C is unable to promote apoptosis in MCF-7 cells, despite its ability to interact with MAP-1. Interestingly, however, RASSF1C cannot interact with ligand-stimulated death receptor complexes (Figure 7B). Further work will be required to resolve the relationship between the tumor suppressor and death receptor signaling functions of RASSF1A. If these functions are related, our identification of MAP-1 as an essential downstream mediator of death receptor signaling via RASSF1A suggests that MAP-1 also may be a tumor suppressor gene. Analysis of the MAP-1 locus and its protein product, particularly in tumor cells in which RASSF1A expression is retained, should address this possibility.

Experimental Procedures

Cell Lysis and Immunoprecipitations

Unless otherwise indicated, cells were lysed in RIPA buffer (50 mM Tris-HCl, [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) and a protease inhibitor cocktail (1 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). Endogenous associations were assessed by immunoprecipitations using 2 μg of antibodies (MAP-1/Bax association, rabbit anti-MAP-1, QED; mouse anti-Bax [5B7] Trevigen; MAP-1/RASSF1A association, mouse anti-RASSF1A [eB14], eBiosciences; RASSF1A or MAP-1/TNF-R1, rabbit anti-TNF-R1 antibody, Santa Cruz [H-271] for immunoprecipitation and immunoblotting in 5% BSA for nonspecific blocking prior to antibody incubation; RASSF1A or MAP-1/TRAIL-R1, goat anti-TRAIL-R1, R&D Biosciences [AF347] for immunoprecipitation; and rabbit anti-TRAIL-R1, Santa Cruz [H-130] for immunoblotting in 5% milk for nonspecific blocking prior to antibody incubation). For Bax immunoprecipitations, lysates and washes were performed by using 1% CHAPS, 10 mM HEPES, (pH 7.4), 100 mM NaCl, and protease inhibitors. After lysis for 20 min at 4°C, samples were clarified by microfuge centrifugation and incubated with antibody overnight. After overnight incubation, samples were added to a 50% slurry of either protein A or protein G Sepharose affinity beads for 2 hr, followed by two washes with 1XPBS and bound proteins resolved by SDS-PAGE. For precipitation experiments using GST fusion expression constructs, lysates were incubated with a 50% slurry of glutathione agarose beads (Sigma) overnight, followed by two washes with 1XPBS and bound proteins resolved by SDS-PAGE. For all whole-cell lysate (WCL) immunoblots, ~100 μg of protein/lane were used.

Immunofluorescence

To detect Bax conformational change, cells were plated onto 22 × 22 mm coverslips in 6-well dishes (Corning) and fixed in 1% paraformaldehyde/1XPBS for 15 min at room temperature, followed by two 1XPBS washes and incubation overnight with monoclonal antibody 6A7 (Trevigen) 1:200 in 0.05% digitonin/1XPBS. Samples were washed twice with 1XPBS, once with water, and mounted on glass slides with VectaShield (Vector Laboratories). For cytochrome c translocation assays, cells were permeabilized in 0.2% Triton X-100/1% BSA/1XPBS for 20 min after fixation in 1% paraformaldehyde/1XPBS, washed twice with 1XPBS, and incubated overnight with monoclonal anti-cytochrome c antibody (BD Pharmingen, 556432), 1:200 in 1% BSA/1X PBS. After overnight incubation, coverslips were washed twice with 1XPBS and incubated for 1 hr with secondary antibodies (for BaxIF: 1:500 of goat anti-mouse Cy3 [Jackson Laboratory]; for cytochrome cIF: 1:500 dilution of Alexa 546 goat anti-mouse, [Molecular Probes] in 1% BSA/1XPBS). Nuclei were stained with TOPRO-3 (Molecular Probes, 0.2 μM in 1% BSA/PBS) for 15 min after secondary antibody staining. Confocal images were acquired by using a Zeiss Laser Scanning Microscope and analyzed with LSM510 software.

Apoptosis Assays

Etoposide (100 μM) or staurosporine (3 μM) were added as indicated. Mouse or human TNFα or TRAIL (50 ng/ml) were added together with 10 μg/ml cyclohexamide for the indicated times (except for MCF-7 cells where 100 ng/ml was used). For Annexin V staining, cells were harvested by trypsinization and centrifuged into a fluorescence-activated cell sorting (FACS) tube to which 100 μl of Annexin V buffer (10 mM HEPES, [pH 7.4], 150 mM NaCl, and 2.5 mM $CaCl_2$ containing 2 μg of Annexin V 647 Fluor [Molecular Probes]) were added. After incubation for 20 min in the dark, samples were diluted with 400 μl of Annexin V buffer, and flow cytometry was carried out on a FACSCaliber (Becton-Dickinson) by sequential gating on the FL3 (PI) and FL4 (Annexin V 647 Fluor) channels.

Caspase activity was assayed fluorometrically by measuring the hydrolysis of appropriate peptide substrates linked to 7-amino-4-trifluoromethyl coumarin (AFC). For caspase-3 assays, cells were lysed in 50 μl of 0.5% Triton X-100 in 1XPBS and clarified by centrifugation at 20,000 × g for 5 min at 4°C. Supernatants were normalized for protein content and incubated in the presence of 10 μM z-DEVD-AFC for 2 hr. Caspase-8 assays using the substrate IETD-AFC (50 μM) were performed by using a commercially available kit (BD Biosciences). Fluorescence of liberated AFC was monitored on a CytoFluor Multi-Well Plate Reader, Series 4000 (PerSeptive Biosystems). All apoptosis assays were performed at least four times. Data for all immunofluoresence and apoptosis assays were evaluated by Student's t test (two tailed), unless otherwise stated. P values were used instead of percent error in order to evaluate the statistical significance between the indicated groups.

Subcellular Fractionation

Mitochondrial and cytosolic fractions were isolated by a modified needle lysis protocol (without digitonin) (Zhivotovsky et al., 1999).

Briefly, cells from two 10 cm^2 dishes were resuspended in 500 μl of 320 mM sucrose, 1 mM EDTA, 50 mM HEPES, (pH 7.3), and 1 μM dithiothreitol and disrupted by 13 strokes through a 25 5/8 gauge needle. Debris and nuclei were pelleted at 1000 × g for 10 min, and the resultant supernatant centrifuged at 100,000 × g for 30 min to obtain P100 (mitochondria and microsomes) and S100 (cytosol) fractions. Purity and recovery of the S100 and P100 were assessed by immunoblotting with anti-α-tubulin and anti-F0F1 ATPase, respectively. For cytochrome c release assays, mouse liver mitochondria were isolated as described previously (Gogvadze et al., 2001) and resuspended at 0.5 mg/ml in 125 mM KCl, 10 mM Tris-MOPS (pH 7.4), 5 mM glutamate, 2.5 mM malate, 1 mM KPO4, and 10 mM EGTA-Tris (pH 7.4). Assays were carried out by using 30 μl of the S100 cytosolic fraction plus 30 μl of mitochondrial suspension for 60 min at 30°C, followed by centrifugation at 10,000 × g at 4°C for 5 min to obtain pellet (P10, containing intact mitochondria) and supernatant (S10, containing released cytochrome c) fractions, respectively. The P10 was solubilized in RIPA buffer for immunoblot analysis.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and three figures and are available with this article online at http://www.molecule.org/cgi/content/full/18/6/637/DC1/.

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Supplemental Data

The Tumor Suppressor RASSF1A and MAP-1

Link Death Receptor Signaling to Bax

Conformational Change and Cell Death

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Supplemental Experimental Procedures

Antibodies and Reagents

Alexa Fluor 546, TOPRO-3, Propidium Iodide (PI) and Mitotracker Orange (Molecular Probes). Mouse anti-α-tubulin, rabbit anti-PARP and Bcl-xL (Cell Signaling Technologies); mouse anti-cytochrome c (BD Pharmingen); rabbit anti-Bid, Erk1, and Erk2 (Santa Cruz Biotechnology), mouse anti-GFP and GST (Santa Cruz Biotechnology); rabbit anti-Bak (Upstate Biotechnology), human TNFα and TRAIL (Peprotech); z-VAD-FMK (Calbiochem). Murine monoclonal anti-HA (12CA5) and anti-Myc (9E10) were purified from their corresponding hybridomas. Pierce Supersignal ECL was utilized for detection of endogenous association with receptor components, while in house ECL detection was used for all other immunostaining analysis.

Cells Lines and Transfections

COS-7 and U2OS cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal calf serum (FCS). MCF-7 cells, RASSF1A$^{-/-}$ primary mouse embryonic fibroblasts (MEFs), and immortalized WT, Bid$^{-/-}$ and Bax$^{-/-}$/Bak$^{-/-}$ and Bak$^{-/-}$ MEFs were maintained in DMEM plus 10% FCS, 1% non-essential amino acids, and 1% L-gluatmine. Fugene6 (Roche) was used for transient transfection of U2OS cells and MEFs. To generate stable MCF-7, U20S and MCF10A cell lines, transfections were carried out using Effectene (Qiagen). Single cell clones and pools were obtained from each transfection. For doxycycline-inducible cell lines, we first generated MCF-7 cells expressing the tetracycline repressor. These cells were transfected with a vector directing RASSF1A expression under control of a tetracycline-responsive element (Invitrogen). Further details are available from S.B. upon request.

Expression Vectors

For shRNA vectors, complementary phosphorylated oligonucleotides were cloned into pSUPER(Brummelkamp et al., 2002) using Bgl II/HindIII. For RASSF1A, primers were: 1A shRNA-#1, 5'-CGTGGACGAGCCTGTGGAG-3' (corresponding to nucleotides 351 - 373 of human RASSF1A); 1A shRNA-#2, 5'-GCTGAGATTGAGCAGAAGA-3' (corresponding to nucleotides 394 - 416 of human RASSF1A), 1A Mismatch (mutant to#1), CGTGGACGA<u>T</u>CCTGT<u>A</u>GAG (underlined residues indicated changed residues). For MAP-1, 5'-TCGAGGAGGCTCTGCAGGC-3' was used (corresponding to nucleotides 101 - 123 of human MAP-1).

RASSF1A expression constructs were generated by PCR. All HA-, Myc-, and GFP-tagged proteins contained single tags at their amino termini. For GFP-RASSF1A, the forward primer was 5'-tgcgaattcactagtatgtcgggggagcct-3'; reverse primer was 5'- ggtggatccatatcacccaagggg-gcaggc-3'. The PCR product was cloned as an EcoR1/Bam H1 fragment into pEGFP-C2 (Clontech), placing GFP N-terminal of RASSF1A. For all HA-RASSF1A constructs, the forward primer was 5'-cag-tgcggatccatcgatatgtcgggggagcct-3'. Reverse primers were: 5'-tatgcggccgctattcacc-caaggggcaggc-3' (WT);

5'-tatgcggccgctattcattcaggcatgctgaa-3' (1-300); 5'-tatgcggccgctattcagaggtgctcctcctc-3' (1-316); and 5'-tatgcggccgctattcacaggatctggcggag-3' (1-320). All PCR products were cloned as BamH1/Not1 fragments into pCDNA3. Myc-MAP-1 mutants (M1, M2, and M3) were generated with the Stratagene Quickchange Kit, using complementary oligonucleotides containing the mutations of interest. To generate carboxyl terminal GFPp22 Bid, pCDNA3-p22 Bid (from S. Korsmeyer) was used as template for PCR using forward primer (5'-tgcgaattcactagtatggactctgaggtcagc-3') and reverse primer (5'-cggtggatcccggtccatctcgtttctaac-3'). The resultant PCR product was cloned as an EcoR1/BamH1 fragment into pEGFP-N1 (Clontech). Further details on the generation of these constructs are available from S.B. upon request. All expression constructs were confirmed by sequencing.

Surface Staining of TNF-R1

Surface TNF-R1 expression was assessed using antibodies against its extracellular domain (mouse anti-TNF-R1, sc-8436 [Santa Cruz Biotechnology]). Cells were harvested by scraping into 1 X PBS, followed by the addition of primary antibodies (100 µl solution of 3 µg/ml antibody in 1 X PBS + 3% FBS) for 30 min on ice. Cells were then washed twice with 1 X PBS, followed by the addition of PE-labeled hamster anti-mouse IgG1 secondary antibodies (BD Pharmingen, 1:50). Staining with secondary antibodies alone was used as a negative control. All experiments were carried out at least four times using single clones and pools of the stable cell lines with similar results

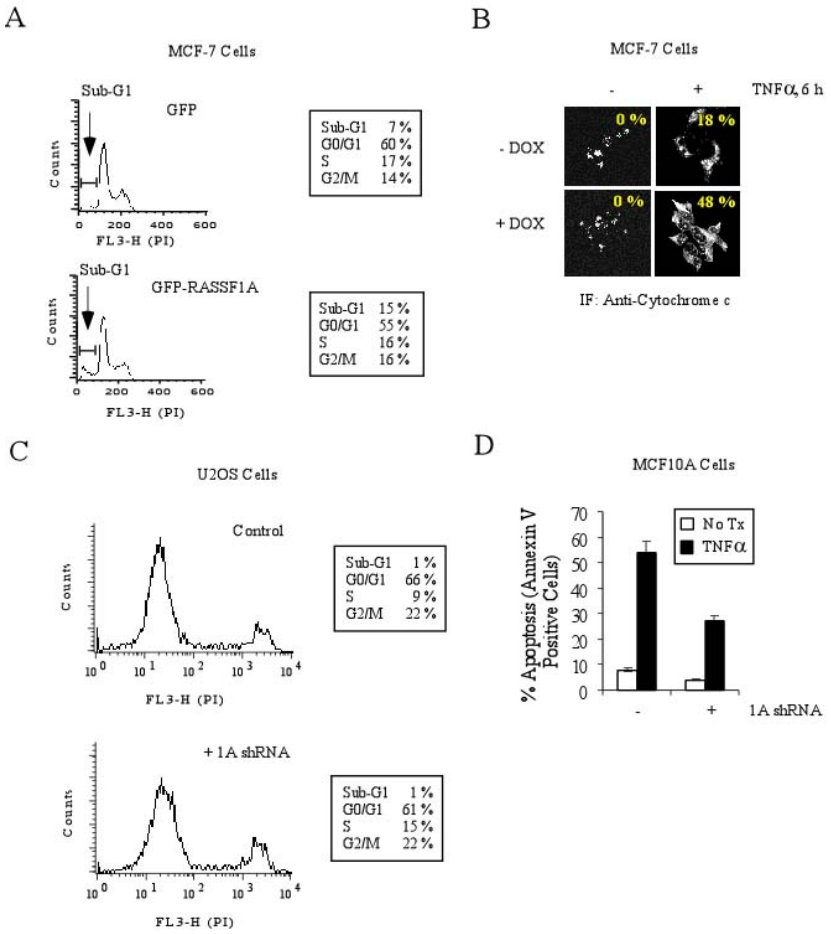

Figure S1. RASSF1A Promotes Apoptosis, not Cell Cycle Arrest, in MCF-7 cells.
(A) Cell cycle analysis of MCF-7 cells transfected with GFP-RASSF1A or GFP, stained with propidium idodide (PI), and subjected to flow cytometric analysis. Note the lack of effect on cell cycle distribution, apart from the appearance of a sub-G1 population.

(B) Precocious release of endogenous cytochrome c in MCF-7 cells expressing RASSF1A (+ DOX) after short exposure to TNFα (6 h). Numbers represent percentage of cells containing diffuse cytochrome c staining.
(C) Asynchronous pools of U2OS cells expressing RASSF1A shRNA (#1) demonstrate no significant changes in cell cycle populations.
(D) Pooled MCF10A cells expressing shRNA#1 (+ 1A shRNA) exhibit reduced TNFα-evoked apoptosis when compared to pooled MCF10A cells expressing shRNA vector only (Control). Apoptosis was quantified by Annexin V staining and MCF10A cells with 1A shRNA had comparable knockdown of RASSF1A as in U2OS cells (data not shown). Error bars correspond to an error of ± 8%.

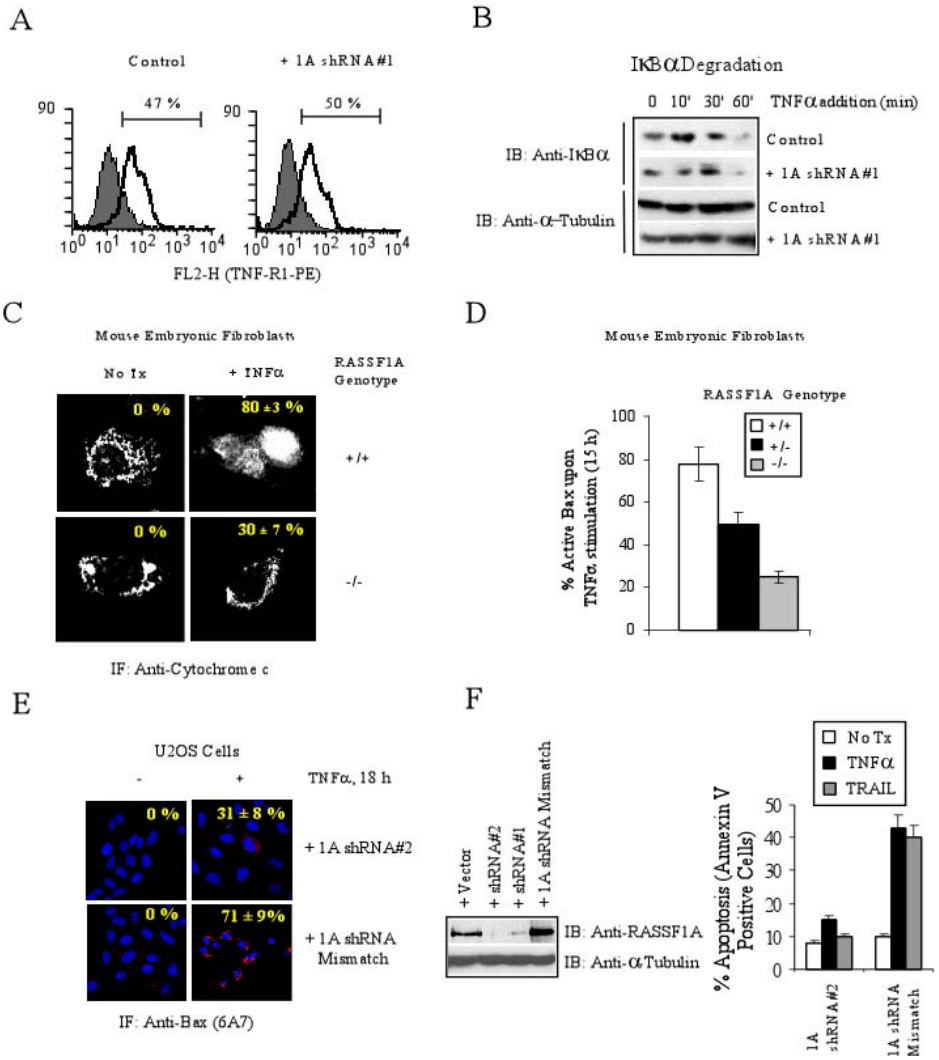

Figure S2. RASSF1A and MAP-1 Influences Bax Conformational Change and Condensed Nuclei Formation, but not Surface TNF-R1 Expression or IκBα Degradation
(A and B) RASSF1A knockdown by RNA interference does not affect surface TNF-R1 expression (A) or IκBα degradation. For (B), only TNFα (100 ng/ml) was added for the indicated times.

(C) Reduced TNFα-stimulated cytochrome c release (monitored as in Figure 1G) in primary RASSF1A$^{-/-}$ MEFs when compared to wild type MEFs (+/+). Numbers represent percentage of cells containing diffuse cytochrome c staining (released cytochrome c).
(D) Bar Graph representing reduction in TNFα-stimulated Bax conformational change in primary MEFs from wild type (+/+), RASSF1A$^{+/-}$, and RASSF1A$^{-/-}$ MEFs (see Fig. 3C for representative fields). Error bars correspond to an error of ± 11%.
(E) Knockdown of RASSF1A in the presence of two shRNAs impedes Bax conformational change (as monitored by Bax 6A7 antibody) in U2OS cells stimulated with TNFα (red); nuclei were stained with TOPRO-3 (in blue). Numbers indicate percentage of cells with conformationally altered Bax. However, a mismatch mutant shRNA does not impede Bax conformational change. The 1A shRNA mismatch contains two base pair changes from the shRNA#1 sequence.
(F) Left panel, immunoblot showing knockdown of RASSF1A in the presence of the two shRNAs and a mismatch mutant. Right panel, Annexin V staining of U2OS containing stable pools or 1A shRNA#2 or 1A shRNA mismatch. TNFα or TRAIL at were added with cycloheximide for 15 h. Error bars correspond to an error of ± 9%.

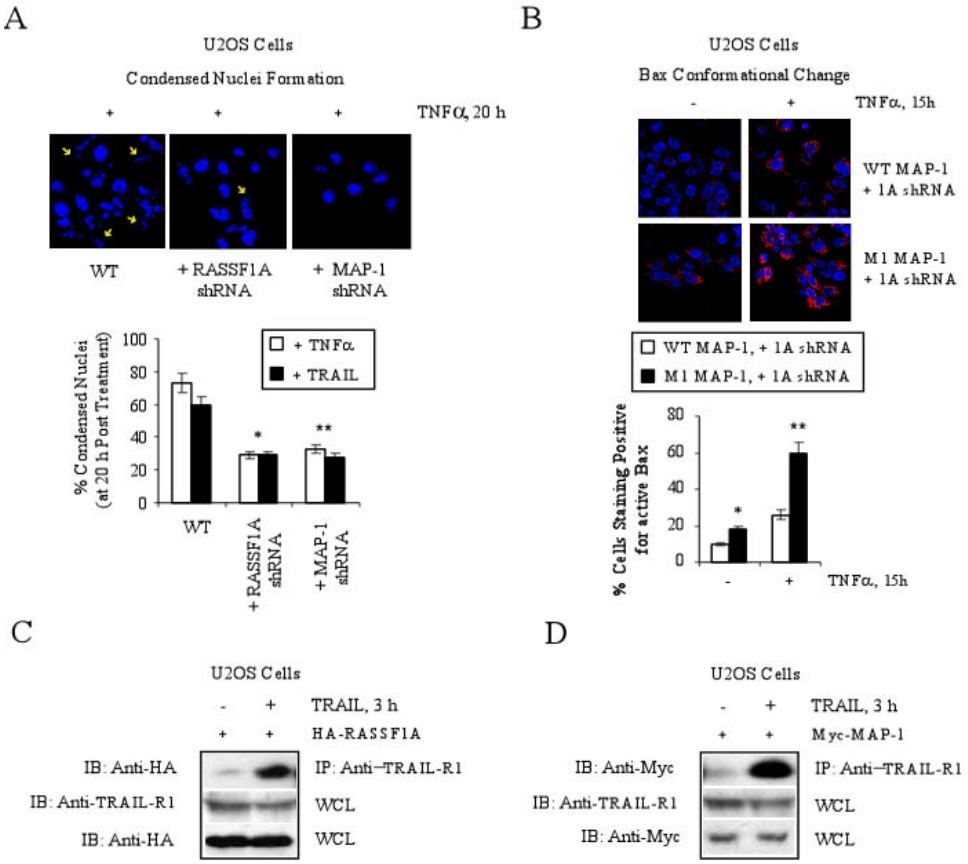

Figure S3. Both RASSF1A and MAP-1 are Required for Condensed Nuclei Formation and Bax Activity and can Associate with the TRAIL receptor R1 (TRAIL-R1).
(A) Pools of U2OS cells containing the indicated shRNA vectors were stimulated with TNFα and TRAIL and the percentages of condensed nuclei formation were quantified; * p value < 0.002; ** p value < 0.009. Arrows indicated examples of condensed nuclei.

(B) The “open” MAP-1 mutant M1 triggers RASSF1A-independent Bax conformational change that is augmented by TNFα treatment. Analysis was carried out in RASSF1A shRNA stable cells; * p value < 0.04 and ** p value < 0.002 for WT versus M1 MAP-1.
(C) Co-immunoprecipitation of transfected RASSF1A with endogenous TRAIL-R1 following TRAIL stimulation (3 h) using the indicated antibodies.
(D) Co-immunoprecipitation of transfected MAP-1 with endogenous TRAIL-R1 following TRAIL stimulation (2 h) using the indicated antibodies.