The death domain kinase RIP1 links the immunoregulatory CD40 receptor to apoptotic signaling in carcinomas

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CD40, a tumor necrosis factor (TNF) receptor family member, is widely recognized for its prominent role in the antitumor immune response. The immunostimulatory effects of CD40 ligation on malignant cells can be switched to apoptosis upon disruption of survival signals transduced by the binding of the adaptor protein TRAF6 to CD40. Apoptosis induction requires a TRAF2-interacting CD40 motif but is initiated within a cytosolic death-inducing signaling complex after mobilization of receptor-bound TRAF2 to the cytoplasm. We demonstrate that receptor-interacting protein 1 (RIP1) is an integral component of this complex and is required for CD40 ligand-induced caspase-8 activation and tumor cell killing. Degradation of the RIP1 K63 ubiquitin ligases cIAP1/2 amplifies the CD40-mediated cytotoxic effect, whereas inhibition of CYLD, a RIP1 K63 deubiquitinating enzyme, reduces it. This two-step mechanism of apoptosis induction expands our appreciation of commonalities in apoptosis regulatory pathways across the TNF receptor superfamily and provides a telling example of how TNF family receptors usurp alternative programs to fulfill distinct cellular functions.

Introduction

Receptor-interacting protein 1 (RIP1) is a death domain–containing kinase with diverse and context-specific roles in inflammation, cell survival, and apoptosis (Festjens et al., 2007; Galluzzi et al., 2009b). Genetic evidence has demonstrated that RIP1 is required for the pro-inflammatory and antiapoptotic functions of TNF receptor 1 (TNFR1) by mediating nuclear factor κB (NF-κB) and MAPK signaling (Kelliher et al., 1998; Vivarelli et al., 2004). whereas other studies have shown that RIP1 is an integral component of a cytoplasmic apoptosis-inducing signaling complex mediated by TNFR1 engagement (Micheau and Tschopp, 2003; Jin and El-Deiry, 2006; O'Donnell et al., 2007; Wang et al., 2008; Legarda-Addison et al., 2009). RIP1 is also required for caspase-8 activation within a Fas ligand (CD95L)-triggered death-inducing signaling complex in epithelial cells (Geserick et al., 2009; Morgan et al., 2009) and for necroptosis triggered by TNF-related apoptosis-inducing ligand (TRAIL), TNF, or anti-Fas Ab (Holler et al., 2000; Hitomi et al., 2008; Cho et al., 2009; Zhang et al., 2009).

CD40, a TNF family receptor, and its cognate ligand, CD154, have long been recognized for their prominent role in the regulation of the immune response (van Kooten and Banchereau, 2000). Humans with CD154 mutations develop a severe immune deficiency called hyper-IgM syndrome, which is clinically manifested by recurrent infections (Callard et al., 1993) and, interestingly, enhanced susceptibility to malignancy (Hayward et al., 1997). Accumulated experimental and clinical evidence suggests that activation of the CD40 pathway exerts tumor regression through a "two-hit" mechanism of action involving an indirect effect of immune activation and a direct cytotoxic effect on the tumor (Vonderheide, 2007; Loskog and Eliopoulos, 2009).

Similar to other TNF receptor family members, CD40 stimulates the activation of competing signals that influence malignant cell survival versus death. Thus, a recessive, death-inducing

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Abbreviations used in this paper: CHX, cycloheximide; cIAP; cellular inhibitor of apoptosis; DED, death effector domain; ERK, extracellular signal-regulated kinase; FADD; Fas-associated death domain protein; NF-κB, nuclear factor κB; RAd, recombinant adenovirus; RIP1, receptor-interacting protein 1; TRAF, TNF receptor-associated factor.

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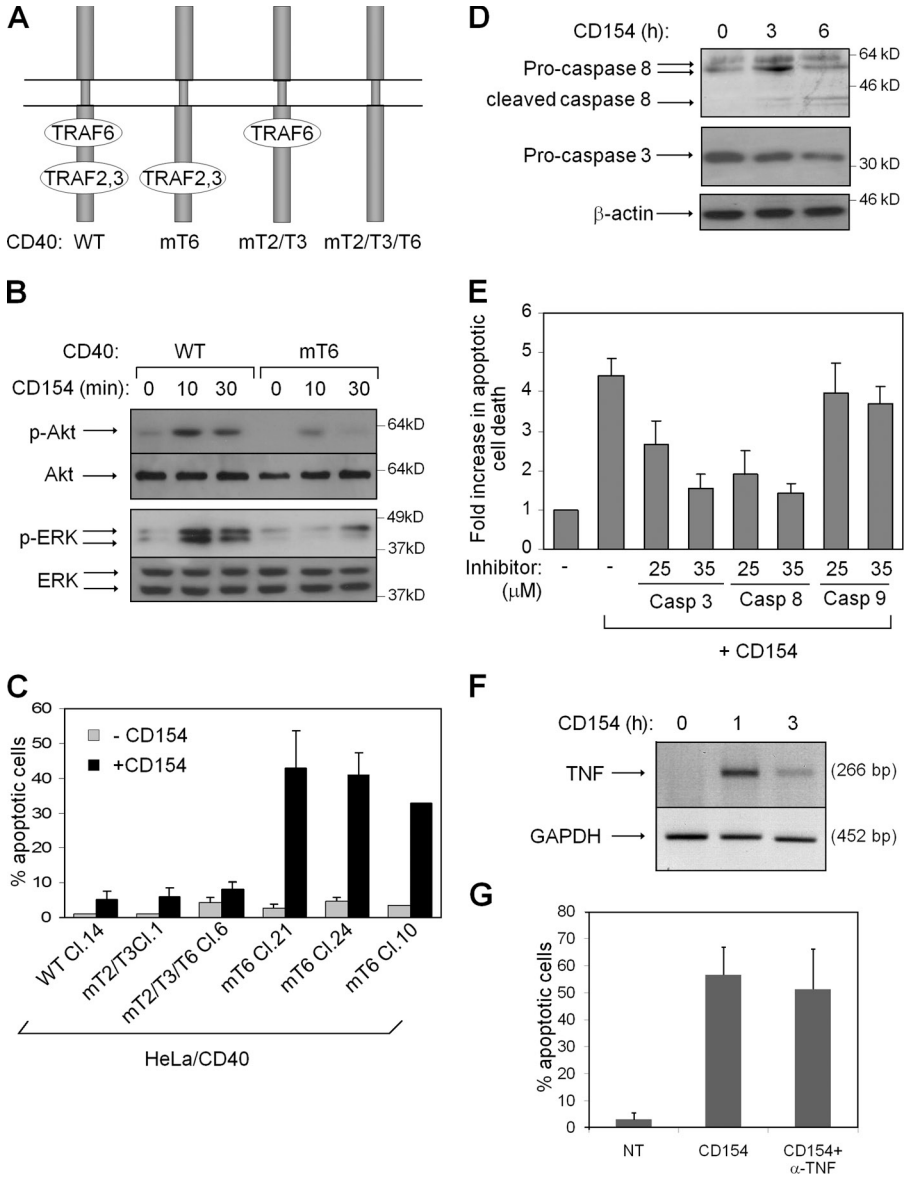

Figure 1. **The TRAF2/TRAF3 binding domain of CD40 mediates CD154-induced death signals.** (A) Graphical representation of CD40 and its TRAF-binding domains. A double $Q^{234}E^{235} \rightarrow AA$ mutation, yielding CD40mT6, selectively abolishes the interaction of TRAF6 with CD40, whereas a $T^{254} \rightarrow A$ mutation (CD40mT2/T3) inhibits TRAF2 and TRAF3 but not TRAF6 binding to CD40. CD40mT2/T3/T6 combines the aforementioned mutations and perturbs the binding of all TRAFs (Davies et al., 2005b). WT, wild type. (B) The TRAF6-interacting domain of CD40 transduces ERK and Akt signaling. Lysates from CD154-stimulated HeLa/CD40 and HeLa/CD40mT6 cells were analyzed for the expression of phosphorylated, active ERK and Akt, or the total proteins. (C) The TRAF2/TRAF3-interacting domain of CD40 mediates CD154-induced death signals. HeLa clones expressing the WT or mutated CD40 sequences described in A were stimulated with CD154 for 12 h before assessment of apoptosis. Mean values of percentage apoptotic cells from at least three independent experiments are shown with the exception of HeLa/CD40mT6 clone 10, where two determinations were performed. (D and E) The TRAF2/TRAF3-binding domain of CD40 mediates cell death via caspase-8 activation. HeLa/CD40mT6 clone 21 cells were stimulated with CD154 for the indicated time points, and lysates were analyzed for the expression of caspase-8, caspase-3, or β-actin (D). Inhibitors of caspase-8 and -3 but not caspase-9 protect HeLa/CD40mT6 cells from CD154-induced apoptosis assessed by cell death ELISA. Data are expressed as fold increase (±SD; $n = 4$) in apoptosis induced by CD154 relative to untreated cultures, which was given the arbitrary value of 1. (F) RT-PCR showing up-regulation of TNF mRNA after treatment of HeLa/CD40mT6 cells with CD154. GADPH, glyceraldehyde 3-phosphate dehydrogenase. (G) Early CD154-mediated death signals are independent of autocrine TNF production. HeLa/CD40mT6 cells were exposed to 0.5 μg/ml neutralizing anti-TNF mAb and then treated as described in C before assessment of apoptosis. Error bars indicate SD.

pathway emerges upon disruption of phosphatidylinositol 3-kinase and extracellular signal-regulated kinase (ERK) survival signaling (Davies et al., 2004; Hill et al., 2005) or treatment with inhibitors of de novo protein synthesis such as cycloheximide (CHX), which target labile antiapoptotic proteins (Hess and Engelmann, 1996; Bugajska et al., 2002; Davies et al., 2004). However, the cytoplasmic tail of CD40 lacks a "death homology domain" that mediates death signals by the TNFR1, Fas, and TRAIL receptors, and so the nature of the CD40-triggered apoptotic pathway has been obscure. Data shown in this report reveal a novel role for RIP1 in linking CD40 to carcinoma cell death.

Results and discussion

The TRAF2/TRAF3-interacting domain of CD40 mediates death signals

CD40 signals through TNF receptor-associated factor (TRAF) proteins (Bishop, 2004, 2007; Eliopoulos, 2008). Specifically, a membrane-proximal region of the receptor cytoplasmic C terminus binds TRAF6, whereas a membrane-distal domain recruits TRAF2 and TRAF3 (Fig. 1 A). To address the impact of specific CD40–TRAF interactions on apoptotic signaling, we used a panel of HeLa cell clones stably expressing wild-type or mutated CD40 sequences that were unable to directly associate with TRAF6 (CD40mT6), TRAF2/TRAF3 (CD40mT2/mT3), or all TRAFs (CD40mT2/T3/T6; Fig. 1 A; Tsukamoto et al., 1999; Jabara et al., 2002; Benson et al., 2006). We have previously used this cell system to demonstrate that the TRAF2/TRAF3-interacting domain of CD40 is primarily responsible for the engagement of NF-κB, JNK, and p38 cascades, whereas the TRAF6-binding region contributes to NF-κB signaling (Davies et al., 2005b).

Other studies have shown that ligation of a CD40 mutant lacking a functional TRAF6 binding site is defective in activation of ERK and Akt in lymphoid cells (Mukundan et al., 2005; Benson et al., 2006) and that ectopic expression of TRAF6 but

not TRAF2 or TRAF3 engages the ERK pathway in epithelial cells and fibroblasts (Kashiwada et al., 1998; Eliopoulos et al., 2003). Fig. 1 B shows that both ERK and Akt phosphorylation are significantly impaired in CD154-stimulated HeLa/CD40mT6 cells.

Akt and ERK are the survival signals that override CD154-induced apoptosis in carcinoma cells (Davies et al., 2004, 2005a). We therefore hypothesized that CD40mT6-expressing HeLa cells would be sensitive to the cytotoxic effects of CD40 ligation because of the defect in ERK/Akt activation. Indeed, treatment of HeLa/CD40mT6 cell clones with CD154 induced significant levels of apoptosis in the absence of protein synthesis inhibition (Fig. 1 C). In contrast, the stimulation of CD40mT2/mT3, CD40mT2/T3/T6, or wild-type CD40 HeLa clones with CD154 failed to increase apoptosis above background levels (Fig. 1 C).

Death signals transduced by the CD40 mutant lacking a functional TRAF6 binding site led to rapid caspase-8 and caspase-3 activation (Fig. 1 D). Caspase activation was required for apoptosis induction because the caspase-8 inhibitor peptide z-IETD.fmk or the caspase-3/7 inhibitor z-DEVD.fmk diminished the cytotoxicity of CD154, whereas the caspase-9 inhibitor z-LEHD.fmk had no effect (Fig. 1 E). Although TNF was upregulated after CD40mT6 stimulation (Fig. 1 F), this early apoptotic response did not depend on autocrine TNF signaling, as viability remained unaffected by coculture with a TNF-neutralizing antibody (Fig. 1 G).

Together, these data reveal distinct roles for the TRAF-interacting CD40 domains in the regulation of carcinoma cell death. They demonstrate that upon CD154 stimulation, the TRAF6 binding site mediates survival signals that dominate over a caspase-dependent death pathway triggered by the TRAF2/TRAF3-interacting domain.

TRAF2 transduces CD40 death signals

On the basis of the aforementioned findings, we used RNAi to define the relative contribution of TRAF2 versus TRAF3 to CD40-mediated apoptosis (Fig. 2 A). HeLa/CD40mT6 cells transfected with a control siRNA (siPOOL) responded to CD154 treatment with elevated apoptosis, which was reduced in TRAF2 siRNA-transfected cells (Fig. 2 B). In contrast, knockdown of TRAF3 did not have a significant effect.

The role of TRAFs in CD154-induced apoptosis was also examined in EJ bladder carcinoma cells (Stamenkovic et al., 1989), which are responsive to CD40-mediated death signals in the presence of CHX (Davies et al., 2004). As shown in Fig. 2 C, TRAF2 knockdown significantly reduced the cytotoxic effects of CD154 and CHX combination treatment compared with TRAF3 or control siRNA. These data demonstrate a novel role for TRAF2 as a positive regulator of CD40-transduced death signals.

TRAF2 interacts with RIP1 in the context of CD40 signaling

TRAF2 is a component of the cytosolic TNFR1 death-inducing signaling complex (Micheau and Tschopp, 2003), and binds RIP1 (Hsu et al., 1996). We thus reasoned that TRAF2 may

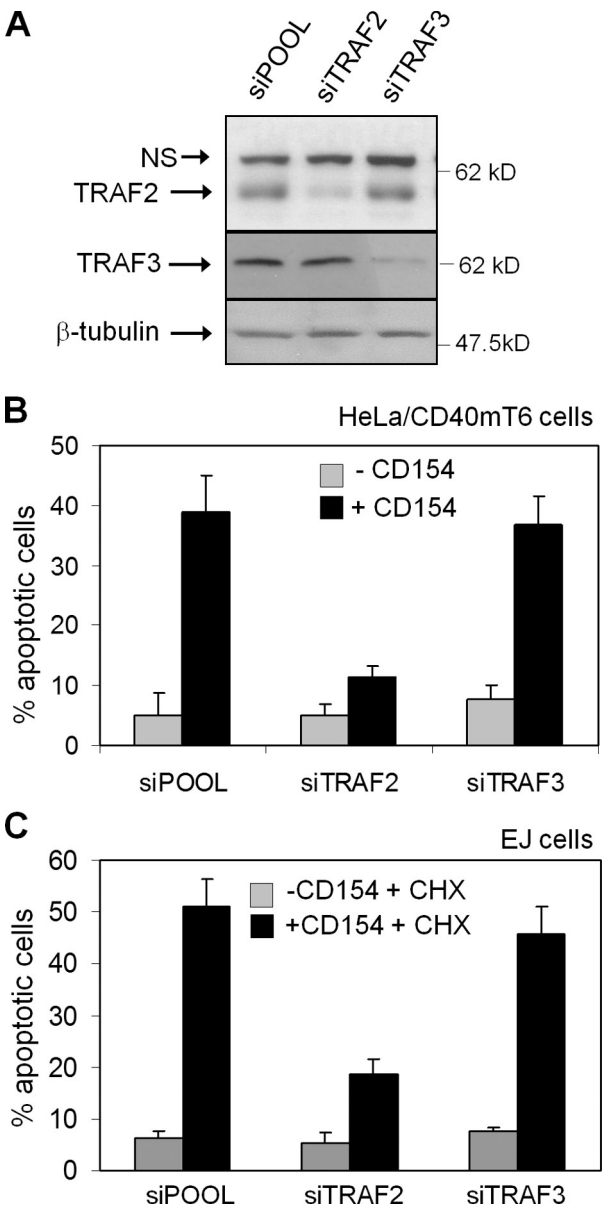

Figure 2. **TRAF2 is required for the transduction of CD40 death signals.** HeLa/CD40mT6 cells were transfected with TRAF2, TRAF3, or control siRNA and either lysed for the assessment of TRAF knockdown efficacy by immunoblotting (A) or exposed to 1 μg/ml soluble CD154 before evaluation of apoptosis (B). (C) Knockdown of TRAF2 in EJ bladder carcinoma cells confers resistance to apoptosis induced by soluble CD154 in the presence of 10 μg/ml CHX. Mean values (±SD) from three independent experiments are shown (error bars).

channel CD40 signals to RIP1. We tested this hypothesis by performing coimmunoprecipitation experiments in lysates from CD40-negative 293 cells transfected with FLAG-tagged RIP1 in the presence or absence of a CD40 receptor expression vector. When overexpressed, CD40 stimulates signal activation through formation of receptor multimers and recruitment of TRAFs in a ligand-independent manner (Rothe et al., 1995; Pullen et al., 1999). As shown in Fig. 3 A, although very little endogenous TRAF2 coprecipitated with FLAG-RIP1 in the absence of CD40, this interaction dramatically increased upon transfection of increasing amounts of CD40 expression vector.

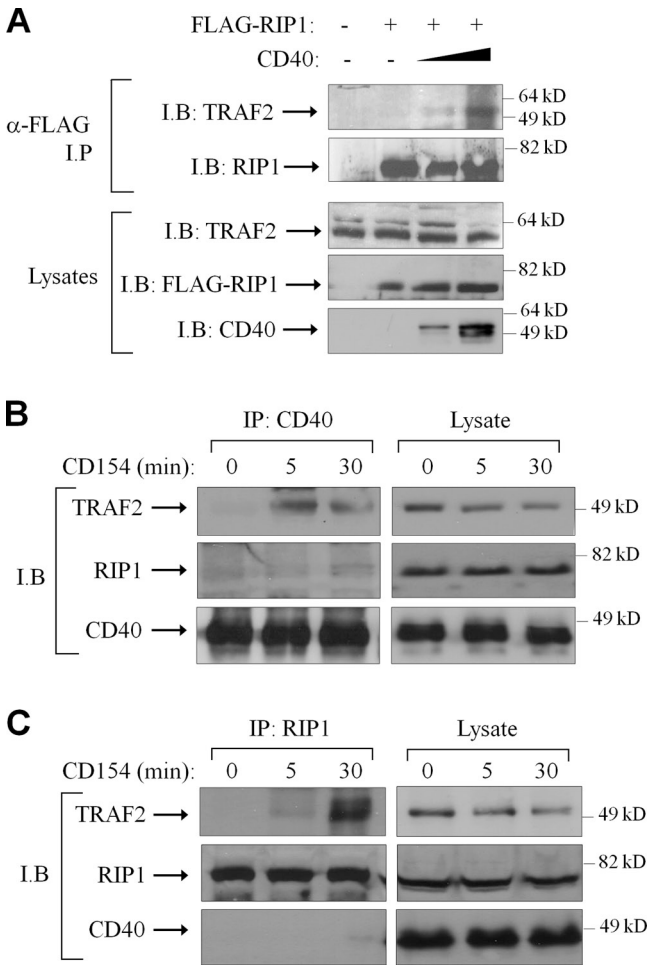

Figure 3. **TRAF2 and RIP1 interact in the context of CD40 signaling.** (A) Ectopic expression of CD40 enhances the interaction of RIP1 with endogenous TRAF2. Two million 293 cells were transfected with 0.5 µg FLAG-tagged RIP1 in the presence of increasing amounts of a CD40 expression vector (0, 1, or 2.5 µg). Lysates were immunoprecipitated (IP) with anti-FLAG and immunoblotted (IB) with an anti-TRAF2 or RIP1 antibody as indicated. (B and C) CD40 ligation induces the association of endogenous RIP1 with TRAF2 but not CD40. HeLa/CD40mT6 cells were stimulated with CD154, then lysates were sequentially immunoprecipitated with anti-CD40 (B) and anti-RIP1 (C) and immunoblotted with TRAF2, RIP1, or CD40 antibodies.

The TRAF2-RIP1 link was further explored in physiological conditions under which endogenous proteins were analyzed. To this end, lysates were prepared from HeLa/CD40mT6 cells before and after stimulation with CD154 and sequentially immunoprecipitated with anti-CD40 and RIP1 antibodies. In anti-CD40 immunoprecipitates, no detectable association of endogenous CD40 and TRAF2 was observed in the absence of stimulation, but this interaction was rapidly induced after CD40 ligation, as described previously (Rothe et al., 1995; Matsuzawa et al., 2008). RIP1 did not coprecipitate with CD40 before or after stimulation (Fig. 3 B). Interestingly, TRAF2 was readily detected in anti-RIP1 immunoprecipitates from CD154-stimulated cultures (Fig. 3 C).

These data suggest that RIP1 is not a component of the CD40-bound TRAF2 signaling complex but forms a separate, cytoplasmic association with TRAF2 after CD40 ligation. We hypothesize that the absence of RIP1 in CD40-bound TRAF2 complexes is the result of the overlapping requirement of the C terminus of TRAF2 for binding to both CD40 (Rothe et al., 1995) and RIP1 (Liu et al., 1996). Once released from CD40, presumably after the CD154-mediated ubiquitination and degradation of TRAF3 (Matsuzawa et al., 2008; for review see Eliopoulos, 2008), the C terminus of TRAF2 could become accessible to RIP1, allowing the formation of a pro-apoptotic signaling complex. The difference in the kinetics of CD40–TRAF2 and RIP1–TRAF2 interactions after CD40 stimulation (Fig. 3, B and C) is compatible with this model.

RIP1 is required for CD40-induced death signaling in tumor cells

We next explored the functional role of RIP1 in CD40 death signaling by using geldanamycin, a compound which induces the degradation of RIP1 by disrupting the function of the RIP1-associating chaperone protein HSP90 (Lewis et al., 2000). Treatment of different tumor lines with geldanamycin induced a time-dependent decrease in RIP1 expression (Fig. S1 A) and protected them from CD154-induced apoptosis (Fig. S1 B).

These results demonstrate a correlation between RIP1 protein levels, modulated by geldanamycin, and sensitivity to CD154-induced apoptosis but do not exclude the possibility that geldanamycin impacts on CD40-induced cell death through molecules other than RIP1. Therefore, we used RNAi to explore more specifically the role of RIP1 in death signaling. The knockdown of RIP1 in both EJ and HeLa/CD40mT6 (Fig. 4 A) dramatically reduced CD154-induced cytotoxicity, as determined by morphological changes (Fig. 4 B), nuclear condensation, and degradation detected by propidium iodide staining (Fig. 4, C and E), oligo-nucleosomal enrichment (Fig. 4, D and F), crystal violet staining (Fig. 5 F), or Annexin V staining and flow cytometry (not depicted). In contrast, diminished RIP1 did not affect CD95L-induced apoptosis (Fig. 5 F; Jin and El-Deiry, 2006).

To confirm that RIP1 is a bona fide regulator of CD40 death signaling, we assessed the effect of RIP1 knockdown on apoptosis induced by CD154 transgene expression. Adenovirus-mediated delivery of the *CD154* gene to carcinoma cells elicits pro-apoptotic effects through membrane-bound CD154 (Gomes et al., 2009; Vardouli et al., 2009), which is likely to mimic CD154 expressed on activated T lymphocytes. EJ cells transfected with control or RIP1 siRNA were transduced with recombinant adenovirus (RAd) expressing *CD154* or, as a control, the *lac*Z gene, then analyzed for transgene expression (Fig. S1 C) or exposed to CHX before assessment of apoptosis (Fig. S1 D). It was found that RIP1 knockdown provided protection from the cytotoxic effect of RAd-*CD154* and CHX treatment (Fig. S1 D). Collectively, these data provide compelling evidence that RIP1 has an essential role in the regulation of CD40-mediated death signaling in tumor cells.

RIP1 is critically involved in TNF-induced JNK, ERK, and NF-κB signaling in most (Kelliher et al., 1998; Devin et al., 2003; Lee et al., 2003) but not all settings (Wong et al., 2010). We have found that the knockdown of RIP1 did not significantly influence the CD154-mediated degradation of IκBα, a hallmark

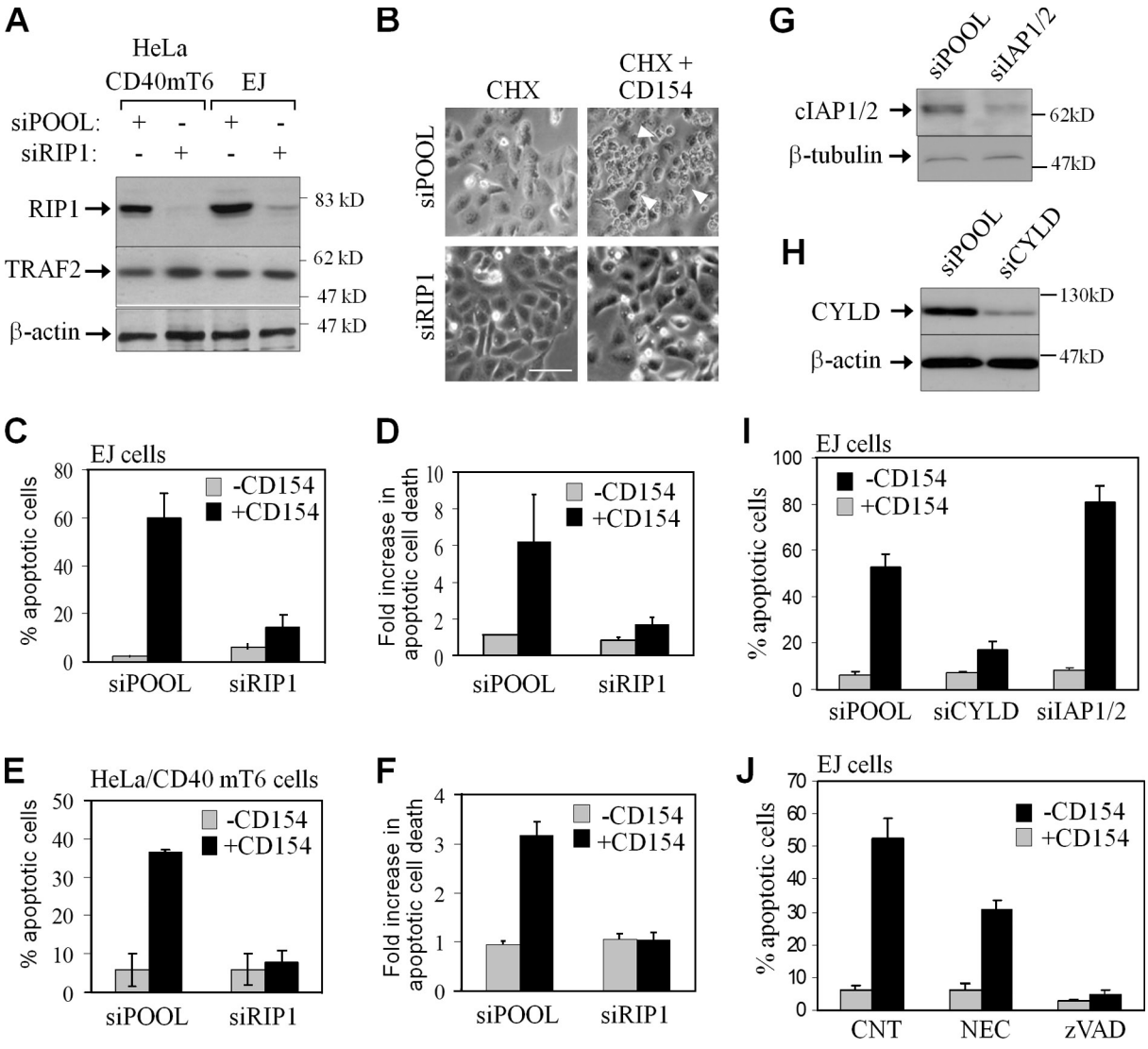

Figure 4. **RIP1 is required for CD40-mediated apoptosis in carcinoma cells.** (A) Knockdown efficiency of RIP1 siRNA. (B) EJ cells treated with CD154 in the presence of CHX display morphology typical of apoptotic cells (see representative arrowheads), whereas RIP1 siRNA-transfected cultures remain unaffected. Bar, 25 µm. (C and D) RIP1 knockdown rescues EJ cells from CD154 and CHX-induced apoptosis, as determined by propidium iodide staining and immunofluorescence microscopy (C) or cell death ELISA (D). Mean values (±SD) from four independent experiments are shown. (E and F) RIP1 knockdown rescues HeLa/CD40mT6 cells from CD154-induced apoptosis, determined as described in the legend of Fig 4 C. (G and H) Expression of cIAP1/2 (G) and CYLD (H) before and after transfection with the respective siRNAs. (I) Effect of cIAP1/2 and CYLD knockdown on CD154-mediated EJ cell death. (J) Necrostatin (50 µM) inhibits and the pan-caspase inhibitor zVAD-fmk (15 µM) abolishes the pro-apoptotic effects of CD154 in EJ cells. Error bars indicate SD.

of canonical NF-κB signaling, ERK or JNK phosphorylation, or the processing of p100 NF-κB2 to p52 (Fig. S2).

CYLD and cellular inhibitors of apoptosis (cIAPs) are involved in CD40-mediated cell death

Ubiquitination and phosphorylation are two RIP1 posttranslational modifications that influence RIP1-mediated cell death. cIAP1/2 are required for TNF-induced K63-linked ubiquitination of RIP1 (Bertrand et al., 2008), which functions to inhibit TNF-induced apoptosis (O'Donnell et al., 2007), whereas RIP1 deubiquitination by CYLD facilitates its direct interaction with caspase-8 and initiation of cell death (Wang et al., 2008). Because cIAP1/2 are involved in CD40-mediated MAPK signaling (Matsuzawa et al., 2008), we hypothesized that they may also function in the CD40 death pathway. In line with this prediction, knockdown of cIAP1/2 increased EJ cell killing by CD154 and CHX treatment (Fig. 4, G and I), whereas the knockdown of CYLD inhibited it (Fig. 4, H and I).

Smac mimetic compounds induce degradation of cIAPs and thus amplify death ligand–induced cancer cell killing (Li et al., 2004; Wang et al., 2008; Geserick et al., 2009). When EJ cells were treated with the Smac mimetic LBW242 (Gaither et al., 2007), cIAP1/2 were rapidly degraded (Fig. S3 A) and the cells became susceptible to CD154-induced apoptosis (Fig. S3 B). This effect was blocked by RIP1 knockdown or the pan-caspase inhibitor zVAD-fmk, further highlighting the critical involvement of the cIAP1/2–RIP1–caspase axis in the CD40 death pathway.

Moreover, the catalytic activity of RIP1 may also contribute to the CD40-trigerred death pathway, as necrostatin-1,

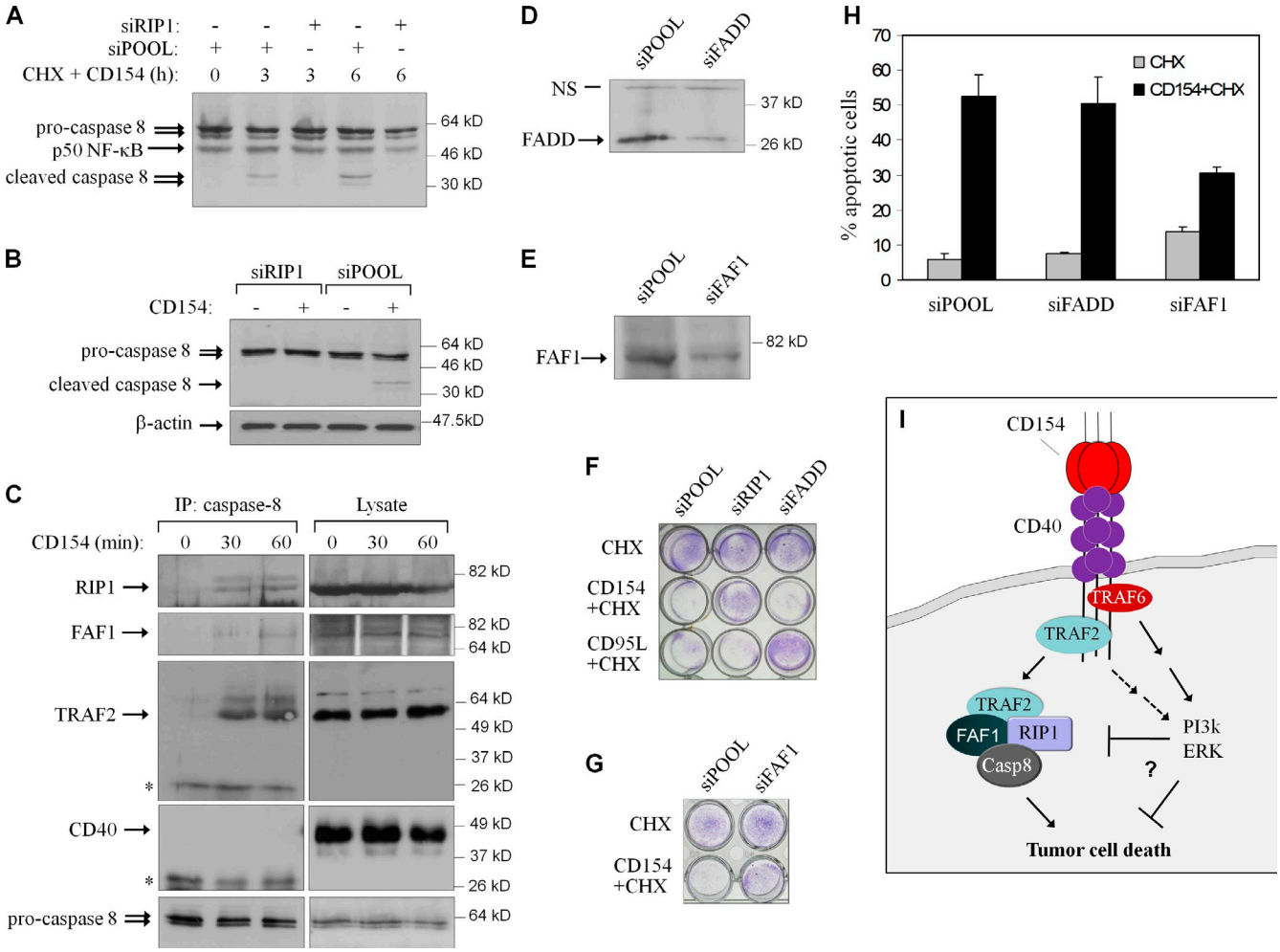

Figure 5. **RIP1 associates with caspase-8 and is required for its activation.** (A and B) RIP1 knockdown suppresses CD154-induced caspase-8 cleavage in EJ (A) or HeLa/CD40mT6 (B) cells. Lysates were immunoblotted for caspase-8 or, as a control, β-actin or p50 NF-κB. (C) RIP1, TRAF2, FAF1, and caspase-8 interact upon CD40 stimulation. Cells were stimulated with CD154, and lysates were immunoprecipitated with a goat polyclonal against caspase-8. Immunoprecipitants were immunoblotted using rabbit polyclonal antibodies against RIP1, TRAF2, CD40, and FAF1 or a monoclonal anti-caspase-8, as indicated. Results are representative of three independent experiments. The Ig light chains are indicted by asterisks. (D and E) Expression of FADD (D) and FAF1 (E) before and after transfection with the respective siRNAs. (F and G) Crystal violet staining of RNAi-transfected EJ cells treated with CHX in the presence or absence of CD154 or CD95L. (H) FAF1 but not FADD knockdown protects from the pro-apoptotic effects of CD40 ligation. After knockdown, EJ cells were exposed to CD154 and CHX for 5 h before assessment of apoptosis. Error bars indicate SD. (I) Proposed model of CD40-induced death signaling in tumor cells. CD40-mediated apoptosis involves the formation of a secondary cytoplasmic complex of TRAF2, RIP1, FAF1 and caspase-8 (Casp-8). RIP1 is required for caspase-8 activation and cell death, whereas apoptosis is antagonized by survival signals predominantly mediated by the TRAF6-binding domain of CD40, which may operate at the level of the death-inducing signaling complex and/or downstream of it.

an allosteric RIP1 kinase inhibitor (Degterev et al., 2008), inhibited CD40-mediated apoptosis by 40% (Fig. 4 J).

RIP1 associates with caspase-8 and is required for its activation

As caspase-8 activation is required for CD40 induced apoptosis (Fig. 1 E), we examined whether RIP1 functions upstream of caspase-8 in this pathway. Cells were depleted of RIP1 and treated with CD154 in the presence (EJ) or absence (HeLa/CD40mT6) of CHX before analysis of caspase-8 by immunoblotting. RIP1 knockdown was found to suppress caspase-8 activation in both cases (Fig. 5, A and B).

This observation prompted us to investigate the hypothesis that caspase-8 interacts with RIP1 and TRAF2. HeLa/CD40mT6 cells were stimulated with CD154, and lysates were subjected to immunoprecipitation using anti–caspase-8 antibody. In control lysates, neither RIP1 nor TRAF2 coprecipitated with caspase-8. However, both proteins (but not CD40) were found in complex with caspase-8 after exposure to CD154 (Fig. 5 C).

RIP1 has a death domain motif, and caspase-8 has a death effector domain (DED). Fas-associated death domain protein (FADD) possesses both motifs and has been proposed to link RIP1 and caspase-8 in death receptor signaling (Chinnaiyan et al., 1995; Kischkel et al., 2000; Sprick et al., 2000). Although FADD knockdown blocked CD95L-induced killing (Fig. 5, D and F), it did not impact on CD40-mediated apoptosis (Fig. 5, F and H). FADD knockdown also fails to influence TNF-induced, RIP1-mediated death signals in tumor cells (Jin and El-Deiry, 2006; Wang et al., 2008). FAF1 is a pro-apoptotic protein that possesses a DED-interacting domain responsible for association with the DED of caspase-8 and an atypical death domain (Ryu et al., 2003). Knockdown of FAF1 in EJ cells (Fig. 5 E) was

found to partially reduce the cytotoxic effect of CD154 and CHX treatment (Fig. 5, G and H), and FAF1 is detected in the RIP1–caspase-8 complex (Fig. 5 C).

We have recently shown that TRAF2 is largely responsible for the CD154-mediated sequential activation of NF-κB and IRF1, which act in concert to ensure the synchronous synthesis of components of the antigen presentation machinery required for the engagement of antitumor immune responses (Moschonas et al., 2008). Results presented in this study demonstrate that TRAF2 is also required for CD40-mediated tumor cell killing (Figs. 1 and 2). Together, these observations suggest that TRAF2 is a master regulator of the antitumor functions of CD40 in malignant epithelial cells.

However, our data also show that apoptosis is not triggered at the level of the receptor, but requires the function of a cytosolic complex containing TRAF2, RIP1, FAF1, and caspase-8, which is antagonized by signals emanating from the TRAF6-binding domain of CD40 (Fig. 5 I). Considering the breadth of CD40 expression and the diversity of its roles, the identification of two signaling complexes regulating cell survival versus death could be exploited to fine-tune CD154-based anticancer strategies.

Materials and methods

Cell culture, adenovirus constructs, and reagents

The bladder carcinoma EJ, the cervical cell line HeLa, and CD40-expressing clones were maintained in RPMI medium supplemented with 10% FCS. The early passage ovarian AGE60 (Vardouli et al., 2009) and the human embryonic kidney (HEK) 293 cell line were cultured in Dulbecco's modified Eagle medium supplemented with 10% FCS (Invitrogen). Parental 293 and HeLa cells are CD40-negative (Davies et al., 2005b). Human recombinant soluble CD40L was kindly provided by Amgen Inc., or purchased from Enzo Life Sciences, Inc. Geldanamycin, kinase, and caspase inhibitors were obtained from EMD and dissolved in dimethyl sulfoxide before use. RAds expressing *CD154* and *lac*Z have been described previously (Vardouli et al., 2009). The Smac mimetic LBW242 was provided by L. Zawel (Novartis Institutes for Biomedical Research, Basel, Switzerland).

Antibodies, immunoprecipitations, and immunoblotting

Phospho-specific antibodies against JNK, ERK, Akt, and the corresponding antibodies that recognize both the phosphorylated and unphosphorylated forms, the FAF1, and monoclonal caspase Abs were purchased from Cell Signaling Technology and used at dilutions of 1:500–1:1000. The IκBα/MAD3 (C21), RIP1 (C20), TRAF2 (C20), CD40 (H120 and C20), and caspase-8 (C20) antibodies were obtained from Santa Cruz Biotechnology, Inc., the β-actin and FLAG M2 antibodies were obtained from Sigma-Aldrich, and the cIAP1/2 Ab was obtained from R&D Systems. Anti–rabbit IgG-HRP and anti–mouse IgG-HRP were obtained from Sigma-Aldrich. Immunoblotting was performed as described previously (Eliopoulos et al., 2003; Moschonas et al., 2008). For immunoprecipitation, cells ($1–2 \times 10^7$) were lysed in 1 ml of DISC immunoprecipitation buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, and 1% Triton X-100) with protease inhibitor cocktail (Roche). Cell lysates (900 µl) were incubated overnight with 2–3 µg of antibody at 4°C. Complexes were precipitated by protein G–agarose (Millipore) and suspended in 50 µl of SDS sample buffer after three washes with DISC immunoprecipitation buffer. Immunoprecipitates were subjected to SDS-PAGE and Western blotting. The neutralizing anti-TNF mAb2101 was purchased from R&D Systems and used at 0.5 µg/ml.

Quantitative measurement of apoptosis

For the assessment of apoptosis, we took into consideration the guidelines for the use and interpretation of assays for monitoring cell death (Galluzzi et al., 2009a) and performed multiple, methodologically unrelated assays to quantify dead cells.

Cytochemical staining. To estimate apoptosis based on nuclear morphology, the fluorescent DNA staining dye propidium iodide (Sigma-Aldrich) was used. Approximately 2×10^4 cells in 25 µl were stained by adding 1 µl of 100 µg/ml propidium iodide. Uptake of the dye was examined by fluorescence microscopy. To estimate the apoptotic index, a minimum of 300 cells was examined by at least two independent investigators and quantified by recording the relative number of cells showing condensed or fragmented chromatin. Unless stated otherwise, data shown depict apoptosis measurements using this assay, with error bars representing SD of *n* independent experiments indicated in the figure legends.

ELISA-based nucleosomal DNA fragmentation enrichment assay. Apoptosis was quantified by direct determination of nucleosomal DNA fragmentation using the Cell Death Detection Elisa Plus kit (Roche), an assay designed to provide relative measurements of apoptosis rather than absolute numbers of dead cells. Cells were plated in 96-well plates at an initial concentration $5–7 \times 10^3$ cells/well depending on the cell line. The following day, cells were treated with CD154 in the presence or absence of CHX. Alternatively, cells were infected with adenoviruses at MOI 200 and allowed to express the transgene for 16 h before application of 10 µg/ml CHX. Cells were then processed according to the manufacturer's instructions. The mono- and oligonucleosomes contained in the cell lysates were determined using the anti–histone-biotin Ab. The concentration of the mono- and oligonucleosomes was determined photometrically using an anti–histone-biotin Ab, followed by incubation with 2,2′-azino-di(3-ethylbenzthiazolin-sulfonate) (ABTS) as substrate for the antibody. The OD was read on a microplate reader (Bio-Rad Laboratories) at a wavelength of 405 nm, and the ratio of OD of the sample to the OD of cells without treatment was estimated to give the fold increase in apoptosis.

Annexin V staining. Cells were lightly trypsinized and incubated with Annexin V-FITC (BD) and propidium iodide for 15 min before assessment of fluorescence intensity on a flow cytometer (BD).

RNAi

For the delivery of siRNAs, 5×10^4 EJ or HeLa/CD40mT6 cells were plated into each well of a 24-well plate (Costar), and two rounds of transfection with siRNA duplexes were performed as described previously (Davies et al., 2005b; Moschonas et al., 2008). The sequences of the siRNAs used were as follows. RIP1 siRNA, 5′-GUACUCCGCUUUCUGUAAA-3′; TRAF2 siRNA, Dharmacon siGENOME SMARTpool M-005198 (Thermo Fischer Scientific); TRAF3 siRNA, Dharmacon siGENOME SMARTpool M-005252 (Thermo Fischer Scientific); FADD siRNA, Dharmacon siGENOME SMARTpool M-003800 (Thermo Fischer Scientific); and FAF1 siRNA, Dharmacon siGENOME SMARTpool M-009106 (Thermo Fischer Scientific). The siRNA sequences for cIAP1, cIAP2, and CYLD were as described previously (Wang et al., 2008). cIAP1 and cIAP2 were knocked down simultaneously.

Light microscopy

Morphological changes related to apoptosis were observed using an inverted microscope (DMIRE2; Leica) equipped with a digital camera (DFC300 FX; Leica). Camera image acquisition was controlled by IM50 software (Leica), and single images were exported as TIFF files. Individual frames were prepared for presentation using Photoshop (Adobe). Cells were seeded into 4-well, chambered coverglass units with coverslip-quality glass bottoms (Laboratory-Tek; Thermo Fischer Scientific) and, after treatment, were examined with a 63× dry objective lens.

Online supplemental material

Fig. S1 shows data supporting the involvement of RIP1 in CD40-mediated apoptosis. Fig. S2 illustrates that RIP1 is dispensable for CD154-stimulated NF-κB and MAPK signaling. Fig. S3 shows that RIP1 is required for apoptosis induced by a Smac mimetic and CD154 combination treatment. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201003087/DC1.

The authors declare no conflict of interest.

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