The Ret Finger Protein Induces Apoptosis via Its RING Finger-B Box-Coiled-coil Motif*

The Ret finger protein (RFP) is a member of the tripartite motif family, which is characterized by a conserved RING finger, a B-box, and a coiled-coil domain (together called RBCC). Although RFP is known to become oncosgenic when its RBCC moiety is connected to a tyrosine kinase domain by DNA rearrangement, its biological function is not well defined. Here we show that ectopic expression of RFP in human embryonic kidney 293 cells causes extensive apoptosis, as assessed by multiple criteria. RFP expression activates Jun N-terminal kinase and p38 kinase and also increases caspase-3-like activity. However, RFP failed to release cytochrome c and, therefore, to increase caspase-9-like activity. RFP-induced apoptosis could be blocked by the caspase-8 inhibitor crmA and dominant negative ASK1 but not by Bcl-2. These results reveal a novel RFP death pathway that recruits mitogen-activated protein kinase and caspases independently of mitochondrial events. Domain mapping showed that the intact RBCC moiety is necessary for the pro-apoptotic function of RFP. Moreover, expression of the RBCC moiety further potentiated the pro-apoptotic activity and resulted in a 7-fold increase of caspase activation compared with that induced by full-length RFP. This suggests that a large number of tripartite motif family members sharing the RBCC moiety may participate in the control of cell survival.

The Ret finger protein (RFP)*1 is a member of the tripartite motif (TRIM) protein family, also known as the RING-B box-coiled-coil (RBCC) family (1, 2). RFP is also designated TRIM 27, based on the 27 known members of the TRIM family (3). The TRIM contains a RING finger (R), one or two B-boxes (B1, B2), followed by a coiled-coil domain (CC); therefore, it is also called the RBCC. In addition to these characteristic features, RFP contains an additional specific carboxyl-terminal region known as the RFP domain.

Several TRIM proteins play key roles in regulating gene expression and cell proliferation. For example, transcriptional intermediary factor 1α (TIF1α)/TRIM24, TIF1γ/KAP1/TRIM28, and promyelocytic leukemia protein (PML)/TRIM19, modulate transcriptional machinery to control specific gene expression during cell proliferation, differentiation, and development (4–6). TRIMs have been implicated in several human diseases; mutations in Pyrin/TRIM20, MID1/TRIM18, and MUL/TRIM37 have been associated with familial Mediterranean fever, X-linked Opitz/GBBB syndrome, and mulibrey nanism, respectively (7–9).

In addition, three members of the TRIM family (PML, RFP, and TIF1α) acquire oncosgenic activity when fused to kinases by chromosomal rearrangements (10–14). The PML-retinoic acid receptor α fusion protein blocks hematopoiesis in acute promyelocytic leukemia cells (10, 11). The RBCC moiety of TIF1α fuses to the kinase domain of B-Raf and Ret tyrosine kinase in mouse hepatocellular carcinoma and human papillary thyroid carcinoma (PTC), respectively (13, 14). The RBCC moiety of RFP was found to be fused to the Ret tyrosine kinase in transformed NIH3T3 cells (12). It has been shown that the RBCC moiety is required for the transforming capacities of these TRIM oncogenes (6, 13–16).

RFP often localizes in discrete nuclear structures called PML nuclear bodies (17, 18), where it binds directly to PML (another TRIM family member) as well as Int-6, expression of which activates Bcl-2 to trigger receptor- or mitochondria-mediated apoptotic pathways. Execution of apoptosis involves activation of signaling by mitogen-activated protein (MAP) kinases and/or caspases. Various stresses activate two well-defined MAP kinase signaling modules, JNK and p38. MAP kinase kinase kinases such as MEKK1 and ASK1 activate two different subgroups of MAP kinase kinase, SEK1 (or MKK4) and MKK3/MKK6, which in turn activate the JNK and p38 subgroups of MAP kinase (MAPK), respectively (23, 24). JNK and p38 up-regulate Fas ligands, activate Bid, and/or inactivate Bcl-2 to trigger receptor- or mitochondria-mediated apoptotic pathways. Caspases, a class of cysteine proteases, are activated by at least two mechanisms. One involves a direct pathway via stimulation of death receptors such as Fas and tumor necrosis factor receptor 1 (TNFR1), recruitment and activation of caspase-8 through the adaptor protein Fas-associated death domain (FADD), and subsequent activation of

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caspase-3, -6, and -7. The other passes through mitochondria (25, 26). Pro-apoptotic Bel-2 family members, once activated by intracellular stresses such as cytokine deprivation and genotoxic damage, permeabilize the outer mitochondrial membrane and release cytochrome c. In the cytosol, cytochrome c controls the assembly of an apoptosome composed of oligomers of Apaf-1 and procaspase-9, thereby triggering activation of caspase-9 and subsequent activation of caspase-3. In addition to MAP kinase, caspase, and Bel-2 families, a variety of signaling molecules have been suggested to regulate apoptosis, and overexpression of some of them induces apoptosis. For instance, overexpression of PML, p53, Daxx, or HEF1 triggers apoptosis through various mechanisms (22, 27–29).

Here, we demonstrate for the first time that the RBCC moiety of RFP triggers a rapid apoptosis through activation of stress-activated MAP kinases and caspases. Considering the known pro-apoptotic activity of PML (21, 22), another TRIM family member, our results suggest that a large number of TRIM members sharing the RBCC moiety may participate in the control of cell survival.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The full-length human RFP cDNA (provided by M. Takahashi) was used as a template to generate deletion constructs by PCR. The cDNA fragments encoding the RING finger (amino acids 1–62), RING finger-B box (amino acids 1–132), RING finger-B box-coiled-coil (amino acids 32–117), and coiled-coil (amino acids 132–331), were subcloned into the pFLAG-CMV-2 plasmid, and confirmed by direct sequencing.

Cell Culture and Immunoblotting—HEK 293 and HEK 293T cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal calf serum (Invitrogen) in a 5% CO₂ atmosphere at 37 °C. Cells were transfected by the calcium phosphate precipitation method. At indicated times after transfection, cells were harvested and lysed in a lysis buffer containing 20 mM HEPES, pH 7.5, 50 mM NaCl, 10% glycerol, and 0.5% Triton X-100. Cell extracts were separated with SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell). After blocking with 5% skim milk in 20 mM Tris, pH 7.4, and 150 mM NaCl containing 0.05% Tween 20, the membranes were probed with rabbit anti-mouse RFP antiserum, mouse anti-FLAG antibody (Sigma), goat anti-actin antibody (Santa Cruz Biotechnology), rabbit anti-phospho-SEK1/MKK4 (Thr-180; New England Biolabs), rabbit anti-phospho-MKK3/MKK6 (Ser-189/207; New England Biolabs), or rabbit anti-phospho-SEK1/MKK4 (Thr-261; New England Biolabs), rabbit antibody (Sigma), goat anti-phospho-p38 MAP kinase (Thr-180/Tyr-182; New England Biolabs), or anti-rabbit IgG antibody (Upstate Biotechnologies), then developed with the use of the chemiluminescence detection system (Pierce).

Caspase Assay—Approximately 10⁶ cells were used for measurement of caspase activities. Caspase-3, -7, and -9-like activities were measured using the synthetic substrates DEVD-7-amino-4-trifluoromethyl coumarin (Calbiochem), IETD-AMC (Peptron), and LEHD-7-amino-4-trifluoromethyl coumarin (Peptron), respectively. Activities were assayed according to the manufacturer's instructions. The fluorescence of the released AMC was measured at an excitation wavelength of 360 nm and an emission wavelength of 400 nm and 505 nm, respectively. The results are expressed as fold-increase in the caspase activity of sample cells compared with control cells transfected with empty vectors.

Immunocomplex Kinase Assays—Cells were washed with phosphate-buffered saline and lysed in a lysis buffer containing 20 mM HEPES, pH 7.5, 50 mM NaCl, 10% glycerol, 0.5% Triton X-100, and 0.1 mM sodium orthovanadate. The lysis buffer was supplemented with a mixture of protease inhibitors (0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 2.0 μM aprotinin, 2.0 μM leupeptin) before use. The cell lysates were immunoprecipitated with anti-JNK1 (BD Pharmingen) or anti-MEKK1 (C-22; Santa Cruz Biotechnology) antibodies, bound to protein G agarose (Sigma) and washed twice with lysis buffer, twice with LiCl buffer (100 mM Tris-HCl, pH 7.6, 500 mM LiCl, and 0.1% Triton X-100) and then twice with kinase reaction buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 10 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mM sodium orthovanadate). To measure the kinase reaction, beads were incubated with 2 μCi of [γ-³²P]ATP and 1 μg of GST-c-Jun or GST-SEK1(K129R). The samples were subjected to 12% SDS-PAGE, and the phosphorylation of substrate proteins was analyzed by exposing the gels to x-ray film or a BAS 1500 phosphorescence image analyzer (Fuji). Other protein kinase activities were examined by immunoblot analysis of the cell lysates with antibodies against phospho-JNK, phospho-SEK1, phospho-p38, and phospho-MEKK3/6.

Annexin V and Propidium Iodide Staining—Apoptosis was monitored by measuring the distribution of plasma membrane phosphatidylserine and hypodiploid DNA content. Cells were trypsinized, collected by centrifugation, and washed with phosphate-buffered saline. Aliquots of cells were incubated with Annexin V-fluorescein isothiocyanate (FITC)-conjugated Annexin V and subsequently analyzed by flow cytometry.

RESULTS

RFP Expression Induces Apoptosis—Given that many TRIM proteins play roles in cell proliferation, we examined the effect...
of RFP overexpression on cell proliferation. Transient transfection of RFP into HEK 293 cells resulted in morphological changes suggestive of apoptosis. On gross examination, we found that cells became round, displayed membrane blebbing, and initiated detachment from the dish within 36 h after transfection. pEGFP (Clontech) was co-transfected with pCMV-RFP to distinguish the transfected cells from nontransfected cells. All EGFP-positive cells showed membrane blebbing, whereas EGFP-negative cells were normal in shape (Fig. 1A). Apoptosis induced by RFP expression was further confirmed by Annexin V assay. pCMV-RFP transfection resulted in increased Annexin V-positive cells compared with empty vector transfection (Fig. 1B), indicating a typical apoptotic event.

**RFP Activates both JNK and p38 Signaling Pathways**—Because JNK and p38 kinase signaling play important roles in apoptotic cell death (23, 24), we investigated JNK and p38 kinase cascades after RFP overexpression in cells. First, we examined the activity of JNK1, the major and ubiquitously expressed JNK, by an in vitro-coupled kinase assay using a recombinant c-Jun protein. An ASK1-expressing plasmid was transiently transfected as a positive control, because ASK1 has been shown to induce JNK activation (30). RFP expression in HEK 293 cells activated JNK1 to an extent similar to that of ASK1 when protein levels were normalized (Fig. 2A). Similarly, we found RFP-mediated activation of both exogenous and endogenous forms of the other stress-activated MAPK, p38. Among the tested MAP kinase kinases, SEK1 and MKK6 were highly activated, whereas MKK3 was weakly activated (Fig. 2, D and E). The activations of MAP kinase kinase kinases such as ASK1 and MEKK1 were also examined to determine which MAP kinase kinase kinase might be involved in phosphorylation of SEK1 and MKK3/ MKK6 during RFP-induced apoptosis. Expression of RFP did not activate MEKK1, in comparison with clear activation by the positive control, UV irradiation (Fig. 2B). Because of technical difficulties, we were unable to observe ASK1 activation in either the experimental or positive control measurements of autophosphorylation and in vitro kinase reaction using a recombinant GST-SEK1(K129R) kinase-inactive protein substrate (data not shown). Therefore, we used a dominant-negative mutant of ASK1 to determine its involvement in RFP signaling. Dominant-negative mutants of ASK1 and SEK1 significantly inhibited RFP-induced apoptosis (Fig. 3A), suggesting that ASK1 activation is required for this apoptosis. Taken together, these results suggest that RFP expression induces stress-activated MAPK signaling cascades likely to originate with ASK1.

**Caspases Are Activated during RFP-triggered Apoptosis**—Caspase induction is a specific indicator of apoptotic cell death. In response to apoptotic stimuli, initiator caspases such as caspase-2, -8, -9, and -10 are activated by self-cleavage and in turn cleave effector caspases such as caspase-3, -6, and -7, leading to degradation of specific cellular components and ultimately to cell death (31, 32). To determine whether caspases are activated by RFP expression, we examined the activities of caspase-3, -8, and -9-like proteases by detecting cleavage of the fluorogenic substrates DEVD-AMC, IETD-AMC and LEHD-7-amino-4-trifluoromethyl coumarin, respectively (33). Expression of RFP in HEK 293 cells strongly induced caspase-3-like activity (4-fold that of control cells; Fig. 3B), and weakly induced caspase-8-like activity (less than 2-fold; data not shown), whereas there was little induction of caspase-9-like activity at 36 h after transfection.

We next assessed whether caspase-8 and/or caspase-3 are required for RFP-induced apoptosis. To test the requirement of caspase-8, we co-expressed RFP with the viral protein crmA, which is known to inhibit caspase-8. To test the requirement of caspase-3, we treated the RFP-expressing cells with the peptidyl caspase-3 inhibitor benzoyloxycarbonyl-DEVD-fluoromethyl ketone. Both inhibitors markedly suppressed RFP-induced apoptosis (Fig. 3A), implying that activation of caspase-8 and the subsequent activation of caspase-3 contribute a great deal to this apoptosis. Taken together, our results suggest that the
MAPK and caspase pathways are both required for RFP-induced apoptosis and that neither of them is sufficient to initiate apoptosis alone.

Two Distinct RFP-mediated Signaling Pathways—We next considered the relationship between the two pathways in RFP-induced apoptosis. For example, MAPKs activated by ASK1 expression can induce caspase activation via cytochrome c release from mitochondria, as shown by the triggering of apoptosis in Mv1Lu and MEF cells by a constitutively active form of ASK1 (34). Conversely, caspase-3 can cleave MEKK1, which in turn activates downstream MAPKs in the Fas-mediated apoptosis of vascular smooth muscle cells (35). Therefore, we questioned whether caspases might be activated by MAPKs or vice versa in RFP-induced apoptosis. However, the observation that caspases were active even when MAPKs were blocked by expression of ASK1- or SEK1 dominant-negative mutants suggests that the RFP-induced caspase cascade does not occur through the activation of MAPKs (Fig. 3B). In addition, the observation that JNK1/2 was not inhibited by caspase inhibitor crmA expression or DEVD treatment suggests that RFP does not initiate MAPK cascades via caspase activation, either (Fig. 3C). Thus, the MAPK and caspase cascades may mediate apoptotic signaling downstream of RFP by two distinct pathways.

Several reports have indicated that JNK activity and caspase activity are both required for apoptosis. For example, the amyloid β-peptide (Aβ 17–42) leads to apoptosis in human neuroblastoma cells through activation of caspase-8/3 as well as JNK (36). Fas- and UV-induced apoptosis in HEK 293 cells is mediated by independent actions of the JNK and FADD/caspase pathways (28, 37), and overexpression of the docking protein HEF1 causes apoptosis by simultaneously activating the JNK and caspase pathways (29).

RFP Expression Leads to Apoptosis Independent of a Mitochondrial Event—Apoptotic signals often lead to mitochondrial dysfunction (25, 26), which includes loss of membrane potential, production of reactive oxygen species, opening of the permeability transition pores, and the release of the intermembrane space protein, cytochrome c.

To investigate whether RFP causes the release of cytochrome c from the mitochondria, we examined the distribution of cytochrome c after RFP transfection. Subcellular fractions including either cytosol or mitochondria-enriched heavy membranes (HM) were prepared, and cytochrome c protein levels were measured by immunoblotting. Cytosolic cytochrome c increased significantly in a positive control experiment using staurosporine as an apoptotic reagent (Fig. 4A). In contrast, there was no significant change of cytosolic cytochrome c release.
was little changed during RFP-induced apoptosis. We therefore suggest that cytochrome c release does not occur during RFP-mediated apoptosis. Death pathways mediated by endogenous gene products such as p53 (27) and MEKK1 (38) are similar examples of apoptotic inductions that are not accompanied by cytochrome c release.

To further investigate the involvement of mitochondria in RFP-induced apoptosis, we next determined whether the expression of Bcl-2 or Bcl-xL, which blocks the mitochondrial permeability transition and subsequent release of cytochrome c (25), inhibited RFP-mediated apoptosis. We found that neither Bcl-2 nor Bcl-xL blocked apoptosis induced by RFP (Fig. 4B), whereas Bcl-2 and Bcl-xL suppressed up to 52 and 58% of staurosporine-induced apoptosis, respectively. In addition, we determined intracellular production of reactive oxygen species, a known marker for mitochondria-dependent apoptosis (25, 26), in RFP-expressing cells by staining with the reactive oxygen species-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate. Reactive oxygen species levels were not changed significantly even after the RFP-transfected cells showed apoptotic morphology (data not shown). Taken together, our results indicate that RFP induces apoptosis in a mitochondria-independent fashion.

RBCC Is Sufficient to Induce Cell Death—To further dissect RFP signaling, we investigated which region of RFP is required for its pro-apoptotic activities of triggering the stress-activated MAPK and caspase cascades. To assess this, we constructed a series of RFP deletion mutants (Fig. 5A) and transfected them into HEK 293 cells (Fig. 5B). First, caspase-3-like activities were examined (Fig. 5C). Deletion of the C-terminal RFP domain to yield a fragment containing only the RBCC moiety caused marked elevation of caspase-3-like activity to 7-fold.
that of full-length RFP. Further deletion of the coiled-coil or amino-terminal RING finger domains abolished this caspase activation (Fig. 5C), and transfection with the RING finger, coiled-coil, or RFP domain alone did not induce caspase-3-like activity. When these RFP mutants were tested for their ability to activate a MAP kinase kinase (SEK1), only the RBCC moiety was able to activate SEK1 to wild-type levels (Fig. 5D). Consistently, drastic apoptotic morphology appeared only in the cells transfected with RBCC moiety (data not shown), whereas no morphological changes occurred in the cells transfected with the other mutants. These results indicate that the intact RBCC moiety is necessary for RFP’s pro-apoptotic function. Induction of apoptosis by the RBCC moiety was verified by increased Annexin V-positive population in RBCC transfected cells compared with those transfected with empty vector (Fig. 6A). Induction of apoptosis was further confirmed by a marked increase of the sub-G1 cell population with hypodiploid DNA content, from 3.7 ± 0.2% in the control to 14.8 ± 0.7% in the RBCC-transfected cells, as quantified by flow cytometry using propidium iodide staining (Fig. 6B). This evidence demonstrates that the RBCC region is sufficient to mediate apoptosis.

Collectively, our results suggest that RFP activates two distinct cell death pathways: one via caspase pathway independent of mitochondrial events, and one via stress-activated MAP kinase pathway that involves JNK and p38 kinase. Neither of them alone is sufficient to initiate apoptosis, but both are required for RFP-induced apoptosis.

**DISCUSSION**

Our results provide the first demonstration that RFP, a member of the TRIM family, triggers apoptosis via stress response kinases and caspases. Unlike many other pro-apoptotic pathways, this pathway does not involve mitochondrial dysfunction.

Despite the absence of related mitochondrial events, caspase-3-like activity was increased during RFP-induced apoptosis. Caspase-3 can be activated not only by the cytochrome c-Apaf-1-caspase-9 complex but also by caspase-8 (32). Caspase-8 activation is mostly associated with apoptosis mediated by members of the TNFR family (31). On ligand binding, these receptors may directly or indirectly recruit the FADD adaptor protein, which in turn oligomerizes and activates caspase-8. However, recent studies have suggested that alternative pathways may also be responsible for caspase-8 activation. It has been observed that tumor growth factor-β- or B-cell receptor-mediated apoptosis in Burkitt’s lymphoma cells and monocytic-derived dendritic cell-mediated apoptosis of Jurkat cells are associated with caspase-8 activation independent of death domain receptors (39–42). Similarly, anticancer drugs can induce apoptosis with caspase-8 cleavage in a FADD-independent manner (43). Even though the nature and regulation of these FADD-independent pathways of caspase-8 activation remains unknown, these findings show that caspase-8 activation is not restricted to death receptors. Therefore, we suggest that RFP may activate caspase-8 and subsequently caspase-3 in an FADD-independent pathway. Further studies will reveal whether RFP activates caspase-8 and whether this activation involves, for example, the recruitment of adaptor molecules that may mediate caspase-8 oligomerization and cleavage.

Activation of JNK and p38 and concurrent inhibition of ERK is important for induction of apoptosis (44, 45). However, the actual roles of each MAPK cascade are highly dependent on cell type and context (45). Many possible targets of JNK and p38 may mediate pro-apoptotic signaling. JNK can up-regulate the Fas ligand, whose promoter contains an AP-1 site under the control of c-Jun phosphorylation (46, 47). JNK and p38 are also suggested to activate Bid, a pro-apoptotic Bcl-2 member that is proteolytically activated to induce cytochrome c release (48, 49). Another possible target is Bel-2, resulting in loss of its anti-apoptotic activity (50). We found that JNK and p38 were highly activated during RFP-induced apoptosis (Fig. 2, A and C). However, induction of the Fas ligand was not observed by RT-PCR or immunoblot analysis. Moreover, co-expression of a dominant-negative c-Jun (TAM67) did not block the RFP-induced apoptosis. These results indicate that AP-1 activation by JNK may not influence this apoptosis. Because c-Jun overexpression did not prevent RFP-induced apoptosis (Fig. 4B), we expect that neither Bel-2 nor Bid is the molecular target for activated JNK or p38 in our system. It will be interesting to investigate how JNK and p38 are activated in this case, and how they influence RFP-induced apoptosis. Because RFP does not contain any known kinase-, death-, death effector domain or caspase recruitment domain, at least one of which is usually required for the activation or recruitment of MAPKs or caspases, we suggest that RFP interacts with an apoptotic domain-containing molecule and activates it to trigger apoptosis. The exact effector molecule that interacts with RFP to trigger apoptosis will be a focus of further investigation.

We observed that the caspase activity induced by the RBCC moiety alone was about 27-fold higher than that of control cells, whereas the caspase activity induced by full-length RFP was 4-fold in HEK 293 cells (Fig. 5C). In addition, the sub-G1 population of RBCC-transfected cells (14.8 ± 0.7%) was significantly larger than that of RFP-transfected cells (9.5 ± 0.1%; Fig. 6B). These results suggest that the C-terminal overhang after the RBCC moiety in the RFP protein may have a negative effect on the pro-apoptotic function of the RBCC moiety. As an initial hypothesis, we propose that interactions with as-yet unidentified cellular proteins may change the folding of the RFP protein to release the inhibitory C-terminal overhang or proteolytic cleavage may remove the inhibitory overhang, resulting in amplification of downstream apoptotic signaling.

RFP was originally isolated as a transforming fusion gene similar to the case of PML, in which the RBCC moiety is oncogenically fused to a receptor tyrosine kinase (12). The PML-retinoic acid receptor α fusion in acute promyelocytic leukemia is thought to disrupt both PML and retinoic acid receptor pathways, thus acting as a double dominant-negative oncogenic product (21). It has been proposed that acute promyelocytic leukemia pathogenesis relies in part on transcriptional silencing of retinoic acid receptor α target genes and in part on the loss of PML-triggered apoptosis (21). We hypothesize that RFP may follow a similar pattern. In the case of the oncogenic RFP-Ret fusion (12), we propose that the pro-apoptotic activity of RFP shown in this study may be disrupted and the protein kinase activity of Ret may be constitutively activated, both of which would act toward the acquisition of transforming capability.

Biological functions of a large majority of TRIM family are still unclear. So far, two members, PML/TRIM19 (21, 22) and RFP/TRIM27 (this study), have been shown to be pro-apoptotic reagents. Because all TRIM proteins share the RBCC moiety, which this study has identified as a potent inducer of apoptosis, we propose that a large majority of TRIM family members might have apoptotic roles in a variety of processes such as development and cell growth. We speculate that the putative tumor-suppressing activities of glioblastoma expressed RING finger protein/TRIM8 and LEU5/TRIM13 (deleted in glioblastoma and B-cell lymphocytic leukemia, respectively (51, 52)) may result from their pro-apoptotic effects. Further studies will be required to clarify the roles of potentially apoptotic TRIM proteins in many biological processes.

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