Scythe Regulates Apoptosis-inducing Factor Stability during Endoplasmic Reticulum Stress-induced Apoptosis

Fabienne Desmots1,5, Helen R. Russell2, Denis Michel1, and Peter J. McKinnon1

From the 1Department of Genetics and Tumor Cell Biology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105 and the 2UMR 6026, CNRS, Université de Rennes 1, 35042 Rennes Cedex, France

Scythe (BAT3; HLA-B associated transcript 3, Bag 6) is a protein that has been implicated in apoptosis because it can regulate the Drosophila melanogaster apoptotic regulator, Reaper. Mice lacking Scythe show pronounced defects in organogenesis and in the regulation of apoptosis and proliferation during mammalian development. However, the biochemical pathways important for Scythe function are unknown. We report here multiple levels of interaction between Scythe and the apoptotic mitochondrial intermembrane protein AIF (apoptosis-inducing factor). Scythe physically interacts with AIF and regulates its stability. AIF stability is markedly reduced in Scythe−/− cells, which are more resistant to endoplasmic reticulum stress induced by thapsigargin. Reintroduction of Scythe or overexpression of AIF in Scythe−/− cells restores their sensitivity to apoptosis. Together, these data implicate Scythe as a regulator of AIF.

Apoptosis is the physiological process responsible for the demise of superfluous, aged, damaged, and ectopic cells and is essential during embryonic development and for maintenance of adult tissue homeostasis (1). Among the numerous proteins implicated in apoptosis is Scythe (BAT3; HLA-B associated transcript; Bag 6). Scythe has been shown to interact with Reaper, a central regulator of developmental apoptosis in Drosophila melanogaster (2, 3). To date, no Reaper homologues have been discovered in vertebrate species, but Reaper and associated Drosophila regulators Hid and Grim can induce apoptosis in mammalian systems (4, 5). The interaction between Scythe and Reaper is required for Reaper-induced apoptosis in Xenopus egg extracts and results in the release of an as yet unidentified Scythe-bound apoptotic-inducing factor, leading to rapid mitochondrial cytochrome c release, caspase activation, and nuclear fragmentation. Immunodepletion of Scythe prevented reaper-induced apoptosis (2, 3). Our previous study also indicated a role for Scythe during apoptosis in a mammalian system. We found that inactivation of Scythe in the mouse resulted in lethality associated with pronounced developmental defects in the lung, kidney, and brain (6). Co-incident with organogenesis defects was widespread aberrant apoptosis and proliferation, and a resistance of Scythe-null cells to apoptosis (6).

A critical step in apoptosis is often the permeabilization of mitochondrial membranes, leading to the release of proteins that are normally localized behind the outer mitochondrial membrane (7). One of those proteins is the apoptosis-inducing factor (AIF)2 (8). In healthy cells, AIF is located in the mitochondrial intermembrane space where its physiological function is not entirely clear, but may involve protection against oxidative stress (8–11). In apoptotic cells, AIF relocates from the mitochondria to the nucleus where it exerts its pro-apoptotic activity via chromatin condensation and large scale DNA fragmentation (12–14). Although AIF is strongly linked to apoptosis, many aspects of its activity remain to be clarified.

Here, we show that Scythe interacts with and regulates the stability and location of AIF after apoptotic stimuli mediated by endoplasmic reticulum stress. Thus, AIF action is impaired in Scythe−/− cells, which could account for their resistance to select apoptotic stimuli. Our data provide new insight into apoptotic regulation by Scythe and its relationship to the pro-apoptotic protein AIF.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—Human embryonic kidney 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 4 mM glutamine, and 100 units of penicillin and streptomycin in a 10% CO2 humidified incubator. Primary culture of MEFs (mouse embryonic fibroblasts) were obtained from E13.5 WT and Scythe−/− embryos and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 4 mM glutamine, 100 units of penicillin and streptomycin, 25 mM Hepes and non-essential amino acid in a 10% CO2 humidified incubator. Primary MEFs were not used beyond passage eight.

DNA Constructs and Transfection—Full-length Scythe (FL-Scythe) and truncated Scythe variants (Scy-NΔ380 or CΔ482), full-length AIF (FL-AIF), and truncated AIF (tAIF) (all HA-

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2 To whom correspondence should be addressed. Tel.: 33-02-23-23-50-93; Fax: 33-02-23-23-50-52; E-mail: fabienne.desmots@univ-rennes1.fr.

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3 The abbreviations used are: AIF, apoptosis-inducing factor; TG, thapsigargin; MEF, primary mouse embryonic fibroblast; WT, wild type; GFP, green fluorescent protein; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; MSCV, murine stem cell virus; HA, hemagglutinin; PBS, phosphate-buffered saline; RT, reverse transcriptase; BisTris, 2-[bis(2-hydroxyethyl)aminomethyl]-2-(hydroxymethyl)propane-1,3-diol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
tagged) cDNA were cloned into a murine stem cell virus (MSCV) vector that contained an internal ribosome entry site to allow coincident expression of a green fluorescent protein (GFP). Human AIF full-length sequence corresponds to amino acids 1–612 (GenBank™ accession number AF 100928), whereas truncated AIF lacks the 101 first amino acids containing the mitochondrial location sequence. For transient transfections, fusion proteins were generated by cloning Scythe and AIF (both full-length sequences) (Sigma). For transient transfections, fusion proteins were generated by cloning Scythe and AIF (both full-length sequences) into pEGFP-C3 (Clontech). Mutants of Scythe cDNA were cloned into a pcDNA3-HA vector. For transient transfection Nucleofector™ technology (Amaza Biosystems) was used according to the manufacturer’s recommendations.

Immunoblotting and Immunoprecipitation—MEFs were homogenized in 200 μl of lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5% Nonidet P-40, 15 mM MgCl2, 1 mM dithiothreitol, 60 mM β-glycerophosphate, 0.1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonl fluoride and protease inhibitor mixtures (Roche)). Proteins were separated on 8 or 4–12% NOVEX Tris glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore) and detected using antibodies to Scythe (6), AIF (Sigma), HA (12CA5, Roche Applied Science), GFP (Molecular probes), FLAG-M2 (Sigma), or Actin (Santa Cruz). To study protein-protein interactions, 293T cells or MEFs were trypsinized 48 h after transfection, washed with PBS, and cell pellets were suspended in lysis buffer (described above) and sonicated. Lysates were immunoprecipitated overnight at 4 °C with 5 μg of the indicated antibodies, followed by a 2-h incubation at 4 °C with Protein A- or G-Sepharose beads and washed three times in lysis buffer. Immune complexes were boiled in 2× sample buffer, separated on an 8 or 4–12% Bis-Tris NuPAGE gel (Invitrogen), and then electroblotted to polyvinylidene difluoride membranes.

Immunofluorescence Studies—Cells grown on tissue culture-treated glassed slides (BD Falcon) were treated or transfected as indicated, washed with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, and washed several times in PBS. For cells transfected with GFP-AIF or Scythe fusion proteins, cells were incubated for 30 min at 37 °C with 100 nM of a red fluorescent dye that stains the mitochondria in live cells (MitoTracker Red CMXRos; Molecular Probes), then loading solution was replaced with prewarmed medium and cells were directly observed using a confocal microscope (Leica TCS Sp2). Co-localization analysis has been performed using ImageJ (WCIF Image) package with the plug-in Intensity Correlation Analysis.

For cells transfected with MSCV- or pcDNA3-HA-tagged protein, cells were incubated for 1 h in blocking solution (5% goat serum, 1% bovine serum albumin in PBS containing 0.1% Triton X-100 (PBST)), followed by anti-HA antibody at 1 μg/ml in blocking solution overnight at 4 °C. Cells were washed three times in PBST for 5 min, and then incubated with secondary antibody (goat anti-mouse Cy3) 1 h in the dark. After being washed three times in PBST for 5 min, cells were mounted in Vectashield solution and examined under a confocal microscope (Leica TCS Sp2).

Metabolic Labeling and Pulse-Chase Assays—For the GFP-AIF stability experiment, WT and Scythe+/− MEFs were transfected with pEGFP or pEFGP-AIF. For endogenous AIF stability experiments, WT, Scythe−/−, or Scythe−/− transduced with MCSV-FL-Scythe MEFs were used. Then cells were washed with PBS and preincubated for 60 min with methionine- and leucine-free Dulbecco’s modified Eagle’s medium, containing 10% dialyzed fetal bovine serum and 4 mM glutamine. Cells were labeled for 2 h in the same medium with 10 μCi/ml of L-[35S]methionine (PerkinElmer Life Science), and chased in complete media. During the chase, in all experiments cells were treated with 1 μM TG. At various times after termination of labeling, cells were isolated in lysis buffer (described above), sonicated, and centrifuged at 10,000 × g at 4 °C for 10 min and soluble proteins were quantified using a Bio-Rad assay. Proteins (200 μg) were immunoprecipitated overnight at 4 °C with 5 μg of polyclonal rabbit anti-GFP or polyclonal rabbit anti-AIF antibodies as indicated. Immune complexes recovered by protein A-Sepharose and washed three times with lysis buffer were denatured and electrophoretically separated on NOVEX 8% Tris glycine denaturing gels (Invitrogen), then transferred to polyvinylidene difluoride membranes. Dried membranes were coated with ENHANCE and subjected to autoradiography. Corresponding lysates (Lysates35S) were loaded as control for an equal amount of proteins, as well as equal incorporation of 35S. Quantification of the bands was performed by the Image processing tool kit software (Reindeer Graphics, Inc.).

Apoptosis Assays—MEFs were cultured at 1 × 105 per 10-cm diameter culture dish. These cells were treated with 1 μM thapsigargin, 100 μM 2-APB, 50 nM staurosporine, 100 ng/ml Fas, 20 ng/ml tumor necrosis factor α, 10 μM camptothecin, and 20 μM hydrogen peroxide, for 24 h. Apoptosis was determined by fluorescence-activated cell sorter analysis of cells stained with Annexin V FITC/propidium iodide (Alexis Biochemicals) or by measuring DEVD-caspase activity (using the fluorogenic substrate DEVD-AMC). For the rescue experiment, after Scythe-GFP transfection, Annexin-V FITC/propidium iodide was counted only on GFP positive cells. In other experiments, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer containing 1% Triton X-100, 1 μg/ml of the indicated antibodies, followed by anti-aquamarine antibody (goat anti-mouse Cy3) 1 h in the dark. After being washed three times in PBST for 5 min, cells were mounted in Vectashield solution and examined under a confocal microscope (Leica TCS Sp2).

Quantitative RT-PCR—WT and Scythe−/− MEFs (0.4 × 106) were treated with 1 μM TG for 24 h. RNA extraction was performed with TRIZol reagent (Invitrogen) and 2.5 μg of extracted RNA were converted into cDNA using random primers (Amersham Biosciences) and reverse transcriptase with Moloney murine leukemia virus RT (Invitrogen). Real-time PCR for GAPDH and AIF were performed in triplicates, 40 cycles at 95 °C for 30 s and 54 °C for 30 s were carried out in a 12-μl volume containing 75 ng of cDNA, 300 nM custom made primers, and 6 μl of IQ™ SYBR Green Supermix (Bio-Rad). All primers were custom made at Sigma-Proligo and tested to ensure amplification of single bands with no primer-dimers. PCR efficiencies for different pairs of primers were verified by a
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RESULTS

Scythe+/− MEFs Are Resistant to Thapsigargin-induced Cell Death—We previously found that Scythe−/− primary cortical neurons were more resistant than WT cells to cell death resulting from endoplasmic reticulum stress signaling induced by TG or menadione (6). To further investigate the role of Scythe in this phenomenon, we examined the sensitivity of WT and Scythe−/− MEFs to treatment with several agents. By analyzing Annexin V positive cells using flow cytometry (Fig. 1A), we found that Scythe−/− MEFs are more resistant to various pro-apoptotic agents, and particularly to TG, compared with WT MEFs. Several time points and concentrations of TG were tested using either Annexin V or crystal violet staining (supplemental Fig. S1). A 24-h treatment with 1 μM TG was then used in this study. Apoptotic resistance of Scythe+/− MEFs to TG was also confirmed by measuring caspase 3/7 activity (Fig. 1B). To confirm that resistance to TG-induced cell death is dependent upon Scythe, we reintroduced Scythe in Scythe−/− MEFs by retroviral transduction and found that apoptosis levels of GFP-Scythe positive cells were similar to WT, whereas vector alone did not rescue apoptosis (Fig. 1C).

Scythe Interacts with the Apoptosis-inducing Factor AIF—TG was shown to induce mitochondrial permeability transition and the release of AIF (15). Genetic ablation of Scythe in cortical neurons (6), and that of AIF in embryonic stem cells, increases resistance to cell death induced by endoplasmic reticulum stress (16). Hence, we investigated the possible relationships between Scythe and AIF. Co-immunoprecipitation experiments revealed that endogenous Scythe and AIF strongly interact together (Fig. 2A). Scythe and eAIF interaction was also obtained in MEFs after transfection of Scythe-FL or certain Scythe mutants (Scythe-CA482, lacking the C-terminal sequence or Scythe-NΔ380, lacking the N-terminal sequence as shown in Fig. 2B). We found an interaction between Scythe-FL and eAIF and also between the C-terminal mutant and eAIF in MEFs, but not with Scythe-NΔ380 mutant (Fig. 2C). We transfected 293T cells with an N-terminal fluorescent full-length AIF fusion protein (GFP-AIF) and FL-Scythe or Scythe mutants, and found a strong interaction between GFP-AIF and Scythe-FL or CA482-, but again not with the NΔ380-Scythe mutant (Fig. 2D). These data indicate that AIF interacts with Scythe within a region encompassing its N terminus. To determine the specificity of this interaction and if it involves the ubiquitin-like N-terminal domain of Scythe, we tested the interaction between Scythe and RAD23, which also contains a ubiquitin-like domain, and found that RAD23, even when highly expressed, does not interact with AIF (data not shown). This suggests that the interaction between AIF and Scythe is mediated by the N-terminal region of Scythe but this interaction is independent of the ubiquitin-like region. Finally, we found an interaction between in vitro translated Scythe and AIF, which suggests a direct physical interaction can occur between these two proteins (Fig. 2E).
Scythe Modulates GFP-AIF Turnover—To further investigate the interplay between Scythe and AIF during apoptosis, we used GFP-AIF fusion protein and checked its expression in WT and Scythe−/− cells. We found that the GFP-AIF protein level was markedly reduced in Scythe−/− cells compared with WT cells, whereas GFP protein expression is similar in both cell types (Fig. 3A). The proteasome inhibitor MG132 fully restored GFP-AIF expression in Scythe−/− cells, indicating that proteasomal degradation of GFP-AIF is increased in the absence of Scythe. Surprisingly, we observed that GFP-AIF overexpression induces apoptosis by itself, without need for an additional apoptotic treatment. Therefore, we checked if the attenuated GFP-AIF protein level in Scythe−/− cells correlated with lower apoptosis. GFP-AIF induced apoptosis in WT cells and to a lesser extent in Scythe−/− cells (Fig. 3B). MG132 treatment restored both GFP-AIF stability and the apoptotic activity in Scythe−/− cells. To confirm that the level of apoptosis induced by GFP-AIF is dependent on Scythe, we reintroduced Scythe in Scythe−/− MEFs. Transfected Scythe restored GFP-AIF expression (Fig. 3C) and GFP-AIF-induced apoptosis (Fig. 3D) in Scythe−/− cells.

To further investigate the connection between Scythe and GFP-AIF protein levels described above, we used pulse-chase analysis to determine whether GFP-AIF fusion protein stability is affected by loss of Scythe. Cells transfected with either GFP or GFP-AIF were metabolically labeled with L-[35S]methionine and chased for various periods of time. Total lysates were immunoprecipitated with the indicated antibodies, resolved on denaturing gels, and visualized by autoradiography (Fig. 4E). The half-life of GFP was around ~8 h in WT and Scythe−/− MEFs. In contrast, that of GFP-AIF was only ~2 h in Scythe−/− cells, but ~5 h in WT cells. These results confirmed that Scythe markedly influences the stability of GFP-AIF.

Decreased Expression of Endogenous AIF after TG Treatment in Scythe−/− Cells Contributes to Their Resistance to Apoptosis—The previous experiments were obtained using transfected AIF. We then checked endogenous expression of AIF (eAIF) in control and TG-treated WT and Scythe−/− cells (Fig. 4A). We observed a decrease in eAIF protein level after TG treatment in WT cells, but this was more pronounced in Scythe−/− cells (Fig. 4A). The decrease in eAIF expression occurred at a post-translational level because RT-PCR analysis showed unchanged Aif gene expression in these cells upon TG treatment (Fig. 4B). The lower eAIF protein level observed in Scythe−/− cells could result from indirect compensatory developmental mechanisms after Scythe inactivation, rather than a direct stabilizing activity of Scythe. Hence, we checked for eAIF expression after re-introduction of Scythe into Scythe−/− MEFs treated with TG and found that eAIF expression was increased in these cells as compared with Scythe−/− cells (Fig. 4C), indicating that eAIF stability is directly influenced by Scythe. We therefore checked the stability of eAIF using pulse-chase analysis in WT and Scythe−/− or Scythe−/− cells transduced with MSCV-FLAG-Scythe (Scythe−/−T.Sc.FL). During the chase, cells were treated with 1 μM TG. eAIF half-life was reduced in Scythe−/− cells treated with TG compared with WT (Fig. 4D). In addition, when Scythe−/− cells were transduced with a Scythe expression vector and treated with TG, eAIF stability was restored (Fig. 4D). To confirm that resistance to TG-induced cell death is dependent on AIF expression, we overexpressed full-length AIF (FL.Flag.AIF) or a truncated mutant of AIF (T.HA.AIF) lacking the N-terminal sequence containing the mitochondrial localization sequence, in Scythe−/− MEFs by retroviral transduction. T.HA.AIF and FL.FLAG.AIF expression were checked by Western blot (Fig. 4E) and as expected, only FL.FLAG.AIF is decreased after TG treatment in Scythe−/− cells. Moreover, full-length AIF overexpression restored sensitivity of Scythe−/− cells to apoptosis (Fig. 4F). These results demonstrated that in Scythe−/− cells, AIF stability is markedly reduced and this potentially contributes to their increased resistance to apoptosis induced by TG.
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A.

|         | CONTROL | MG132 |
|---------|---------|-------|
| V-GFP  | Scythe<− | Scythe<− |
| GFP-AIF| Scythe<− | Scythe<− |

B.

|         | % TUNEL positive cells (GFP-AIF) |
|---------|---------------------------------|
| WT      | C                               |
| MG132   | *                               |

C.

| T.V. | T.SCY. |
|------|--------|
| V-GFP|        |
| GFP-AIF|      |
| GFP  |        |
| ACTIN|        |

D.

|         | % TUNEL positive cells (GFP-AIF) |
|---------|---------------------------------|
| WT      | *                               |
| T.V.    | *                               |
| T.SCY.  | *                               |

E.

|         | V-GFP |
|---------|-------|
| WT      |       |
| Scythe<−|       |
| Lysates 35S| |

**FIGURE 3.** GFP-AIF degradation in Scythe<−/− MEFs occurs via the proteasome pathway and is dependent on Scythe. A. Western blot (WB) analysis using GFP antibody in Scythe<−/− and WT MEFs transfected with either pEGFP (V-GFP) or GFP-AIF, and treated or not with MG132. GFP-AIF expression decreased in Scythe<−/− MEFs restored by MG132 treatment. The amount of proteins was equivalent as controlled with actin expression. B. percentage of cell death determined using TUNEL in WT and Scythe<−/− MEFs that were transfected with either V-GFP or GFP-AIF and treated or not with MG132. Data are expressed as a percentage of apoptotic cells in GFP-AIF expressing cells relative to GFP expressing cells. Low level of AIF expression correlates with less apoptosis in Scythe<−/− cells. C and D. Scythe restores GFP-AIF stability and correlates with a higher level of apoptosis. Western blot analysis using GFP and HA antibodies (C) and percentage of cell death determined using TUNEL (D) in Scythe<−/− MEFs co-transfected with either pEGFP (V-GFP) or GFP-AIF and pcDNA3-HA (T.V.) or pHA-FL-Scythe (T.SCY). E. WT and Scythe<−/− MEFs were transfected with pEGFP (V-GFP) or pEGFP-AIF and grown in methionine- and leucine-free medium and then metabolically labeled with [35S]methionine and chased in fresh complete medium for the indicated time. Labeled proteins were immunoprecipitated with the anti-GFP antibody subjected to denaturing electrophoresis, dried, and analyzed by autoradiography. As a control for an equal amount of proteins and incorporation of 35S, whole cell lysates were also analyzed (Lysates 35S). All data are representative of at least two different experiments. Asterisk indicates statistically significant (mean ± S.D. of three experiments; p < 0.05).

**DISCUSSION**

Inactivation of Scythe leads to a variety of developmental defects involving the lungs, kidneys, and nervous system, resulting in either midgestational or perinatal lethality (6). Coincident with these abnormalities are widespread apoptosis and proliferation defects (6). Very little is known about how this important protein functions as an apoptotic regulator in mammalian cells. Initial biochemical evidence that Scythe plays a role in apoptosis comes from the formation of a caspase-3-cleaved Scythe C-terminal fragment with pro-apoptotic activity after ricin treatment (17), proteins alone and different versions of Scythe (HA-Scy-FL or the cytosolic mutant of Scythe, HA-Scy-CA482) within GFP-AIF and checked their location using confocal microscopy in control or TG-treated WT and Scythe<−/− cells. The results obtained with WT and Scythe<−/− cells were similar. Representative images are shown in Fig. 5. GFP-AIF clearly co-localized with MitoTracker and the mitochondria in control cells and to the nucleus after TG treatment (Fig. 5A). GFP-Scythe was nuclear in control cells and partially co-localized with MitoTracker in TG-treated cells, suggesting that it is subject to nuclear export during apoptosis (Fig. 5B). We observed a similar nuclear export of endogenous Scythe after TG treatment (supplemental Fig. S2). Then, we transfected MEFs with HA-FL-Scythe or HA-Scy-CA482 (mutant of Scythe that co-localized with the mitochondria as shown in supplemental Fig. S3) with GFP-AIF, and cells were treated with TG. In control cells, HA-FL-Scythe, immunodetected using HA antibody, was predominantly nuclear (Fig. 5C), whereas HA-Scy-CA482 and GFP-AIF co-localized in the mitochondria and in the cytoplasm of these cells (Fig. 5D). After TG treatment, FL-Scythe or the cytosolic mutant of Scythe and GFP-AIF co-localized in the cytosol. These results suggest that the cytosol is a main site of interaction between Scythe and AIF in cells undergoing apoptosis.

**Scythe and AIF Co-localize in the Cytosol after TG Treatment—**

To determine the site of action of Scythe on AIF stability, we examined the location of these two proteins in control and TG-treated MEFs. We transfected GFP-Scythe and GFP-AIF fusion proteins alone and different versions of Scythe (HA-Scy-FL or the cytosolic mutant of Scythe, HA-Scy-CA482) within GFP-AIF and checked their location using confocal microscopy in control or TG-treated WT and Scythe<−/− cells. The results obtained with WT and Scythe<−/− cells were similar. Representative images are shown in Fig. 5. GFP-AIF clearly co-localized with MitoTracker and the mitochondria in control cells and to the nucleus after TG treatment (Fig. 5A). GFP-Scythe was nuclear in control cells and partially co-localized with MitoTracker in TG-treated cells, suggesting that it is subject to nuclear export during apoptosis (Fig. 5B). We observed a similar nuclear export of endogenous Scythe after TG treatment (supplemental Fig. S2). Then, we transfected MEFs with HA-FL-Scythe or HA-Scy-CA482 (mutant of Scythe that co-localized with the mitochondria as shown in supplemental Fig. S3) with GFP-AIF, and cells were treated with TG. In control cells, HA-FL-Scythe, immunodetected using HA antibody, was predominantly nuclear (Fig. 5C), whereas HA-Scy-CA482 and GFP-AIF co-localized in the mitochondria and in the cytoplasm of these cells (Fig. 5D). After TG treatment, FL-Scythe or the cytosolic mutant of Scythe and GFP-AIF co-localized in the cytosol. These results suggest that the cytosol is a main site of interaction between Scythe and AIF in cells undergoing apoptosis.
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and also the interaction of Scythe with immediate early gene X-1 that is involved in the control of apoptosis and cellular growth (18). More recently, Scythe has been identified as a regulator of p53 transcripitional activity, suggesting a role in the response to genotoxic stress (19). In the present study, we identified a novel role of Scythe relevant for its apoptotic function. This conclusion is supported by our finding that Scythe expression restores the level of AIF expression in Scythe-/- cells and increases AIF-driven apoptosis. The proteasome is involved in this phenomenon proposed to sequester an unknown apoptotic factor that is sufficient, when released upon Reaper addition, to induce apoptosis (3). The present study suggests that AIF could be such a factor in a mammalian system. AIF was originally linked to a caspase-independent death pathway (14), although it has been proposed that mitochondrial release of AIF occurs downstream of cytochrome c release, either independently or concomitantly with caspase activation (20).

Previously, Scythe has been implicated in the regulation of apoptosis. In Xenopus egg extracts, the binding of (Drosophila) Reaper to Scythe promoted cytochrome c-mediated caspase activation leading to cell death (2, 3). Moreover, Scythe was these domains are required for normal development of the Xenopus embryo (23). The proteasome machinery is implicated in the normal control of apoptosis or proliferation via its ability to regulate degradation of a wide variety of molecules involved in these functions (24–27). In Scythe-/- cells, endogenous AIF protein half-life was shorter after pro-apoptotic treatment, further supporting a role for Scythe in regulating AIF function. This conclusion is supported by our finding that Scythe expression restores the level of AIF expression in Scythe-/- cells and increases AIF-driven apoptosis. The proteasome is involved in this phenomenon.

FIGURE 4. Low endogenous AIF (eAIF) level in Scythe-/- MEFs contributes to their resistance to TG. A, Western blot analysis of eAIF expression in WT and Scythe-/- MEFs after TG (1 μM; 24 h). For each experiment actin blotting was done as protein loading controls. B, AIF gene expression in Scythe-/- and WT MEFs treated with TG (1 μM; 24 h) in comparison with control. Total RNA was isolated and subjected to quantitative real-time RT-PCR. Data are expressed as a -fold change in mRNA levels in treated cells relative to control cells after normalization to GAPDH using the formula 2 -ΔΔCt, means of three different experiments are shown. C, Western blot analysis of Scythe and eAIF in Scythe-/- MEFs transduced with MSCV-GFP-Vector (V), or MSCV full-length Scythe (Sc. FL) and treated with TG (1 μM; 24 h). D, TG modifies steady-state levels of endogenous AIF in Scythe-/- cells and Scythe expression restores AIF stability in Scythe-/- cells. WT, Scythe-/-, or MSCV-GFP-FL Scythe (Sc. FL) transduced Scythe-/- MEFs were treated during the chase by TG (1 μM) and labeled proteins after pulse-chase were immunoprecipitated with anti-AIF antibody or anti-β-tubulin antibody as control. All the data are representative of at least two different experiments. E, Western analysis of WT and Scythe-/- MEFs after transduction with AIF or truncated AIF. F, percentage of TUNEL apoptotic cells was determined in control and TG (1 μM; 24 h) WT and Scythe-/- MEFs transduced with MSCV-GFP-Vector (V), MSCV full-length AIF (FL.Flag.AIF), or a mutant of AIF (T.HA.AIF). Numerical values were expressed as mean of two different experiments.
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FIGURE 5. Scythe co-localized with AIF in the cytosol after TG treatment. Scythe \( ^{−/−} \) MEFs, control and TG-treated (1 \( \mu \)M; 24 h), were transfected with pEGFP-AIF (A) or pEGFP-Scythe (B). Following the treatment, cells were incubated with MitoTracker (100 nM; 30 min) before observation using a confocal microscope. GFP-AIF fusion protein was co-localized with MitoTracker in untreated cells. Accumulation of the mitochondrial GFP-AIF was observed in the nucleus after treatment. Whereas Scythe is nuclear in control cells, it partially co-localized with the MitoTracker in treated cells. Cells were transiently transfected with pEGFP-AIF and pH-A-Scythe.Fl (C) or pH-A-Scythe.C482 (D) expression plasmids with or without TG treatment (1 \( \mu \)M; 24 h). Immunocytochemistry using anti-HA antibody were performed to detect FL-Scythe and mutants of Scythe localization (red) and overlay with GFP-AIF (green). Representative images of at least three different experiments are shown.

because we could maintain AIF protein level and consequently increase apoptosis in Scythe \( ^{−/−} \) cells using a proteasome inhibitor. Moreover, overexpression of AIF in Scythe \( ^{−/−} \) cells restores their sensitivity to apoptosis. Together, our data indicate that Scythe is important for regulating AIF protein stability, via a mechanism involving the proteasome and that the decreased level of AIF in Scythe \( ^{−/−} \) cells is likely to be involved in their resistance to apoptosis. Scythe co-localized with AIF in the cytosol of TG-treated cells suggesting that Scythe transiently affects the nuclear translocation of AIF during apoptosis. A plausible hypothe-
sis is that Scythe could protect AIF from degradation and perhaps favor its apoptotic action in the cytosol. Indeed, whereas the function of AIF in the nucleus is well documented (13), its action in the cytoplasm has recently emerged where it has been shown to activate caspase 7 (28). Scythe, by stabilizing and maintaining AIF in the cytosol may function as a central regulator of the action of AIF. In summary, our data describing a novel regulatory complex between Scythe and AIF thereby provide new insights into the basic physiology of cell death.

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