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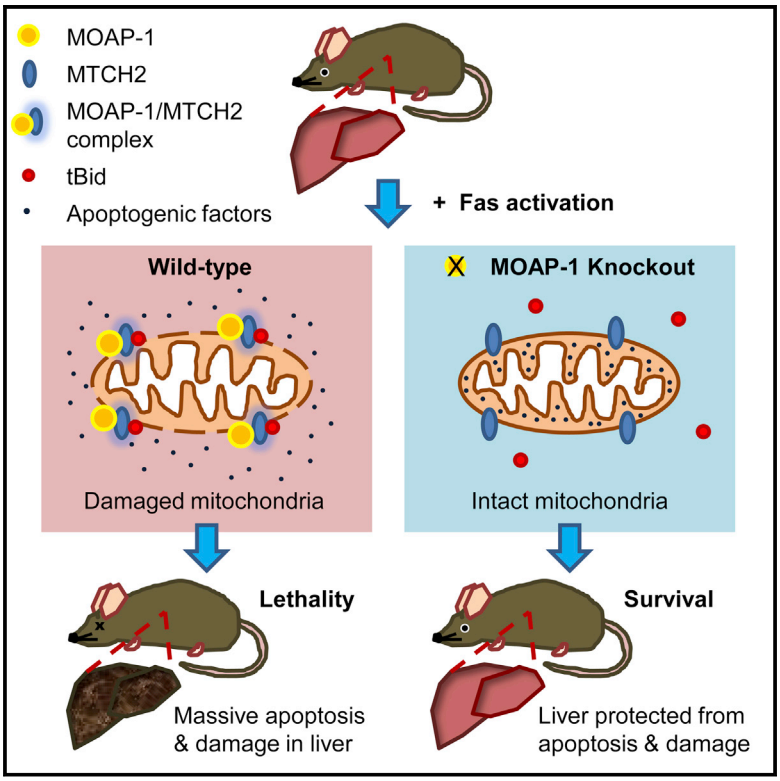
MOAP-1 Mediates Fas-Induced Apoptosis in Liver by Facilitating tBid Recruitment to Mitochondria

Graphical Abstract



Highlights

- MOAP-1 is required to mediate Fas apoptotic signaling in liver
- Loss of *MOAP-1* protects mice from Fas-induced liver injury and lethality
- MOAP-1 is required for efficient tBid recruitment to mitochondria
- Binding of MOAP-1 to MTCH2 engages the receptor function of MTCH2 for tBid

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In Brief

Activation of Fas apoptotic signaling triggers acute liver injury. Tan et al. show that deletion of the *MOAP-1* gene protects mice from this injury. MOAP-1 binds to MTCH2, and the interaction appears necessary for MTCH2 recruitment of tBid to mitochondria, an essential step for executing Fas-induced apoptosis in the liver.

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MOAP-1 Mediates Fas-Induced Apoptosis in Liver by Facilitating tBid Recruitment to Mitochondria

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SUMMARY

Fas apoptotic signaling regulates diverse physiological processes. Acute activation of Fas signaling triggers massive apoptosis in liver. Upon Fas receptor stimulation, the BH3-only protein Bid is cleaved into the active form, tBid. Subsequent tBid recruitment to mitochondria, which is facilitated by its receptor MTCH2 at the outer mitochondrial membrane (OMM), is a critical step for commitment to apoptosis via the effector proteins Bax or Bak. MOAP-1 is a Bax-binding protein enriched at the OMM. Here, we show that MOAP-1-deficient mice are resistant to Fas-induced hepatocellular apoptosis and lethality. In the absence of MOAP-1, mitochondrial accumulation of tBid is markedly impaired. MOAP-1 binds to MTCH2, and this interaction appears necessary for MTCH2 to engage tBid. These findings reveal a role for MOAP-1 in Fas signaling in the liver by promoting MTCH2-mediated tBid recruitment to mitochondria.

INTRODUCTION

Fas (also known as CD95 and APO-1) signaling for apoptosis activation plays important roles in diverse physiological processes including regulating homeostasis of the hematopoietic and immune systems (Peter et al., 2007; Strasser et al., 2009; Watanabe-Fukunaga et al., 1992). Dysfunction of Fas signaling has been implicated in a multitude of human diseases such as autoimmune disorders and liver diseases (Guicciardi and Gores, 2005; Peter et al., 2007). Indeed, elevated expression of Fas receptor or ligand in livers was frequently observed among patients suffering from fulminant hepatitis (Galle et al., 1995; Ryo et al., 2000). Fas activation triggers apoptosis via two distinct mechanisms dependent on cell types (Jost et al., 2009; Scaffidi et al., 1998). In type I cells (e.g., thymocytes and lymphocytes), Fas-induced activation of caspase-8 is sufficient to execute apoptosis by activating effector caspase-3 and caspase-7. In type II cells (e.g., hepatocytes and β-pancreatic cells), Fas-mediated apoptosis requires involvement of the mitochondrial pathway. Upon activation of the Fas receptor, activated caspase-8 cleaves the BH3-only protein, Bid (Wang et al., 1996), which is an essential step for inducing Fas-mediated apoptosis in liver (Lazic et al., 2014; Yin et al., 1999), to yield the activated form, tBid (also known as p15 Bid) (Li et al., 1998; Luo et al., 1998). tBid accumulates at outer mitochondrial membrane (OMM) to promote activation and oligomerization of Bax and Bak (Gross et al., 1999; Wei et al., 2001). This eventually leads to mitochondrial outer membrane permeabilization (MOMP) and releasing of multiple apoptogenic factors including cytochrome *c* and Smac/DIABLO to the cytosol (Du et al., 2000; Luo et al., 1998; Verhagen et al., 2000). Cytochrome *c* forms a complex with Apaf-1 and caspase-9 that triggers activation of caspase-3 (Li et al., 1997), Smac in turn releases activated caspase-3 and caspase-7 from inhibition by binding to the inhibitor of apoptosis proteins (IAPs) (Du et al., 2000; Verhagen et al., 2000). The recruitment of tBid to mitochondria is thought to be facilitated by the mitochondria-specific phospholipid cardiolipin (Esposti et al., 2003; Lutter et al., 2000). More recently, Gross and colleagues demonstrated that mitochondrial accumulation of tBid is dependent on its recruitment by an OMM protein, mitochondrial carrier homolog 2 (MTCH2, also known as MIMP) (Yerushalmi et al., 2002), which acts as a receptor-like protein for tBid (Grinberg et al., 2005; Zaltsman et al., 2010).

Modulator of apoptosis-1 (MOAP-1) is a Bax-binding protein residing at OMM (Fu et al., 2009; Tan et al., 2001, 2005). It is a short-lived protein that is constitutively regulated by the ubiquitin-proteasome system (UPS) (Fu et al., 2007). Apoptotic stimuli stabilize MOAP-1 by blocking its poly-ubiquitination process (Fu et al., 2007) and stimulate its interaction with Bax (Tan et al., 2005). Levels of MOAP-1 protein can modulate sensitivity of cancer cells to apoptotic stimuli, as knockdown of MOAP-1

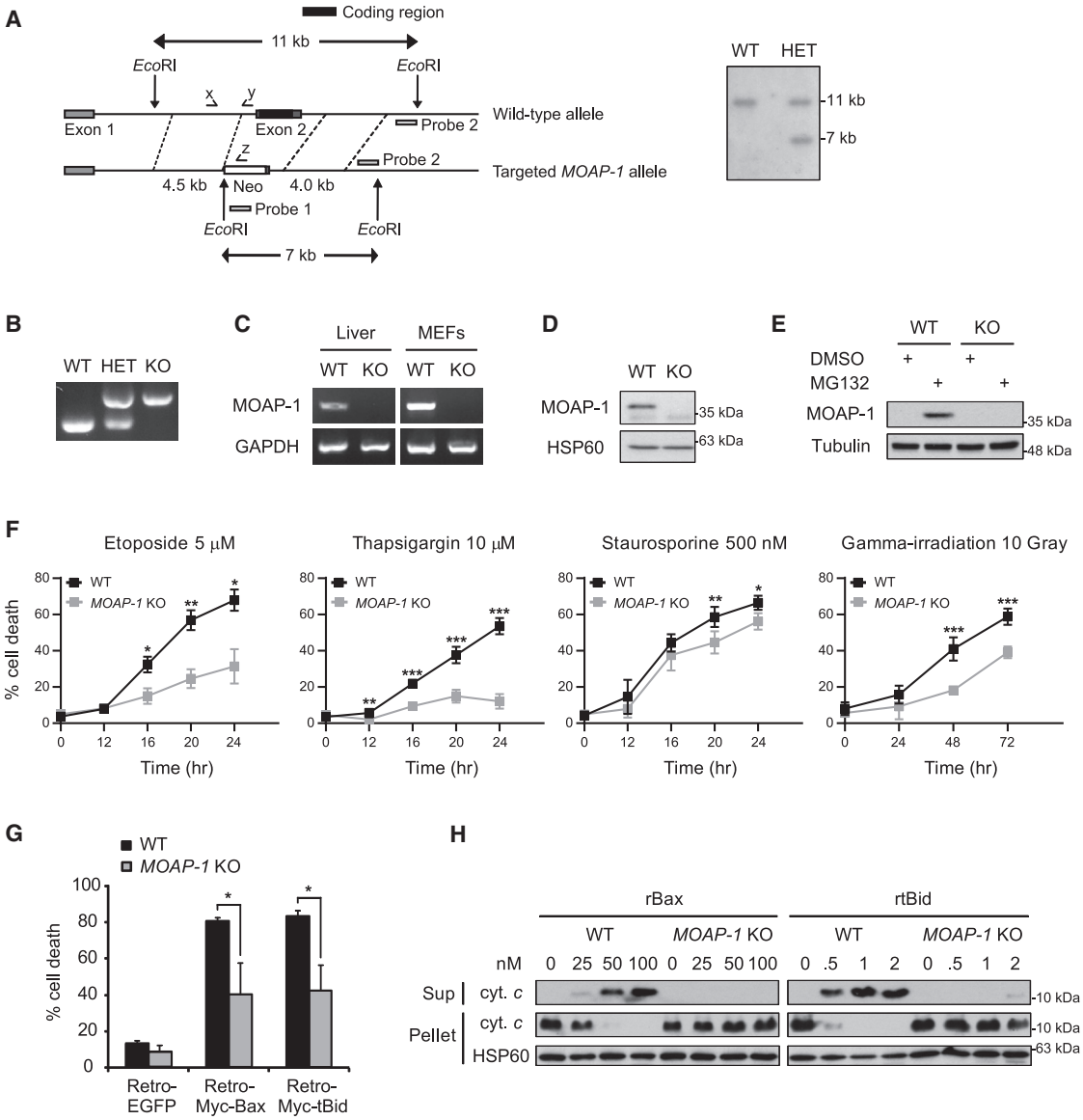

Figure 1. Generation of *MOAP-1* Knockout Mouse and Evaluation of Sensitivity of the MOAP-1-Deficient MEFs to Multiple Apoptotic Stimuli

(A) Left panel: targeting scheme for the *MOAP-1* KO mice. Exon 2 of *MOAP-1* gene, which contains the entire coding region, was replaced with neomycin (Neo) resistance gene via homologous recombination. EcoRI restriction sites were used for southern blotting with the length of the excised fragment stated in kb pairs, and the southern blot probes are indicated as Probe 1 and 2 (Probe 1 is specific for the targeted *MOAP-1* allele whereas Probe 2 hybridizes with both WT and targeted *MOAP-1* alleles). x, y, and z are the primers used for PCR-based genotyping. Right panel: southern blot analysis demonstrating homologous recombination within *MOAP-1* gene locus in the first generation of heterozygous (HET) mouse.

(B) PCR-based genotyping of WT, heterozygous, and *MOAP-1* KO animals.

(C) RT-PCR analysis of WT and *MOAP-1* KO livers (left) and MEFs (right).

(D) Western analysis of MOAP-1 immunoprecipitated from total liver lysates of the WT and *MOAP-1* KO mice. HSP60 was probed to show equal amount of lysates used in the IP.

(E) Expression of MOAP-1 in MEFs derived from the WT or *MOAP-1* KO littermates. MOAP-1 protein is regulated by the proteasome and was readily detected only upon treatment with the proteasome inhibitor MG132 (10 μM) for 16 hr.

(F) MOAP-1 deficiency dampens the sensitivity of MEFs to apoptotic stimuli that act via the mitochondrial pathway. WT and *MOAP-1* KO MEFs were treated with etoposide (5 μM), thapsigargin (10 μM), staurosporine (500 nM), or gamma irradiation (10 gray) for the indicated durations. Cell death was determined using propidium iodide (PI) exclusion-based flow cytometric analysis. Percentages of cell death (cells uptaking PI) (mean ± SEM of three independent experiments) are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's t test.

(G) MOAP-1-deficient MEFs are less sensitive to the pro-apoptotic activity of Bax and tBid. WT and *MOAP-1* KO MEFs were infected with retrovirus encoding Myc-Bax or Myc-tBid for 24 hr. Cell death was determined using PI exclusion-based flow cytometric analysis as in (F). Percentages of cell death (mean ± SEM of three independent experiments) are shown. *$p < 0.05$, Student's t test.

(legend continued on next page)

desensitizes whereas overexpression enhances the sensitivity of cancer cells to chemotherapeutic drugs (Baksh et al., 2005; Fu et al., 2007; Tan et al., 2005). Furthermore, effect of recombinant Bax or tBid on inducing release of cytochrome *c* from isolated mitochondria was impaired in mitochondria prepared from the MOAP-1 knockdown cells (Tan et al., 2005), suggesting MOAP-1 plays an important role in promoting activation of Bax to regulate mitochondrial pathway of apoptosis.

Here, we reveal that MOAP-1-deficient mice are markedly resistant to Fas-induced hepatocellular apoptosis and lethality. Upon Fas induction, cleavage of Bid into tBid remains unaffected in the MOAP-1-deficient livers. Interestingly, tBid is unable to associate with MTCH2 and fails to accumulate at mitochondria in the absence of MOAP-1. MOAP-1 associates with MTCH2, but not tBid. Re-expression of MOAP-1, but not the MOAP-1 mutant defective in binding MTCH, in the MOAP-1-deficient animals, restores the binding of tBid to MTCH2 and hepatocellular apoptosis upon Fas activation. These data together suggest that MOAP-1 serves as a critical component of the Fas signaling in liver by promoting the mitochondrial receptor function of MTCH2 for tBid.

RESULTS

Generation of *MOAP-1* Knockout Mice and Evaluation of Sensitivity of MOAP-1-Deficient MEFs to Multiple Apoptotic Stimuli

To support investigation of the roles of MOAP-1 in regulating mitochondrial apoptosis signaling pathway in vivo, targeting vector to delete the exon 2 of *MOAP-1* covering the entire coding region, was used for generating the knockout (KO) mice (Figure 1A). Genomic integration of the targeting vector was confirmed by southern blotting (Figure 1A, right panel) and by PCR-based genotyping (Figure 1B). Heterozygous parents produced offspring in accordance with the Mendelian ratio. Deletion of the gene in the KO mice was confirmed by RT-PCR (Figure 1C) and western blotting of MOAP-1 protein in the liver (Figure 1D) and in the mouse embryonic fibroblasts (MEFs) (Figure 1E). *MOAP-1* KO mice develop normally to adulthood and are fertile. Similar to the observations made in the MOAP-1 knockdown cells (Tan et al., 2005), MEFs derived from the *MOAP-1* KO mice showed a moderate reduction in sensitivity to multiple apoptotic stimuli (Figure 1F). *MOAP-1* KO MEFs also demonstrated resistance to apoptosis induced by overexpression of Bax or tBid (Figure 1G). Notably, isolated mitochondria prepared from the *MOAP-1* KO liver were markedly resistant to the cytochrome *c* releasing effect of recombinant Bax and tBid (Figure 1H).

MOAP-1-Deficient Mice Are Resistant to Fas-Induced Lethality and Hepatocellular Apoptosis

In vivo activation of Fas signaling by tail vein injection with the anti-Fas antibody (clone Jo-2) into mice is known to trigger massive apoptosis in liver via the mitochondrial pathway, followed by lethality within hours (Ogasawara et al., 1993; Wei et al., 2001; Yin et al., 1999). Because release of cytochrome *c* from isolated mouse liver mitochondria induced by recombinant Bax and tBid appeared critically dependent on MOAP-1, we subjected the *MOAP-1* KO mice to evaluation with this in vivo paradigm. Young adult male wild-type (WT) and *MOAP-1* KO mice were given tail vein injection of the Jo-2 antibody. Remarkably, while almost all the WT mice died within 16 hr (survival rate = 2/13), nearly all the *MOAP-1* KO mice survived (survival rate = 13/15) at 24 hr post-injection (Figure 2A; Table S1).

We then analyzed the liver injury profile of the WT and *MOAP-1* KO mice injected with Jo-2 antibody. In contrast to the WT mice, serum levels of the liver injury marker, alanine aminotransferase (ALT), were only slightly elevated in the *MOAP-1* KO mice (Figure 2B). Histopathologic analysis of the H&E-stained sections revealed significant hemorrhagic lesions in livers of the WT but not *MOAP-1* KO mice (Figure 2C, upper panels; Table S1). Similarly, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay demonstrated massive apoptosis only in the livers of the WT mice (Figure 2C, lower panels). Analysis of total liver lysates prepared from the *MOAP-1* KO mice subjected to Jo-2 injection showed that caspase-8 activation remained unaffected, while activation of caspase-3 and caspase-7 was significantly impaired (Figure 2D). Activation of caspase-9 (Figure 2D), release of cytochrome *c*, Smac, and AIF to the cytosol (Figure 2E), and oligomerization of Bax and Bak (Figure 2F) were all inhibited in the livers of the MOAP-1-deficient mice.

To confirm that MOAP-1 is required for mediating Fas signaling in hepatocytes (type II cells), hepatocytes from the WT and *MOAP-1* KO mice were prepared and subjected to treatment with Jo-2 antibody plus actinomycin D (Act.D), which is an established in vitro paradigm for Fas activation in hepatocytes (Ni et al., 1994). Fas-induced activation of caspase-3, but not caspase-8, was inhibited in the *MOAP-1* KO hepatocytes (Figure S1A). Likewise, *MOAP-1* KO hepatocytes treated with increasing concentrations of recombinant Flag-tagged soluble Fas ligand (FasL) plus anti-Flag antibody to activate Fas receptor by aggregation (Jost et al., 2009), exhibited significant reduction in cell death and caspase-3 activation (Figure S1B). In contrast to hepatocytes, no notable difference in the sensitivity to the FasL plus anti-Flag antibody treatment between the WT and *MOAP-1* KO thymocytes (type I cells) was noted (Figure S1C), suggesting that MOAP-1 is primarily involved in facilitating Fas signaling in the type II cells.

tBid Fails to Accumulate at Mitochondria in the Absence of MOAP-1

Upon cleavage of Bid to tBid by activated caspase-8, tBid accumulates at OMM to participate in the activation of the mitochondrial pathway of apoptosis that involves Bax and Bak (Gross et al., 1999; Hikita et al., 2011; Lovell et al., 2008; Wei et al., 2000, 2001). Because activation of caspase-8 upon Fas

(H) MOAP-1-deficient mitochondria from livers are markedly resistant to the cytochrome *c* releasing effect of recombinant Bax and tBid. Mitochondria prepared from livers of the WT and *MOAP-1* KO mice were incubated with recombinant Bax (rBax) and tBid (rtBid) protein at the indicated concentrations at 30°C for 30 min before segregating the supernatant (Sup), which harbors the released cytochrome *c* protein, from the pellet that contains mitochondria.

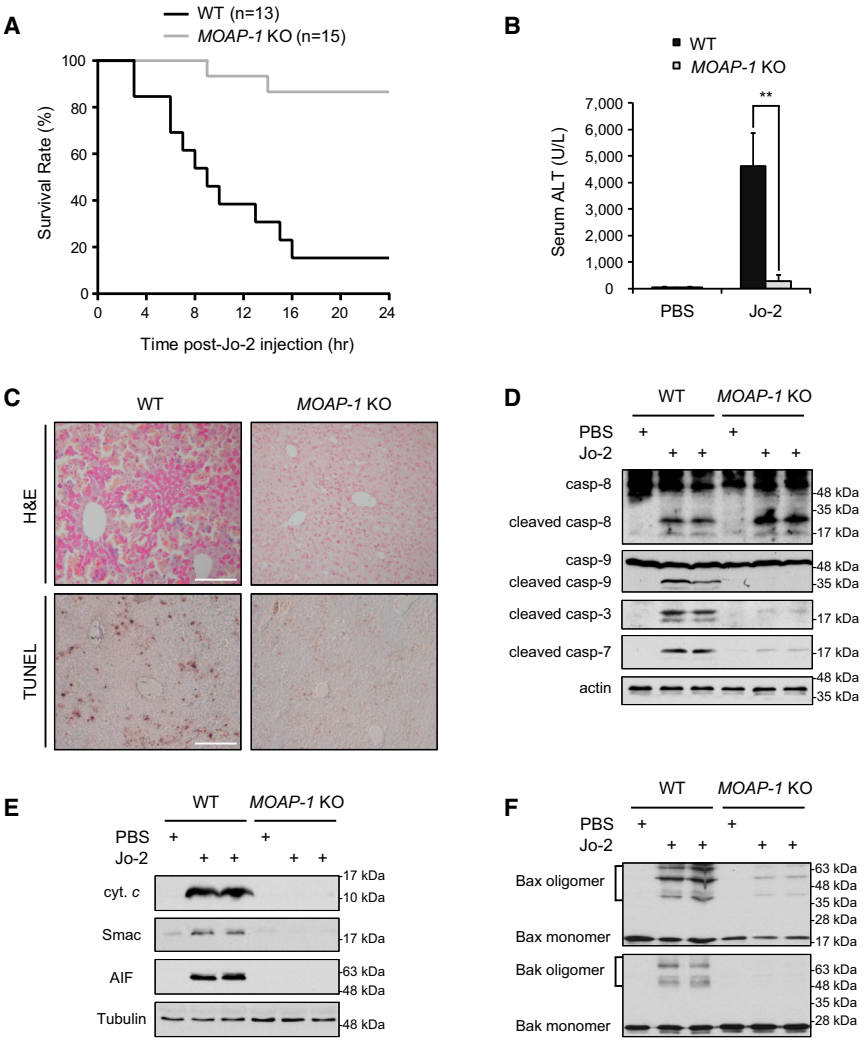

Figure 2. MOAP-1-Deficient Mice Are Resistant to Fas-Induced Lethality and Hepatocellular Apoptosis

(A) Kaplan-Meier survival curve of the 10- to 12-week-old WT and *MOAP-1* KO mice injected with the Fas-activating antibody, Jo-2 (0.25 μg/g).

(B) Degree of liver injury in the WT and *MOAP-1* KO mice mediated by in vivo Fas activation was assessed by measuring serum alanine aminotransferase (ALT) levels. WT and *MOAP-1* KO mice were injected with PBS (n = 3 per group) or Jo-2 (0.25 μg/g) (n = 6 for WT and n = 8 for *MOAP-1* KO) and the mice were euthanized at 200 min post-injection. Error bars represent SEM. **p < 0.01, Student's t test.

(C) H&E staining (upper panels) and TUNEL labeling (lower panels) of liver sections from the mice subjected to Jo-2 treatment as in (B). Scale bar, 100 μm.

(D) MOAP-1 is required for efficient activation of caspase(casp)-3/7/9 but not casp-8. Western analysis was conducted to assess the cleavage of casp-8, casp-9, casp-3, and casp-7 in total liver lysates from the WT and *MOAP-1* KO mice sacrificed at 200 min post-PBS or post-Jo-2 injection (0.25 μg/g). Actin was probed to confirm uniform loading.

(E) Cytosolic fraction of the liver lysates from mice subjected to Jo-2 treatment as in (D) was analyzed by western blotting for cytochrome *c* (cyt. *c*), Smac, and AIF. Tubulin was used as an internal loading control.

(F) Bax and Bak oligomerization in the mitochondrial lysates prepared from livers of mice subjected to Jo-2 treatment as in (D) were determined via crosslinking using bismaleimidohexane (BMH).

See also Figure S1 and Table S1.

induction appeared unaffected in the absence of MOAP-1, we examined levels of tBid in the mitochondrial and cytosolic fractions of lysates prepared from livers and hepatocytes of the WT and *MOAP-1* KO mice injected with Jo-2 and treated with Jo-2 plus Act.D, respectively. Interestingly, while generation of tBid upon Fas activation was not affected, mitochondrial accumulation of tBid was markedly hindered in the absence of MOAP-1 (Figures 3A and 3B). When the time of analysis was extended to 6 hr post-injection, similar observation was noted (Figure S2A). To determine whether the requirement of MOAP-1 for facilitating mitochondrial recruitment of tBid is applicable to other apoptotic stimuli and cell types, we examined mitochondrial localization of tBid in HCT116 human colorectal cancer cells and MEFs upon treatments with apoptotic stimuli including TNF-related apoptosis-inducing ligand (TRAIL), thapsigargin (THA), Jo-2 plus Act.D, or simply by overexpressing tBid. In HCT116 cells treated with TRAIL or THA, knocking down MOAP-1 did not affect production of tBid, but accumulation of mitochondrial tBid as well as caspase-3 activation were inhibited (Figures 3C, S2B, and S2C). In the *MOAP-1* KO MEFs, diffuse staining pattern of Myc-tBid in the cytosol were seen, which was distinct from the punctate pattern associated with mitochondria staining in the WT MEFs (Figure 3D). Moreover, in the MOAP-1-deficient MEFs treated with Jo-2 plus Act.D, tBid also failed to accumulate at mitochondria (Figures 3E and S2E). Importantly, re-expression of MOAP-1 in the MOAP-1-deficient MEFs restored the mitochondrial recruitment of tBid (Figure 3E). Similarly, recombinant tBid (rtBid) associated avidly only with the isolated mitochondria prepared from the WT, but not *MOAP-1* KO livers (Figure 3F). Addition of recombinant GST-MOAP-1, but not GST, to the MOAP-1-deficient mitochondria, was sufficient to restore the association (Figure 3F). Furthermore, in HCT116 cells treated with TRAIL or in MEFs treated with Jo-2 plus Act.D, higher levels of mitochondrial tBid were detected in the cells overexpressing MOAP-1 (Figures S2D and S2F).

MOAP-1 Does Not Serve as a Direct Mitochondrial Receptor for tBid

As MOAP-1 deficiency recapitulates loss of MTCH2 in preventing tBid recruitment to mitochondria (Zaltsman et al., 2010), we hypothesized that MOAP-1 may also act as a mitochondrial receptor for tBid. To test the idea, we expressed Flag-MOAP-1

in the WT MEFs and subjected the cells to treatment with Jo-2 plus Act.D, followed by co-immunoprecipitation (coIP) assay. Cells expressing Flag-MTCH2 were included as a positive control. In agreement with the previous observation that apoptotic stimuli upregulate MOAP-1 (Fu et al., 2007), higher levels of MOAP-1 was detected upon Fas induction (Figure 4A). In contrast to MTCH2 that associates with tBid avidly, no notable interaction between MOAP-1 and tBid was detected (Figure 4A). Similarly, in HCT116 cells expressing Flag-MOAP-1 or Flag-MTCH2 treated with TRAIL, only association of MTCH2 with tBid could be detected (Figure 4B). Moreover, in HEK293T cells overexpressing Myc-tBid and Flag-MTCH2 or Flag-MOAP-1, only weak interaction was noted between MOAP-1 and tBid (Figure 4C). Likewise, in vitro analysis also failed to detect any binding between tBid and GST-MOAP-1, even though robust tBid/Bcl-xL association was detected readily (Figure 4D), as previously reported (Cheng et al., 2001; Li et al., 1998; Willis et al., 2007).

MTCH2 Requires the Presence of MOAP-1 to Function as a Mitochondrial Receptor for tBid

While MOAP-1 does not seem to facilitate mitochondrial recruitment of tBid by serving as its receptor, it may modulate the activity of MTCH2. Interestingly, in mice injected with Jo-2, MTCH2/tBid interaction could only be detected in liver lysates prepared from the WT but not *MOAP-1* KO mice (Figures 5A and S3A). Furthermore, knockdown of MOAP-1 in the HEK293T cells substantially reduced the interaction between Myc-tBid and endogenous MTCH2 (Figure 5B). In vitro binding assay by incubating total cell lysates with increasing concentrations of recombinant tBid also demonstrated a dose-dependent binding of rtBid to MTCH2 in the total cell lysates prepared from the WT but not the *MOAP-1* KO MEFs (Figures 5C). In MEFs treated with Jo-2 plus Act.D, MTCH2/tBid interaction was detected only in the WT MEFs (Figures 5D and S3B), and the interaction was further enhanced by overexpressing MOAP-1 (Figures 5D and S3C). Moreover, MTCH2/tBid interaction in the *MOAP-1* KO MEFs was restored by re-expressing MOAP-1 (Figure 5D).

MOAP-1 Associates with MTCH2 and Fas Activation Promotes Their Interaction

To understand how MOAP-1 regulates MTCH2/tBid interaction, we studied the functional relationship between MOAP-1 and MTCH2. First, we investigated whether MOAP-1 regulates the abundance and mitochondrial localization of MTCH2 protein. MTCH2 protein levels in the mitochondrial lysates from livers (Figures S2A and S4F) and its mitochondrial localization patterns in MEFs (Figures S2E and S4G) all appeared unaffected by the absence of MOAP-1. To test whether MOAP-1 interacts with MTCH2, we carried out coIP analysis. Interaction between MOAP-1 and MTCH2 was readily detected in mouse liver lysates (Figure 6A). In HEK293T cells transfected with Flag-MTCH2 and Myc-MOAP-1, MTCH2/MOAP-1 interaction could be demonstrated in the coIP assays using either anti-Flag antibody (Figure S4A, upper panels) or anti-Myc antibody (Figure S4A, lower panels). To confirm the interaction via an alternative approach, we performed the Duolink proximity ligation assay (PLA) in the MCF-7 cells. As working anti-MOAP-1 antibody for detecting endogenous MOAP-1 by immunofluorescence is not yet available, we expressed Flag-MOAP-1 in these cells and performed PLA using anti-Flag and anti-MTCH2 antibodies. As a negative control, we included MTCH2 knockdown cells (Figures 6B and S4B). Due to low transfection efficiency (~15%), Duolink signals, which represent the MOAP-1/MTCH2 interaction, were detected on average two cells per view only in the Scramble but not MTCH2 small hairpin RNA (shRNA)-expressing cells (Figure 6B). The signals from the Duolink system co-localized with MitoTracker (Figure S4C), suggesting MOAP-1 associates with MTCH2 at mitochondria. Interestingly, upon Fas activation, MOAP-1/MTCH2 interaction was significantly enhanced in the mouse livers (Figures 6C and S4D) and MEFs (Figure S4E). MOAP-1/MTCH2 interaction was also enhanced by other apoptotic inducers, TRAIL and THA, in HCT116 cells (Figure 6D).

Interaction of MOAP-1 with MTCH2 Is Necessary for Mediating the Mitochondrial Receptor Function of MTCH2 for tBid

To address the question whether MOAP-1/MTCH2 interaction is required for mediating mitochondrial recruitment of tBid, we began by using mutagenesis analysis to identify regions of amino acids (aa) of MOAP-1 that are required for the interaction with MTCH2. A series of deletion mutants with 8–16 aa residues of MOAP-1 removed at a time, were tested for their ability to associate with MTCH2 (Figure S5A). The regions spanning aa 1–119 (Figure S5B), aa 163–252 (Figures S5D and S5E) and aa 294–351 (Figure S5E) were dispensable for the binding of MOAP-1 to MTCH2, but aa 120–162 and aa 253–293 regions of MOAP-1 were necessary for mediating the interaction (Figures S5C and S5E). Deletion mutants of MOAP-1 with only aa 115–255 or aa 255–351 remaining, however, were unable to bind MTCH2, although the aa 115–351 mutant, with the N-terminal (aa 1–114) domain deleted, interacted efficiently with MTCH2 (Figure S5F), providing further evidence that both regions (aa 120–162 and aa 253–293) of MOAP-1 are required for establishing essential contact points for stable binding with MTCH2. We reported previously that aa 120–127 of MOAP-1 shares similarities with the BH3 motif present among members of the Bcl-2 family including Bid (Tan et al., 2001). The amino acid sequence in this region is 57% conserved among the MOAP-1 protein in several mammalian species (Figure 7A). To define the key residues in this region required for the interaction with MTCH2, several substitution mutants were evaluated (Figure 7A). Mutations of the L120E and GHE/VLA but not the H126E in MOAP-1 were shown to abolish its interaction with Bax (Tan et al., 2001). Interestingly, the L120E and GHE/VLA, but not H126E, mutants of MOAP-1 also failed to interact with MTCH2, even though their ability in associating with Bcl-xL (Figure 7B) and localization to mitochondria (Figure 7D) remained unaltered. In MCF-7 cells expressing Flag-tagged MOAP-1 or the L120E mutant, the Duolink signals were detectable only in cells expressing MOAP-1, but not the L120E mutant (Figure S5G).

Next, we evaluated MOAP-1, the L120E, GHE/VLA, or H126E mutant for their ability in rescuing the defect in MTCH2/tBid association in the *MOAP-1* KO MEFs. MOAP-1 and the H126E, but

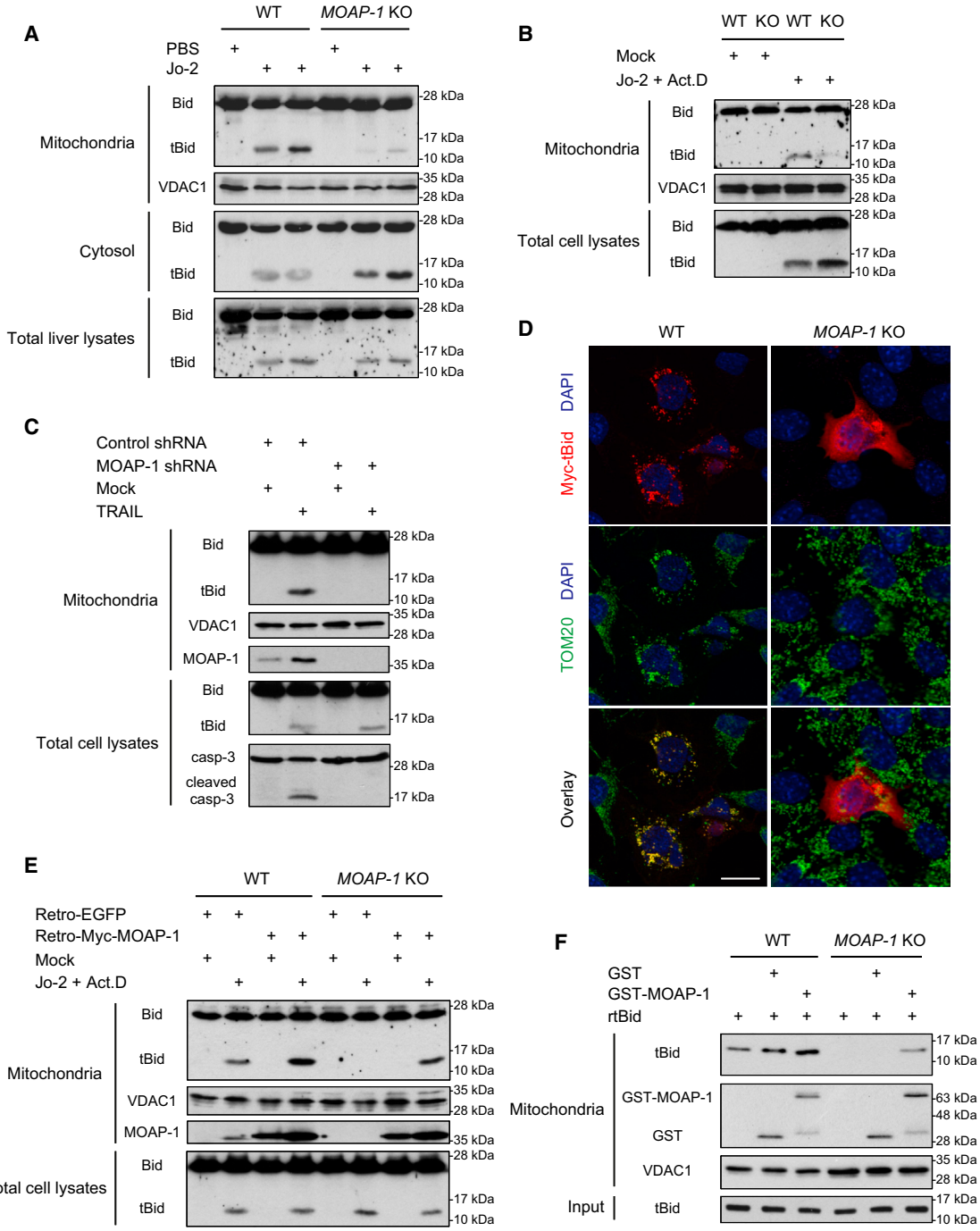

Figure 3. MOAP-1 Is Required for Mitochondrial Accumulation of tBid

(A) Western analysis of tBid in the mitochondria, cytosol, and total lysates prepared from livers of the WT and *MOAP-1* KO mice sacrificed at 200 min post-PBS or post-Jo-2 injection (0.25 μg/g). VDAC1 was probed as mitochondrial loading control.

(B) Hepatocytes prepared from the 10-week-old WT and *MOAP-1* KO mice were mock-treated or with Jo-2 (1 μg/ml) plus actinomycin D (Act.D) (0.1 μg/ml) for 4 hr before total and mitochondrial lysates were prepared for western analysis.

(C) HCT116 cells were transfected with control or MOAP-1 shRNA. Two days post-transfection, cells were mock-treated or with TNF-related apoptosis-inducing ligand (TRAIL) (100 ng/ml) for 3 hr and analyzed as in (A).

(D) tBid co-localizes with TOM20 (an OMM protein) only in the WT, but not *MOAP-1* KO MEFs. WT and *MOAP-1* KO MEFs were transiently transfected with Myc-tBid and subjected to immunofluorescence with anti-Myc (red), anti-TOM20 (green), and DAPI (blue), followed by confocal analysis. Data from a single focal plane is shown. Scale bar, 20 μm.

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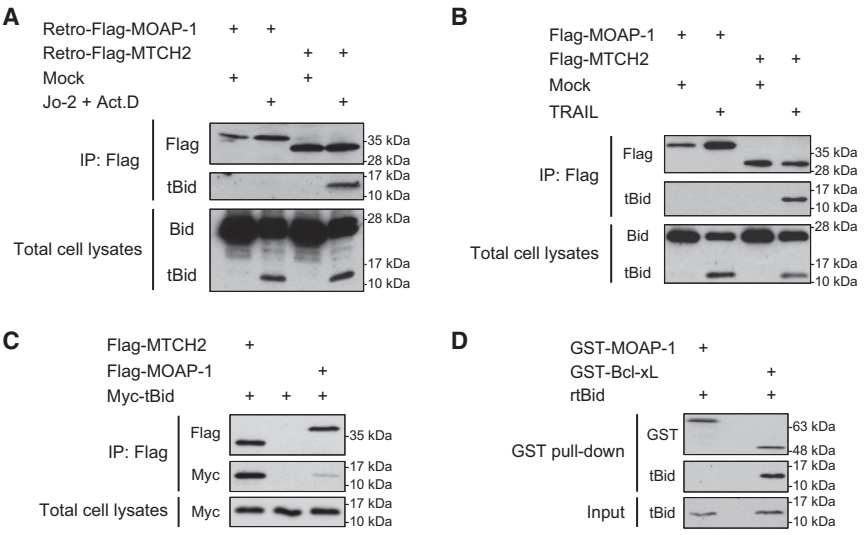

Figure 4. MTCH2, Not MOAP-1, Is a Mitochondrial Receptor for tBid

(A) MTCH2, not MOAP-1, associates with tBid. WT MEFs were infected with retrovirus encoding Flag-MOAP-1 (Retro-Flag-MOAP-1) or Flag-MTCH2 (Retro-Flag-MTCH2) for 24 hr. Cells were then mock-treated or with Jo-2 (1 μg/ml) and Act.D (0.1 μg/ml) for 16 hr before preparing the total lysates for immunoprecipitation (IP) analysis.

(B) HCT116 cells were transfected with Flag-MOAP-1 or Flag-MTCH2 for 24 hr. Cells were then mock-treated or with TRAIL (100 ng/ml) for 3 hr before preparing the total lysates for IP analysis.

(C) HEK293T cells were transfected with Myc-tBid and Flag-MTCH2 or Flag-MOAP-1. Sixteen hours post-transfection, total cell lysates were subjected to IP, followed by western blotting.

(D) MOAP-1 does not bind tBid in vitro. GST-MOAP-1 or GST-Bcl-xL (2 μg/ml) was incubated with 2 nM of rtBid for 2 hr before subjecting the mixtures to GST pull-down. Eluates from the pull-down and input (1% of the pre-pull-down mixtures) were analyzed by western blotting.

not the L120E or GHE/VLA mutant, restored the MTCH2/tBid interaction in the *MOAP-1* KO MEFs (Figure 7C). As mitochondrial accumulation of tBid is facilitated through binding to MTCH2, its recruitment to mitochondria was indeed restored in the MOAP-1-deficient MEFs by re-expressing MOAP-1 and the H126E, but not the L120E or GHE/VLA mutant or EGFP (Figure 7D). Moreover, in the *MOAP-1* KO mice re-expressing MOAP-1 but not the L120E mutant or EGFP, MTCH2/tBid interaction in liver upon in vivo Fas activation was re-established (Figure 7E). Importantly, mitochondrial accumulation of tBid in livers and hepatocellular apoptosis in these KO mice were also effectively restored by re-expressing MOAP-1, but not the L120E mutant or EGFP (Figure 7F).

DISCUSSION

Fas-mediated activation of mitochondrial apoptosis signaling could be regulated at multiple steps, including activation of Fas receptor (Peter et al., 2007), assembly of death-inducing signaling complex (DISC) (Kischkel et al., 1995), induction of caspase-8 (Muzio et al., 1996), processing of Bid to tBid (Li et al., 1998; Luo et al., 1998), mitochondrial accumulation of tBid (Gross et al., 1999; Zaltsman et al., 2010), tBid-mediated activation and oligomerization of Bax and Bak (Kim et al., 2009; Lovell et al., 2008; Wei et al., 2000, 2001) and releasing of cytochrome *c*, Smac, and AIF from mitochondria into cytosol (Du et al., 2000; Li et al., 1997; Susin et al., 1999; Verhagen et al., 2000). Protein-protein interaction is a key regulatory paradigm among members of Bcl-2 family as both activation and inhibition of their activities are frequently controlled by the proteins they interact with (Czabotar et al., 2014). In this study, we uncovered a critical regulatory mechanism in the Fas-Bid signaling axis. Our data demonstrate that MOAP-1 is a crucial component of Fas signaling in liver. Recent data from Zaltsman et al. (2010) suggest that mitochondrial accumulation of tBid is not a spontaneous process, but is dependent on MTCH2 that serves as a receptor-like protein for tBid. Surprisingly, our data show that the receptor function of MTCH2 in binding tBid requires interaction with MOAP-1. It is likely that association of MOAP-1 with MTCH2 induces conformation change in MTCH2 allowing it to bind tBid avidly. While MOAP-1 appears to interact with MTCH2 in resting cells, Fas and other apoptotic stimuli (that were able to induce production of tBid) were shown to have a significant effect for enhancing their interaction. The conformation states of MOAP-1 and MTCH2 in stimulated cells may undergo further changes in MOAP-1, MTCH2, or both, and such changes may serve to facilitate binding of tBid to MTCH2, thereby recruitment of tBid to mitochondria. Further investigation via structural analysis is necessary to gain deeper understanding of the conformation states of MOAP-1/MTCH2 complex in the resting and apoptotic cells.

Because apoptotic stimuli also promote MOAP-1/Bax interaction (Tan et al., 2005), it is conceivable that protein complexes containing unknown and known factors including tBid, MTCH2, MOAP-1, or Bax may exist at different stages of the signaling process to coordinate sequential molecular events leading to

(E) Mitochondrial recruitment of tBid in the MOAP-1-deficient MEFs is restored by re-expressing MOAP-1. WT and *MOAP-1* KO MEFs were infected with retrovirus encoding enhanced green fluorescence protein (Retro-EGFP) or Myc-tagged MOAP-1 (Retro-Myc-MOAP-1) for 24 hr before subjecting the MEFs to mock treatment or with or Jo-2 (1 μg/ml) and Act.D (0.1 μg/ml) for 16 hr. Total and mitochondrial lysates were then prepared from the MEFs for western analysis.

(F) MOAP-1 is required for in vitro association of tBid with mitochondria. Mitochondria prepared from the WT and *MOAP-1* KO mouse livers were incubated with recombinant tBid (rtBid) (1 nM) and GST (500 ng/ml) or GST-MOAP-1 (100 ng/ml) at 30°C for 30 min. Because GST-MOAP-1 is readily accumulated at mitochondria, a higher amount of GST compared to GST-MOAP-1 was added to achieve comparable levels at mitochondria. Mitochondria were subsequently pelleted and washed to remove unbound proteins before being subjected to western analysis. One percent of the pre-binding mixtures were included as the input to ensure equal amount of tBid added to each sample.

See also Figure S2.

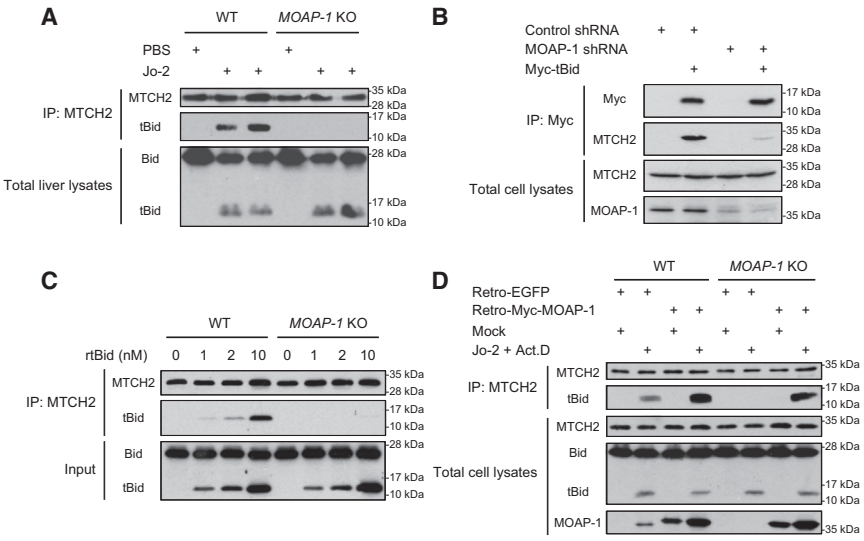

Figure 5. Association of tBid with MTCH2 Requires the Presence of MOAP-1

(A) tBid is unable to associate with MTCH2 in the absence of MOAP-1. Total liver lysates prepared from WT and *MOAP-1* KO mice at 200 min post-PBS or post-Jo-2 injection (0.25 μg/g) were subjected to IP, followed by western analysis.

(B) Association of tBid with MTCH2 is substantially weakened in the MOAP-1 knockdown cells. HEK293T cells were transfected with the control or MOAP-1 shRNA. Forty-eight hours later, cells were transfected with Myc-tBid for 16 hr before subjecting the total cell lysates to IP analysis and western blotting.

(C) MOAP-1 is required for in vitro association of tBid with MTCH2. Total cell lysates of WT or *MOAP-1* KO MEFs were incubated with the indicated concentrations of rtBid for 2 hr before subjecting to IP, followed by western analysis. Inputs (1% of the pre-IP mixtures) were shown to document the increasing amounts of rtBid added to the lysates.

(D) Association of tBid with MTCH2 in MOAP-1-deficient MEFs is restored by re-expressing MOAP-1. WT and *MOAP-1* KO MEFs were infected with Retro-EGFP or Retro-Myc-MOAP-1 for 24 hr before subjecting the MEFs to mock treatment or with Jo-2 (1 μg/ml) and Act.D (0.1 μg/ml) for 16 hr, followed by IP and western blotting.

See also Figure S3.

oligomerization of Bax. Failure in detecting MOAP-1/tBid association raises the question of the presence of a stable protein complex containing MOAP-1, MTCH2, tBid, and Bax. However, protein signaling complexes involving members of Bcl-2 family such as tBid and Bax have also been shown to exist in transient states or in a "hit/kiss-and-run" manner (Chen et al., 2015; Czabotar et al., 2014; Lovell et al., 2008; Shamas-Din et al., 2014; Willis et al., 2007), which create technical challenges for detecting and identifying individual component in the protein complexes with conventional biochemical techniques. Interestingly, the BH3-like motif in MOAP-1 required for binding Bax (Tan et al., 2001) is also found to be necessary for mediating interaction with MTCH2, raising the question whether the two proteins bind to similar regions of MOAP-1. Engagement of Bax in the apoptotic signaling process is known to occur in a sequential manner, from conformational change, translocation to mitochondria, insertion into OMM, to oligomerization (Czabotar et al., 2013; Kim et al., 2009; Lovell et al., 2008). Inhibition of Bax and Bak oligomerization in the absence of MOAP-1 could be due to deficiency in activation of upstream signaling facilitated by mitochondrial tBid, but it may also be possible that MOAP-1 interaction with Bax during apoptosis or in co-operation with tBid/Bax plays a role in promoting Bax oligomerization. Interestingly, MOAP-1 deficiency appears to be more protective than MTCH2 deficiency from the Fas-induced lethality (Zaltsman et al., 2010). Furthermore, in contrast to the *MOAP-1* KO MEFs (Figure 1G), *MTCH2* KO MEFs were not protected against Bax-induced apoptosis (Zaltsman et al., 2010), providing support to the argument that MOAP-1 may regulate the Fas apoptotic signaling through both upstream (mitochondrial targeting of tBid) and downstream (oligomerization of Bax) signaling events.

Martinou and colleagues (Raemy et al., 2016) have recently confirmed that MTCH2 is critical for tBid recruitment in HeLa cells, but in HCT116 cells, MTCH2 and cardiolipin appear to play a redundant role in facilitating mitochondrial recruitment of tBid. However, our data demonstrated that depletion of MOAP-1 is sufficient to abolish mitochondrial accumulation of tBid and inhibit apoptosis in several type II cells including hepatocytes and HCT116 cells (Figures 3A–3C, S1B, S2B, and S2C). While this observation offers evidence to support the critical role of MOAP-1 in promoting the receptor function of MTCH2 for tBid, additional mechanisms in facilitating mitochondrial accumulation of tBid and Bax activation such as regulation of cardiolipin levels or its transport across the mitochondrial membranes during apoptosis, which may also be dependent on the presence of MOAP-1, cannot be ruled out.

In contrast to the *Bid* KO (Yin et al., 1999) and *MOAP-1* KO mice, which display no developmental or fertility defects, *MTCH2* KO is embryonic lethal (Zaltsman et al., 2010), underscoring its essential role during embryonic development. MTCH2 has recently been shown to play a role in regulating mitochondrial oxidative phosphorylation (OXPHOS) and mitochondrial size/volume in hematopoietic stem cells and muscles (Buzaglo-Azriel et al., 2016; Maryanovich et al., 2015). It remains to be studied whether MOAP-1 can co-operate with MTCH2 to participate in the regulation of metabolism and mitochondrial OXPHOS and size/volume.

Identifying the role of MOAP-1 in regulating Fas signaling in mitochondrial pathway of apoptosis would open up interesting opportunities for investigating the roles of MOAP-1 in the development of human liver diseases underpinned by Fas signaling mechanism such as fulminant hepatitis, cirrhosis, and Wilson's disease (Guicciardi and Gores, 2005; Peter et al., 2007). Owing to the potentially druggable property of MOAP-1 by being a direct substrate of the UPS (Fu et al., 2007), MOAP-1 can be explored as a therapeutic target for treatment of certain liver diseases associated with dysfunction of Fas signaling.

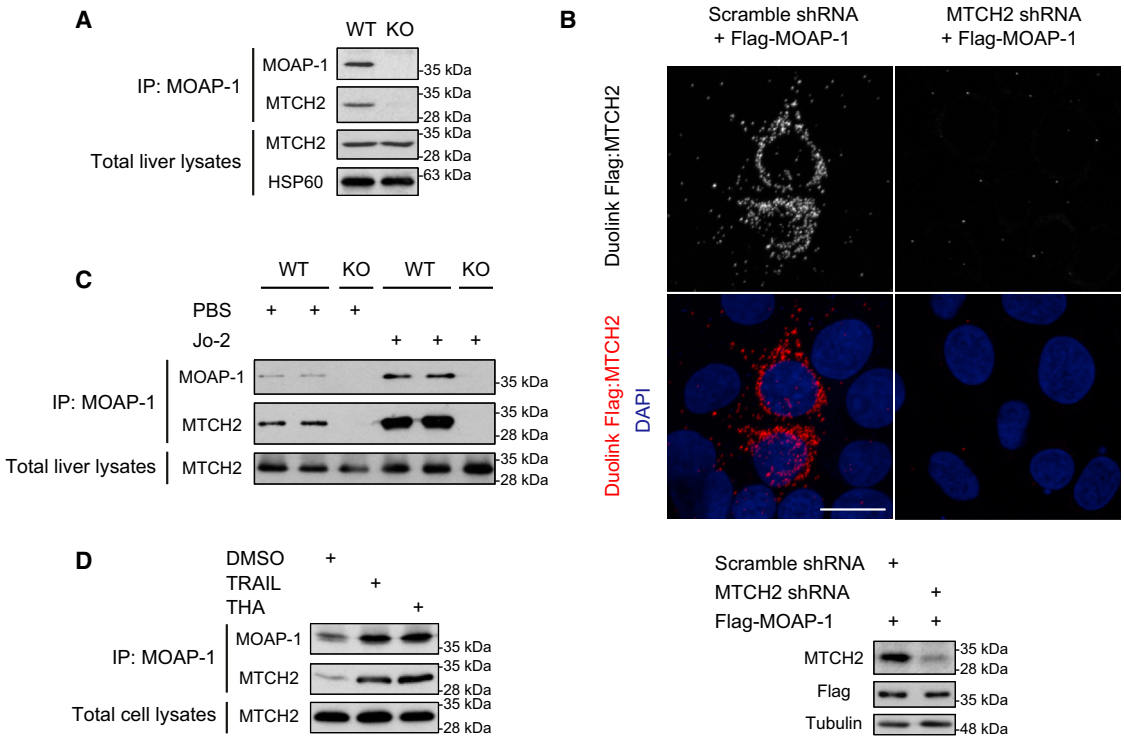

Figure 6. MOAP-1 Associates with MTCH2 and the Interaction Is Enhanced by Fas Activation

(A) MOAP-1 associates with MTCH2 in liver. Total lysates prepared from livers of the WT and *MOAP-1* KO mice were subjected to IP, followed by western analysis. HSP60 was probed to ensure equal input for IP.

(B) Interaction between MOAP-1 and MTCH2 is demonstrated by the proximity ligation assay (PLA). MCF-7 cells expressing Scramble or MTCH2 shRNA were transfected with Flag-MOAP-1 for 24 hr before subjecting the cells to the PLA via the Duolink method, followed by confocal microscopic analysis. Maximal intensity projection of a z stack containing twenty 0.5-μm thick focal planes is shown (top panels). Nuclei were stained with DAPI (in blue). Scale bar, 20 μm. Total cell lysates of MCF-7 cells expressing Scramble or MTCH2 shRNA and transfected with Flag-MOAP-1 were analyzed by western blotting to confirm comparable Flag-MOAP-1 expression and efficient MTCH2 knockdown (bottom panels).

(C) Fas activation enhances MOAP-1/MTCH2 interaction. Total liver lysates prepared from WT and *MOAP-1* KO mice at 200 min post-PBS or post-Jo-2 (0.25 μg/g) were subjected to IP, followed by western analysis.

(D) Apoptotic stimuli enhance the MOAP-1/MTCH2 interaction. HCT116 cells were treated with DMSO, TRAIL (100 ng/ml) for 3 hr or THA (10 μM) for 12 hr. Total cell lysates were prepared for IP and western analysis.

See also Figure S4.

EXPERIMENTAL PROCEDURES

Mice

MOAP-1 KO mice were first generated in the 129 genetic background. The targeting vector was designed to replace the exon 2 of *MOAP-1*, which contains the entire coding region of the gene, with the neomycin cassette via homologous recombination. The linearized targeting vector was introduced into murine embryonic stem cells via electroporation. Southern blot analysis was carried out to confirm homologous recombination within the *MOAP-1* gene locus in the neomycin-resistant clones before blastocyst injection as previously described (Yin et al., 1999). The mutant allele in the *MOAP-1* locus was confirmed in the first generation of *MOAP-1*$^{+/-}$ mice derived from the chimeras by southern blotting and the subsequent genotyping of mice was performed using PCR-based assay as described (Zaltsman et al., 2010). Heterozygous mice were backcrossed with the C57/BL6 strain for more than ten generations. Both wild-type and *MOAP-1* KO mice used for experiments were from the same heterozygous founders in pure C57/BL6 genetic background and were in-bred for no more than six generations. Mice were weaned at the age of 21 days and maintained on the Chow diet and 12 hr light/12 hr dark cycle at 23°C.

For the in vivo paradigm of Fas activation, 10- to 12-week-old *MOAP-1* knockout or wild-type control male mice were injected with anti-Fas antibody (clone Jo-2, BD Biosciences) via tail vein at the dose of 0.25 μg/g body weight. Animal survival was monitored every hour for 24 hr. For tissue harvest, mice were euthanized at the indicated times post-injection by CO_2 asphyxiation. All animal procedures were carried out with evaluation and oversight from the Institutional Animal Care and Use Committee (IACUC) and conducted in compliance with the National Advisory Committee for Laboratory Animal Research (NACLAR) guidelines.

Mitochondria Isolation

Mitochondria-enriched heavy membrane fraction was isolated as previously described (Sukumaran et al., 2010). Briefly, ~30 mg of liver tissues (or near confluent 15 cm dish of MEFs or 10 cm dish of hepatocytes or HCT116 cells) were homogenized in 1 ml of mitochondrial isolation buffer (50 mM HEPES-KOH [pH 7.5], 320 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail) by passing through a Dounce tissue grinder eight times. Cell suspension was disrupted by 20 expulsions through a 25G needle and centrifuged at 800 × *g* for 10 min to remove cell debris and nuclei. The supernatant was then centrifuged at 10,000 × *g* for 20 min to pellet the mitochondria. The supernatant was collected as the cytosolic fraction.

Immunoprecipitation

Immunoprecipitation (IP) assays were performed as previously described (Sukumaran et al., 2010). Liver tissues (~30 mg) were homogenized in 1 ml IP lysis

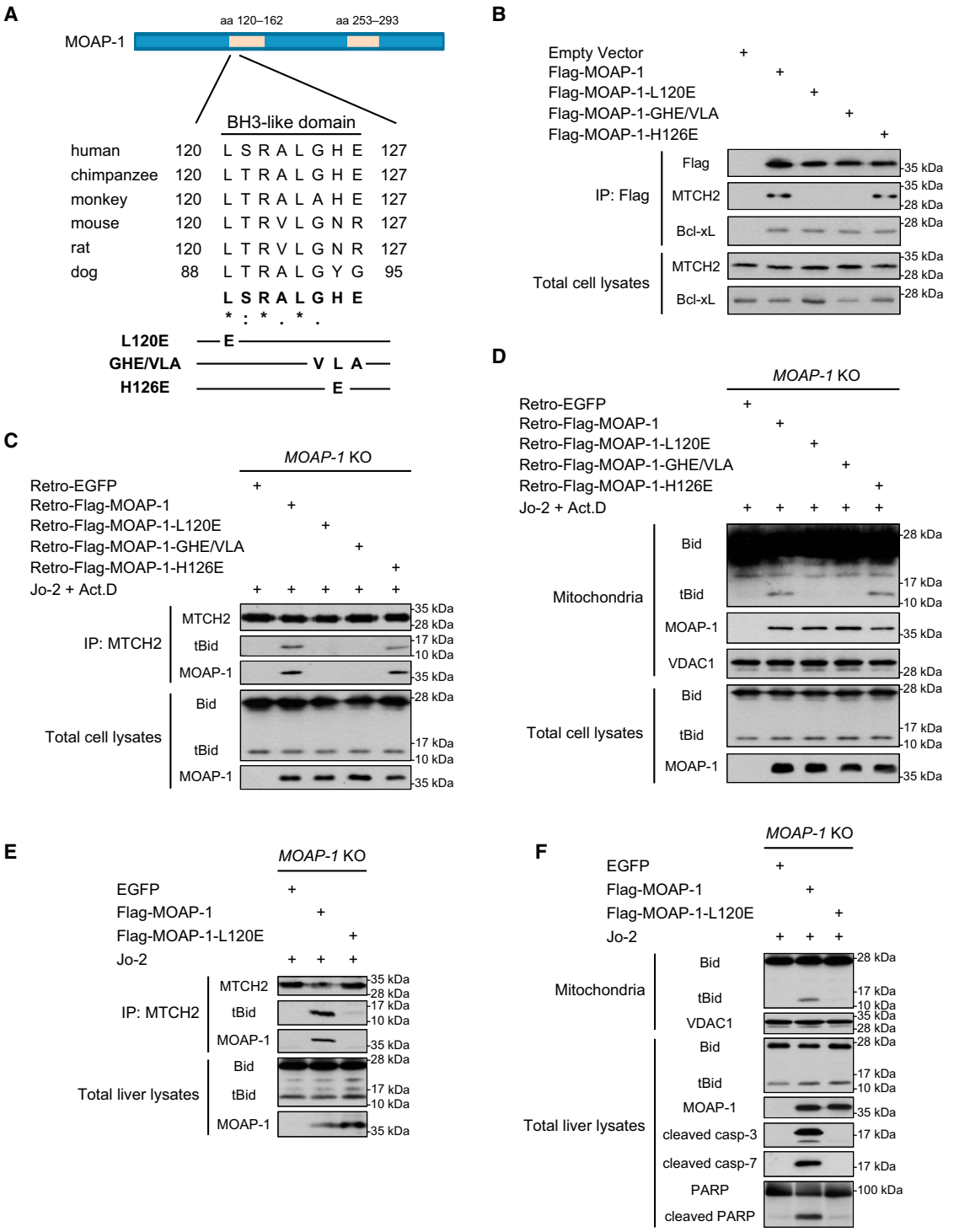

Figure 7. Association of MOAP-1 with MTCH2 Is Required for Engaging the Receptor Function of MTCH2 for tBid

(A) Illustration depicting the two regions of MOAP-1 required for mediating interaction with MTCH2. ClustalW2 alignment of the BH3-like domain of MOAP-1 among the indicated mammalian species. *Indicates the positions that have a single, fully conserved residue; whereas (:) and (.) indicate conservation between groups of strongly similar and weakly similar properties, respectively. The coordinates of the substitution mutants, L120E, GHE/VLA, and H126E are shown.

(B) The L120E and GHE/VLA, but not H126E, mutants of MOAP-1 within the BH3-like domain fail to interact with MTCH2. HEK293T cells were transfected with empty vector, Flag-tagged MOAP-1 or the mutants (L120E, GHE/VLA, and H126E). Total cell lysates were subjected to IP, followed by western analysis.

(legend continued on next page)

buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM $Na_2H_2PO_4$, 10% glycerol and protease inhibitor cocktail) using a Dounce tissue grinder and incubated on ice for 2 hr. For cells, they were scrapped off in 1 ml IP lysis buffer and incubated on ice for 30 min. Tissue/cell lysates were centrifuged at 21,000 × *g* for 20 min to remove cell debris, and 5 mg of the lysates were incubated at 4°C overnight with Protein-A agarose beads (Roche) pre-bound with 5–10 μg of antibodies. Beads were washed four times with IP lysis buffer and eluted in 1× sample buffer.

Statistical Analysis

Statistical differences between two sets of data were analyzed in GraphPad Prism using Student's t test (unpaired, non-parametric) or χ^2 test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.068.

AUTHOR CONTRIBUTIONS

V.C.Y. conceived and supervised the project. C.T.T. and V.C.Y. wrote the manuscript. C.T.T. and V.C.Y. designed and C.T.T. performed most of the experiments. Q.-L.Z. and C.-D.Y. designed and Q.-L.Z. performed the animal survival and liver injury experiments. Y.-C.S. contributed to the analysis of Fas signaling cascade in liver. N.Y.F. contributed to the generation of the MOAP-1 KO mice and provided technical advice throughout the work. H.-C.C. performed the flow cytometry-based cell death analysis. R.N.T. participated in generating the mouse colonies for experiments. S.K.S. carried out the in vitro cytochrome c release experiment. Y.-J.T. raised the mouse polyclonal anti-MOAP-1 antibody. K.S. and S.B. made contributions in helping with the experimental design and providing technical advice. All authors reviewed the manuscript.

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(C) MOAP-1 and its H126E mutant, but not its MTCH2-binding defective mutants, restore MTCH2/tBid interaction in the MOAP-1-deficient MEFs. *MOAP-1* KO MEFs were infected with retrovirus encoding MOAP-1 or the indicated mutants for 24 hr, before treatment with Jo-2 (1 μg/ml) plus Act.D (0.1 μg/ml) for 16 hr. Total cell lysates were prepared and subjected to IP and western blotting.
(D) MOAP-1 and the H126E mutant, but not the MTCH2 binding incompetent mutants, L120E or GHE/VLA, are able to restore mitochondrial recruitment of tBid in MOAP-1-deficient cells. *MOAP-1* KO MEFs were infected with retrovirus encoding MOAP-1 or the indicated mutants for 24 hr, followed by treatment with Jo-2 plus Act.D as in (C). Total and mitochondrial lysates were processed for western analysis.
(E) MOAP-1, but not its L120E mutant, restored the MTCH2/tBid interaction upon Fas activation in the MOAP-1-deficient mice. *MOAP-1* KO mice were injected with plasmids encoding EGFP, Flag-tagged MOAP-1, or the L120E mutant. Twenty-four hours later, mice were injected with Jo-2 (0.25 μg/g) and euthanized at 200 min post-injection. Total liver lysates from the WT and *MOAP-1* KO mice were subjected to IP, followed by western blotting.
(F) Mitochondrial recruitment of tBid and hepatocellular apoptosis were restored in the MOAP-1-deficient mice by re-expressing MOAP-1, but not the L120E mutant. *MOAP-1* KO mice were injected with plasmids encoding EGFP, Flag-MOAP-1, or the L120E mutant. Twenty-four hours later, the mice were subjected to in vivo Fas activation as in (E). Total and mitochondrial lysates were prepared from livers of these mice for western analysis. Hepatocellular apoptosis was assessed by western analysis of the levels of cleaved forms of casp-3 and casp-7 and their substrate PARP.
See also Figure S5.

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