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ACOX1 destabilizes p73 to suppress intrinsic apoptosis pathway and regulates sensitivity to doxorubicin in lymphoma cells.

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Keywords: ACOX1, Lymphoma, Apoptosis, p73, Protein degradation, Drug resistance, Doxorubicin.

ABSTRACT

lymphoma is one of the most curable types of cancer. However, drug resistance is still the main challenge faced in lymphoma treatment. Peroxisomal acyl-CoA oxidase 1 (ACOX1) is the rate-limiting enzyme in fatty acid β-oxidation. Deregulation of ACOX1 has been linked to peroxisomal disorders and carcinogenesis in the liver. Currently, there is no information about the function of ACOX1 in lymphoma. In this study, we found that upregulation of ACOX1 promoted proliferation in lymphoma cells, while downregulation of ACOX1 inhibited proliferation and induced apoptosis. Additionally, overexpression of ACOX1 increased resistance to doxorubicin, while suppression of ACOX1 expression markedly potentiated doxorubicin-induced apoptosis. Interestingly, downregulation of ACOX1 promoted mitochondrial location of Bad, reduced mitochondrial membrane potential and provoked apoptosis by activating caspase-9 and caspase-3 related apoptotic pathway. Overexpression of ACOX1 alleviated doxorubicin-induced activation of caspase-9 and caspase-3 and decrease of mitochondrial membrane potential. Importantly, downregulation of ACOX1 increased p73, but not p53, expression. p73 expression was critical for apoptosis induction which was induced by ACOX1 downregulation. Moreover, overexpression of ACOX1 significantly reduced the stability of p73 protein thereby reducing p73 expression. Thus, our study indicated that suppression of ACOX1 could be a novel and effective approach for the treatment of lymphoma.

INTRODUCTION

Malignant lymphoma is one of the most common hematologic malignancy and comprises 3.37% of all malignancy worldwide (1). The incidence rate of all lymphoma, Non-Hodgkin or Hodgkin lymphoma is approximately 20.3%, 17.37% or 2.92% (2), respectively. The incidence of malignant lymphoma around the world increased at a rate of about 3-4% per year (1). The overall 5-year survival rate for people with Non-Hodgkin or Hodgkin lymphoma is 53.7% or 78.9% (2), respectively. Standard treatments for lymphoma are anthracycline-based combinatorial chemotherapy regimens. Although patients achieve a complete response after initial treatment, a substantial proportion of patient relapse. Relapsed lymphomas are refractory to subsequent treatments and exhibit cross-resistance to a wide variety of anticancer drugs (3, 4). The emergence of acquired chemoresistance poses a challenge in the successful treatment of lymphomas. Thus, understanding the mechanism of drug resistance is important for the successful treatment of lymphoma.

Acyl-CoA oxidase 1 (ACOX1) is a flavoenzyme that catalyzes the first and rate-limiting reaction of the desaturation of very-long-chain acyl-CoAs to 2-trans-enoyl-CoAs and transfers electrons to molecular oxygen to generate hydrogen peroxide (5). ACOX1 deficiency usually causes peroxisomal disorders and has been linked to human diseases, such as pseudoneonatal adrenoleukodystrophy (6). Recent studies indicate that ACOX1 might involve in cancer development. The *ACOX1* knockout mice display growth retardation, infertility, excess very-long-chain fatty acids in the blood, microvesicular steatohepatitis, apoptosis, liver regeneration, and oxidative stress (7, 8). The *ACOX1* knockout mice eventually develop hepatocellular carcinomas (9). These findings suggest that peroxisomal ACOX1 dysfunction contributes to the development of chronic liver disease and hepatocarcinogenesis. Currently, little is known about the role of ACOX1 in other cancers, including lymphoma, and the mechanism behind it remain to be elucidated.

p73, a member of the p53 family of tumor suppressors, shares a remarkable homology in DNA sequence and protein structure with p53 (10). p73 displays a

certain degree of functional overlap with p53 (10). *p53* is usually inactivated in human cancer by point mutations, but the *p73* gene is rarely mutated in human cancers (10). Currently, p73 was found to be suppressed through various mechanisms including epigenetic silencing and post-translational modifications (11). In malignant lymphoma, the mechanisms of epigenetic silencing or deletion are commonly responsible for the inactivation of the *p73* gene (12). However, how post-translational modifications regulate p73 protein stability is not fully elucidated in malignant lymphoma.

In the present study, we examined the role of ACOX1 in lymphoma cells. We found that ACOX1 was important for the proliferation of lymphoma cells. Moreover, overexpression of ACOX1 reduced the sensitivity of lymphoma cells to doxorubicin. While downregulation of ACOX1 significantly enhanced doxorubicin-induced apoptosis. Additionally, ACOX1 participated in the regulation of apoptosis by regulating the activation of caspase-9 and caspase-3, and mitochondrial membrane potential. Importantly, p73, but not p53, was critical for mediating ACOX1 regulated apoptosis response. ACOX1 reduced p73 expression by destabilizing the p73 protein. Our data indicated that ACOX1 could be a novel target for increasing drug sensitivity and improving the treatment of lymphoma.

RESULTS

ACOX1 regulates proliferation and apoptosis.

To evaluate the role of ACOX1 in lymphoma, ACOX1-Flag or ACOX1 shRNA were stably expressed via lentivirus-mediated gene transfer in lymphoma cells. As shown in Fig. 1A and Supplementary Fig. 1A, ACOX1-Flag was overexpressed in lymphoma cells. Overexpression of ACOX1-Flag significantly promoted proliferation in lymphoma cells (Fig. 1B, Supplementary Fig. 1B). While knockdown of ACOX1 expression (Fig. 1C, Supplementary Fig. 1C) markedly suppressed the proliferation of lymphoma cells (Fig. 1D, Supplementary Fig. 1D). These data indicated that ACOX1 was important for regulating lymphoma cell proliferation. We further examined whether ACOX1 might participate in the regulation of apoptosis. As shown in Fig. 1E, F and Supplementary Fig. 1E, F, overexpression of ACOX1-Flag did not cause apoptosis. While downregulation of ACOX1 slightly induced apoptosis as compared with negative control (NC) group (Fig. 1G, H, and Supplementary Fig. 1G, H). To further confirm the effect of ACOX1 on apoptosis, TUNEL assay was performed. Consistently, upregulation of ACOX1 did not induce apoptosis (Fig. 1I, J), while downregulation of ACOX1 induced apoptosis (Fig. 1K, L). These data implied that ACOX1 might participate in the regulation of apoptosis in lymphoma cells.

ACOX1 modulates doxorubicin-induced cytotoxic effect.

Anthracycline-based combinatorial chemotherapy regimens are the most commonly used and efficacious regimens in lymphoma. Due to ACOX1 participated in the regulation of apoptosis (Fig. 1G, H, and Supplementary Fig. 1G, H). We next evaluated whether ACOX1 might affect the cytotoxic effects of doxorubicin. As shown in Fig. 2A, B and Supplementary Fig. 2A, B, 0.6μM doxorubicin treatment alone induced significant apoptosis in lymphoma cells. However, overexpression of ACOX1 markedly reduced doxorubicin caused apoptosis (Fig. 2A, B and Supplementary Fig. 2A, B). Furthermore, our data showed that the combination of ACOX1 downregulation and doxorubicin treatment resulted in greater apoptosis in

lymphoma cells than was achieved with either ACOX1 downregulation or doxorubicin alone (0.1μM) (Fig. 2C, D and Supplementary Fig. 2C, D). To further confirm the above results, the TUNEL assay was performed. Consistently, upregulation of ACOX1 reduced doxorubicin-induced apoptosis (Fig. 2E, F), while downregulation of ACOX1 enhanced doxorubicin-induced apoptosis (Fig. 2G, H). These data indicated that ACOX1 could be a potential target to enhance the cytotoxic effects or prevent drug resistance of doxorubicin.

Downregulation of ACOX1 induced apoptosis through the intrinsic pathway.

We next examined the mechanism of ACOX1 regulated apoptosis. As shown in Fig. 3A and Supplementary Fig. 3A, downregulation of ACOX1 significantly increased the activities of caspase-9 and caspase-3, correlating with increased PARP cleavage. The cleavage of procaspase-8 was not significantly altered after downregulation of ACOX1 (Fig. 3A and Supplementary Fig. 3A). We confirm this by utilizing different caspase inhibitors, including LEHD (inhibition of caspase-9 or caspase-3), DEVD (inhibition of caspase-3) and IETD (inhibition of caspase-8). As shown in Fig. 3B, LEHD or DEVD effectively reversed shACOX1 induced apoptosis. While IETD did not reverse shACOX1 induced apoptosis (Fig. 3B). These data implied that ACOX1 mainly regulated apoptosis through the intrinsic apoptotic pathway. Consistently, the combination of ACOX1 downregulation and doxorubicin treatment induced a more significant increase of activated caspase-9 and caspase-3, and PARP cleavage as comparing with either ACOX1 downregulation or doxorubicin alone (Fig. 3A and Supplementary Fig. 3A). Bad translocation induces the permeabilization of the outer mitochondrial membrane and leads to the reduction of mitochondrial membrane potential (MMP) and cytochrome C releasing (13). Indeed, we observed that downregulation of ACOX1 induced mitochondrial translocation of Bad and pBad (Ser112) (Fig. 3C and Supplementary Fig. 3B), cytoplasmic translocation of cytochrome C (Fig. 3C and Supplementary Fig. 3B), and reduction of MMP (Fig. 3D and Supplementary Fig. 3C). We further evaluated whether overexpression of ACOX1

affected the above effects. As shown in Fig. 3E and Supplementary Fig. 3D, overexpression of ACOX1 reversed doxorubicin-induced activation of caspase-9 and caspase-3 and PARP cleavage. Consistently, overexpression of ACOX1 effectively rescued doxorubicin-induced reduction of MMP (Fig. 3F and Supplementary Fig. 3E).

ACOX1 destabilizes p73 to reduce apoptosis.

We further evaluated the downstream target of ACOX1. We found that p53 expression was not induced by downregulating ACOX1 (Fig. 4A and Supplementary Fig. 4A), while p73 expression was enhanced by downregulating ACOX1 (Fig. 4A and Supplementary Fig. 4A). We also examined the expression of two p53 phosphorylated sites (Ser15 and Ser20) which is required for p53-dependent apoptosis (14). Our data showed that the phosphorylation of these sites did not change when ACOX1 was downregulated (Supplementary Fig. 4B). These data indicated that p73 was critical for ACOX1 regulated apoptosis. We inhibited p73 expression under downregulation of ACOX1 to evaluate apoptosis. Our data showed that downregulation of ACOX1 failed to induce apoptosis when the p73 expression was inhibited (Fig. 4B, C and Supplementary Fig. 4C, D). We further confirmed the regulation of p73 expression by ACOX1. As shown in Fig. 4D and Supplementary Fig. 4E, doxorubicin treatment activated the expression of p53 and p73. Overexpression of ACOX1 abolished doxorubicin-induced p73 expression but not the p53 expression (Fig. 4D and Supplementary Fig. 4E). We also examined the downstream targets of p73. As shown in Fig. 4E and Supplementary Fig. 4F, downregulation of ACOX1 promoted the expression of p73 downstream targets, including p21, Bax, and GADD45 (15). We also found that ACOX1 did not regulate p73 expression at the transcription level (Fig. 4E) or translation level (Supplementary Fig. 4G). We found that MG132 treatment abolished ACOX1 induced reduction of p73 expression (Fig. 4F), suggesting that ACOX1 might regulate p73 expression at the post-translational level. Interestingly, our data showed that overexpression of ACOX1 significantly reduced the protein stability of p73, while downregulation of ACOX1 enhanced the

protein stability of p73 (Fig. 4G, H, and Supplementary Fig. 4H, I). To confirm this, a pulse-chase assay was performed. Consistently, p73 stability was reduced by overexpressing ACOX1 (Supplementary Fig. 4J, K). We also found that ACOX1 interacted with p73 protein (Supplementary Fig. 4L), indicating that ACOX1 might regulate p73 stability via protein interaction. Taken together, our data suggested that ACOX1 regulated apoptosis by modulating the protein stability of p73. Previous study showed that PPARα was critical for ACOX1 regulated effects. Indeed, downregulation of ACOX1 upregulated PPARα expression (Supplementary Fig. 5A). We found that increasing of PPARα expression reduced doxorubicin-induced apoptosis (Supplementary Fig. 5B, C), suggesting that ACOX1 induced PPARα expression might counteract the apoptotic effect.

DISCUSSION

In the present study, we elucidated the oncogenic role of ACOX1 in lymphoma. Our data showed ACOX1 was essential for the proliferation of lymphoma cells. Additionally, overexpression of ACOX1 alleviated doxorubicin-induced apoptosis, while downregulation of ACOX1 aggravated doxorubicin-induced apoptosis. Moreover, ACOX1 participated in the regulation of apoptosis by modulating the mitochondrial membrane potential and activation of caspase-9 and caspase-3. Importantly, ACOX1 destabilizes p73 to suppress the activation of the intrinsic apoptosis pathway.

Peroxisomes play a critical role in the metabolism of reactive oxygen species, bile acids, very-long-chain, and branched-chain fatty acids (16). Cancer cells usually reprogram metabolism to support their malignant phenotype. Dysfunction of peroxisomes might lead to metabolic alterations and contribute to cancer development (17). Current study suggest that the deregulation of peroxisome function might contribute to drug resistance in lymphoma cells (18). Peroxisomal ACOX1 is commonly expressed in most tissues and is critical for the desaturation of very-long-chain acyl-CoAs to 2-trans-enoyl-CoAs. ACOX1 deficiency caused endoplasmic reticulum stress, accumulation of reactive oxygen species, VLCFAs and enlarged peroxisomes and is connected with human disease (19, 20). In this study, we showed that ACOX1 functioned as an oncogene in lymphoma cells to promote proliferation and counteract doxorubicin-induced apoptosis. Our data implied that suppression of ACOX1 could be a novel and effective approach for the treatment of lymphoma. Deficiency of ACOX1 results in activation of PPARα and induces progressive endoplasmic reticulum stress which contributes to hepatocarcinogenesis (9). The lack of both ACOX1 and PPARα causes the absence of spontaneous peroxisome proliferation in aged mice (21), indicating that PPARα is critical for ACOX1 regulated effects. In our study, the upregulation of PPARα was also observed when ACOX1 expression was downregulated. In this situation, the upregulation of PPARα might be a protective mechanism for lymphoma cell to escape apoptosis. This

phenomenon implied that the combination of ACOX1 and PPARα suppression could achieve more significant anti-cancer effects.

The expression of p73 is maintained at low levels in mammalian cells and is usually controlled through mechanisms including epigenetic silencing and post-translational modifications (11). In the current study, our data suggested that p73 was destabilized via a proteasome related-degradation mechanism during ACOX1 overexpression in malignant lymphoma cells. The mechanism of p73 degradation is not fully elucidated. Previous study shows that Itch, a Nedd4-like HETC-E3 ubiquitin ligase, selectively binds and ubiquitinates p73 to induce the proteasome-dependent degradation of p73 (22). Moreover, NEDL2 bind to p73, p73 is ubiquitinated and stabilized in a NEDL2-dependent manner (23). Additionally, NQO1 physically interacts with p73 in an NADH-dependent manner and protects it from 20S proteasomal degradation (24). Furthermore, DNA damage can induce phosphorylation of p73 by c-Abl, leading to stabilization of the p73 protein (25). Our data provided a novel ACOX1-related mechanism to demonstrate the deregulation of p73 expression in lymphoma. p73 is induced in response to DNA damage. Previous study shows that deficiency of ACOX1 results in increased intracellular H2O2 levels (9), which usually causes DNA damage and stress. We speculated that the destabilization of p73 might be caused by downregulating ACOX1 induced DNA damage and stress response. Besides, our data indicated that ACOX1 interacted with p73, implying that the interaction between ACOX1 and p73 might interfere with the degradation of p73 protein via proteasome related-mechanism. However, more data about the physical interaction between ACOX1 and p73 was needed to elucidate the mechanism.

In conclusion, our study uncovers a previously unknown oncogenic function of ACOX1 in lymphoma. We anticipate that our findings will provide a new target for overcoming drug resistance of traditional chemotherapy in lymphoma.

MATERIALS AND METHODS

Reagents and cell culture

Doxorubicin, Cycloheximide (CHX), MG132, WY-14643, and Annexin V-FITC Apoptosis Detection Kit were purchased from Sigma-Aldrich (MA, USA). Antibodies against Flag, Caspase-3, Caspase-8, Caspase-9, PARP, Bax, phospho-Bad (Ser112), Cytochrome C, p53, p53(Ser15), p53(Ser20), p73, and GAPDH were purchased from Cell Signaling Technology (Boston, USA). Antibodies against ACOX1 was purchased from Abcam (Cambridge, U.K.). Antibody against puromycine was purchased from Merk (Darmstadt, Germany). LEHD, DEVD, and IETD were purchased from R&D Systems (MN, USA). Raji and Daudi cells were obtained from the ATCC (Manassas, VA, USA) and were cultured in RPMI-1640 (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA). Cells were maintained at 37℃ in a humidified 5% CO2 atmosphere.

Western blot (WB) analysis

Cells were lysed in RIPA buffer contained protease inhibitors. Equal amounts of protein were analyzed by electrophoresis in SDS-PAGE gels. Proteins were transferred to nitrocellulose membrane. Nitrocellulose membranes were blocked by 3% BSA in TBST at RT and then were incubated with indicated antibodies at 4℃ overnight. Nitrocellulose membranes were incubated for 1 h at room temperature with secondary antibodies. Antibody binding was evaluated with an enhanced chemiluminescence kit (Pierce, USA).

Lentivirus preparation and transfection

Lentivirus preparation and transfection were described previously (26). 5×10^6 293T cells were transfected with 12 μg lentiviral vector, 9 μg psPAX2 (Addgene, MA, USA) and 3 μg pMD2.G (Addgene, MA, USA). Supernatants were collected every 24 h after 12 h transfection and concentrated via ultracentrifugation. The viral titer was determined by serial dilutions. The multiplicity of infection for transfection was 10.

Statistical analysis

Statistical analyses were performed using the SPSS software, version 16.0 (SPSS Inc.). The ANOVA test, followed by Least Significant Difference test, was used when performing multiple comparisons (27). The level of significance was set at $p<0.05$.

Detailed information is provided in the Supplementary Information.

ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

REFERENCES

1. Huh J (2012) Epidemiologic overview of malignant lymphoma. Korean J Hematol 47, 92-104
2. Smith A, Crouch S, Lax S et al (2015) Lymphoma incidence, survival and prevalence 2004-2014: sub-type analyses from the UK's Haematological Malignancy Research Network. Br J Cancer 112, 1575-1584
3. Shanbhag S and Ambinder RF (2018) Hodgkin lymphoma: A review and update on recent progress. CA Cancer J Clin 68, 116-132
4. Armitage JO, Gascoyne RD, Lunning MA and Cavalli F (2017) Non-Hodgkin lymphoma. Lancet 390, 298-310
5. Zeng J and Li D (2004) Expression and purification of his-tagged rat peroxisomal acyl-CoA oxidase I wild-type and E421 mutant proteins. Protein Expr Purif 38, 153-160
6. Fournier B, Saudubray JM, Benichou B et al (1994) Large deletion of the peroxisomal acyl-CoA oxidase gene in pseudoneonatal adrenoleukodystrophy. J Clin Invest 94, 526-531
7. Fan CY, Pan J, Chu R et al (1996) Hepatocellular and hepatic peroxisomal alterations in mice with a disrupted peroxisomal fatty acyl-coenzyme A oxidase gene. J Biol Chem 271, 24698-24710
8. Fan CY, Pan J, Usuda N, Yeldandi AV, Rao MS and Reddy JK (1998) Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor alpha natural ligand metabolism. J Biol Chem 273, 15639-15645
9. Huang J, Viswakarma N, Yu S et al (2011) Progressive endoplasmic reticulum stress contributes to hepatocarcinogenesis in fatty acyl-CoA oxidase 1-deficient mice. Am J Pathol 179, 703-713
10. Melino G, De Laurenzi V and Vousden KH (2002) p73: Friend or foe in tumorigenesis. Nat Rev Cancer 2, 605-615
11. Oberst A, Rossi M, Salomoni P et al (2005) Regulation of the p73 protein stability and degradation. Biochem Biophys Res Commun 331, 707-712
12. Nemajerova A, Petrenko O, Trumper L, Palacios G and Moll UM (2010) Loss of p73 promotes dissemination of Myc-induced B cell lymphomas in mice. J Clin Invest 120, 2070-2080
13. Zorova LD, Popkov VA, Plotnikov EY et al (2018) Mitochondrial membrane potential. Anal Biochem 552, 50-59
14. Chao C, Herr D, Chun J and Xu Y (2006) Ser18 and 23 phosphorylation is required for p53-dependent apoptosis and tumor suppression. EMBO J 25, 2615-2622
15. Jones EV, Dickman MJ and Whitmarsh AJ (2007) Regulation of p73-mediated apoptosis by c-Jun N-terminal kinase. Biochem J 405, 617-623
16. Antonenkov VD, Grunau S, Ohlmeier S and Hiltunen JK (2010) Peroxisomes are oxidative organelles. Antioxid Redox Signal 13, 525-537
17. Dahabieh MS, Di Pietro E, Jangal M et al (2018) Peroxisomes and cancer: The role of a metabolic specialist in a disease of aberrant metabolism. Biochim Biophys Acta Rev

Cancer 1870, 103-121

18. Dahabieh MS, Ha Z, Di Pietro E et al (2017) Peroxisomes protect lymphoma cells from HDAC inhibitor-mediated apoptosis. Cell Death Differ 24, 1912-1924
19. Ferdinandusse S, Denis S, Hogenhout EM et al (2007) Clinical, biochemical, and mutational spectrum of peroxisomal acyl-coenzyme A oxidase deficiency. Hum Mutat 28, 904-912
20. Yu S, Rao S and Reddy JK (2003) Peroxisome proliferator-activated receptors, fatty acid oxidation, steatohepatitis and hepatocarcinogenesis. Curr Mol Med 3, 561-572
21. Hashimoto T, Fujita T, Usuda N et al (1999) Peroxisomal and mitochondrial fatty acid beta-oxidation in mice nullizygous for both peroxisome proliferator-activated receptor alpha and peroxisomal fatty acyl-CoA oxidase. Genotype correlation with fatty liver phenotype. J Biol Chem 274, 19228-19236
22. Rossi M, De Laurenzi V, Munarriz E et al (2005) The ubiquitin-protein ligase Itch regulates p73 stability. EMBO J 24, 836-848
23. Miyazaki K, Ozaki T, Kato C et al (2003) A novel HECT-type E3 ubiquitin ligase, NEDL2, stabilizes p73 and enhances its transcriptional activity. Biochem Biophys Res Commun 308, 106-113
24. Asher G, Tsvetkov P, Kahana C and Shaul Y (2005) A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. Genes Dev 19, 316-321
25. Agami R, Blandino G, Oren M and Shaul Y (1999) Interaction of c-Abl and p73alpha and their collaboration to induce apoptosis. Nature 399, 809-813
26. Zheng F, Yue C, Li G et al (2016) Nuclear AURKA acquires kinase-independent transactivating function to enhance breast cancer stem cell phenotype. Nat Commun 7, 10180
27. Zheng FM, Long ZJ, Hou ZJ et al (2014) A novel small molecule aurora kinase inhibitor attenuates breast tumor-initiating cells and overcomes drug resistance. Mol Cancer Ther 13, 1991-2003

FIGURE LEGENDS

Fig. 1. ACOX1 regulates proliferation and apoptosis. A. ACOX1 was stably expressed in Raji lymphoma cells via lentivirus-mediated gene transfer. Cells were subjected to WB analysis. B. Cells (1×10^5) stably expressed ACOX1 were culture for an indicated time. Then MTT assay was used to evaluate the proliferation. C. ACOX1 shRNA was stably expressed in Raji lymphoma cells via lentivirus-mediated gene transfer. Cells were subjected to WB analysis. B. Cells (1×10^5) stably expressed ACOX1 shRNA were culture for an indicated time. Then MTT assay was used to evaluate the proliferation. E. and F. Cells (1×10^6) stably expressed ACOX1 were subjected to apoptosis analysis (E). The statistical results were shown (F). G. and H. Cells (1×10^6) stably expressed ACOX1 were subjected to apoptosis analysis (G). The statistical results were shown (H). I. and J. The treatment was the same as (E) and (F). Cells were subjected to TUNEL assay analysis (I). The statistical results were shown (J). K. and L. The treatment was the same as (K) and (L). Cells were subjected to TUNEL assay analysis (K). The statistical results were shown (L). The bar represents mean ± SD of three independent experiments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, The ANOVA test, followed by Least Significant Difference test, were used to make statistical comparisons.).

Fig. 2. ACOX1 modulates doxorubicin-induced cytotoxic effect. A. and B. ACOX1 expressed Raji cells and control cells (Con.) were treated with doxorubicin (0.6μM) or control solvent for 48 h. Cells were subjected to apoptosis analysis (A). The statistical results were shown (B). C. and D. shACOX1 expressed Raji cells and control cells (NC) were treated with doxorubicin (0.1μM) or control solvent for 48 h. Cells were subjected to apoptosis analysis (C). The statistical results were shown (D). E. and F. The treatment was the same as (A) and (B). Cells were subjected to TUNEL assay analysis (E). The statistical results were shown (F). G. and H. The treatment was

the same as (C) and (D). Cells were subjected to TUNEL assay analysis (G). The statistical results were shown (H). The bar represents mean ± SD of three independent experiments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, The ANOVA test, followed by Least Significant Difference test, were used to make statistical comparisons.).

Fig. 3. Downregulation of ACOX1 induced apoptosis through the intrinsic pathway. A. shACOX1 expressed Raji cells (sh) and control cells (NC) were treated with doxorubicin (0.1μM) or control solvent for 48 h. Cells were subjected to WB analysis. B. shACOX1 expressed Raji cells (sh) and control cells (NC) were treated with LEHD, DEVD, IETD or control solvent for 24 h. Cells were subjected to apoptosis analysis. The statistical results were shown. C. shACOX1 expressed Raji cells (sh) and control cells (NC) were subjected to total protein extraction or cytoplasmic/mitochondria protein extraction. These proteins were subjected to WB analysis. D. The treatment was similar to (A). Cells were subjected to mitochondrial membrane potential analysis. E. ACOX1 expressed Raji cells and control cells (Con.) were treated with doxorubicin (0.6μM) or control solvent for 48 h. Cells were subjected to WB analysis. F. The treatment was similar to (E). Cells were subjected to mitochondrial membrane potential analysis. The bar represents mean ± SD of three independent experiments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, The ANOVA test, followed by Least Significant Difference test, were used to make statistical comparisons.).

Fig. 4. ACOX1 destabilizes p73 to reduce apoptosis. A. shACOX1 expressed Raji cells (sh) and control cells (NC) were subjected to WB analysis. B. and C. shACOX1 expressed Raji cells (sh) and control cells (NC) were transfected with p73 siRNA for 48 h. Cells were subjected to WB analysis (B) or apoptosis analysis (C). D. ACOX1 expressed Raji cells and control cells (Con.) were treated with doxorubicin (0.6μM) or control solvent for 48 h. Cells were subjected to WB analysis. E. shACOX1

expressed Raji cells (sh) and control cells (NC) were subjected to RNA extraction and PCR analysis. F. Cells were treated with MG132 (10 μM). Cells were subjected to western blot analysis. G. and H. ACOX1 expressed, shACOX1 expressed Raji cells and control cells were treated with CHX for the indicated time. WB analysis of p73, ACOX1, and GAPDH was performed (G). The densitometric quantification of p73 normalized to GAPDH was plotted against various time points to determine its half-life (H).

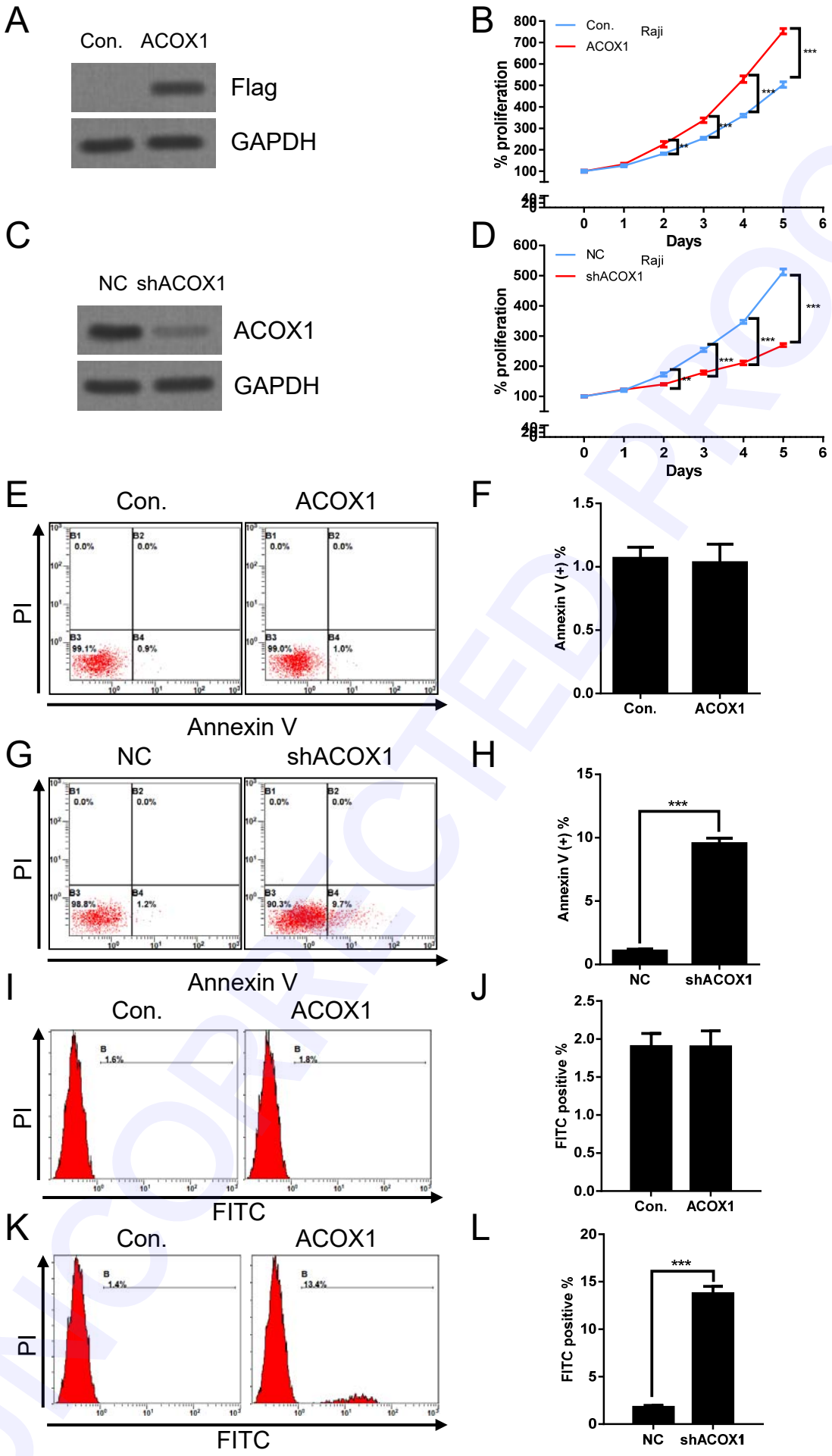


Fig. 1. ACOX1 regulates proliferation and apoptosis.

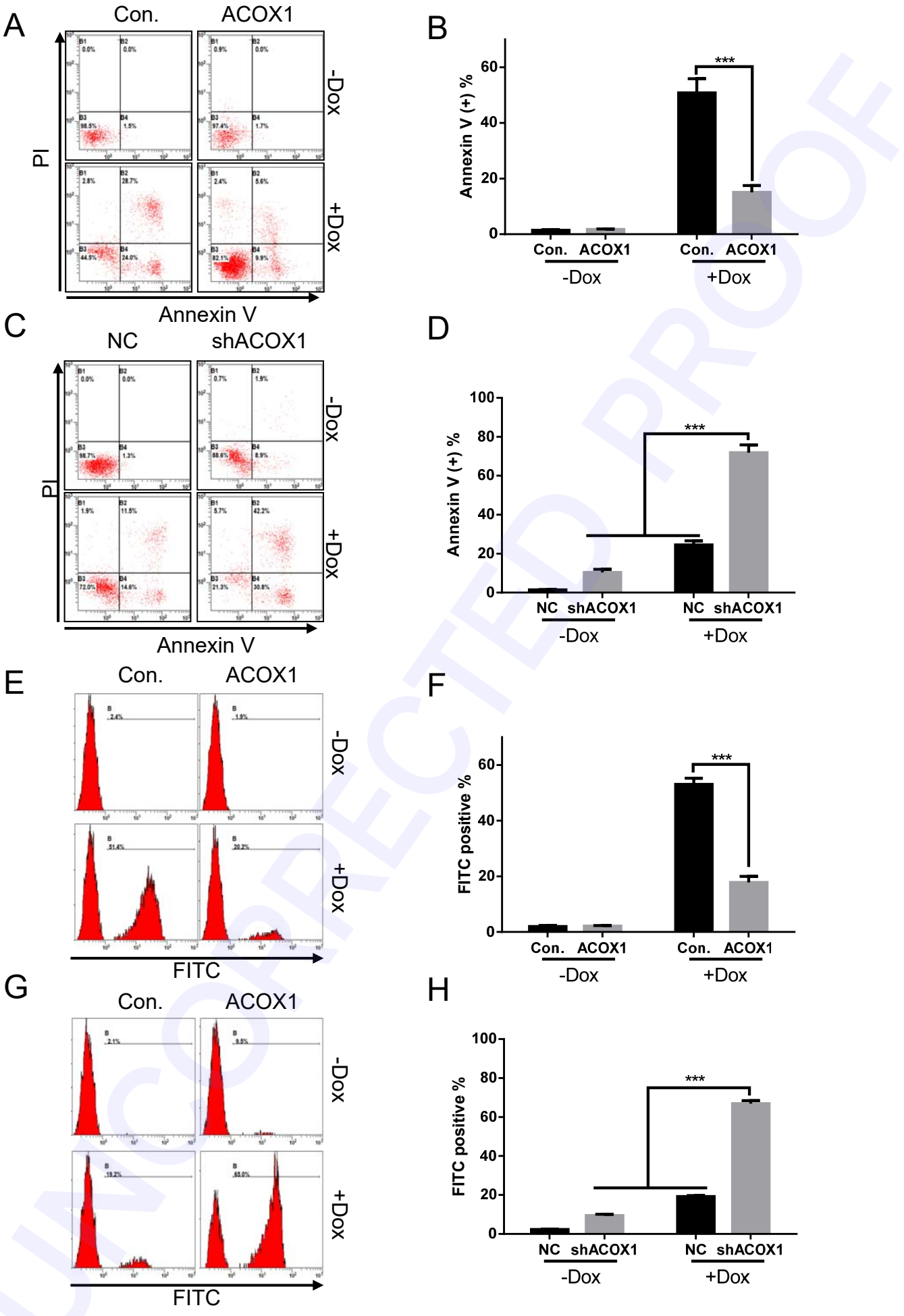


Fig. 2. ACOX1 modulates doxorubicin-induced cytotoxic effect.

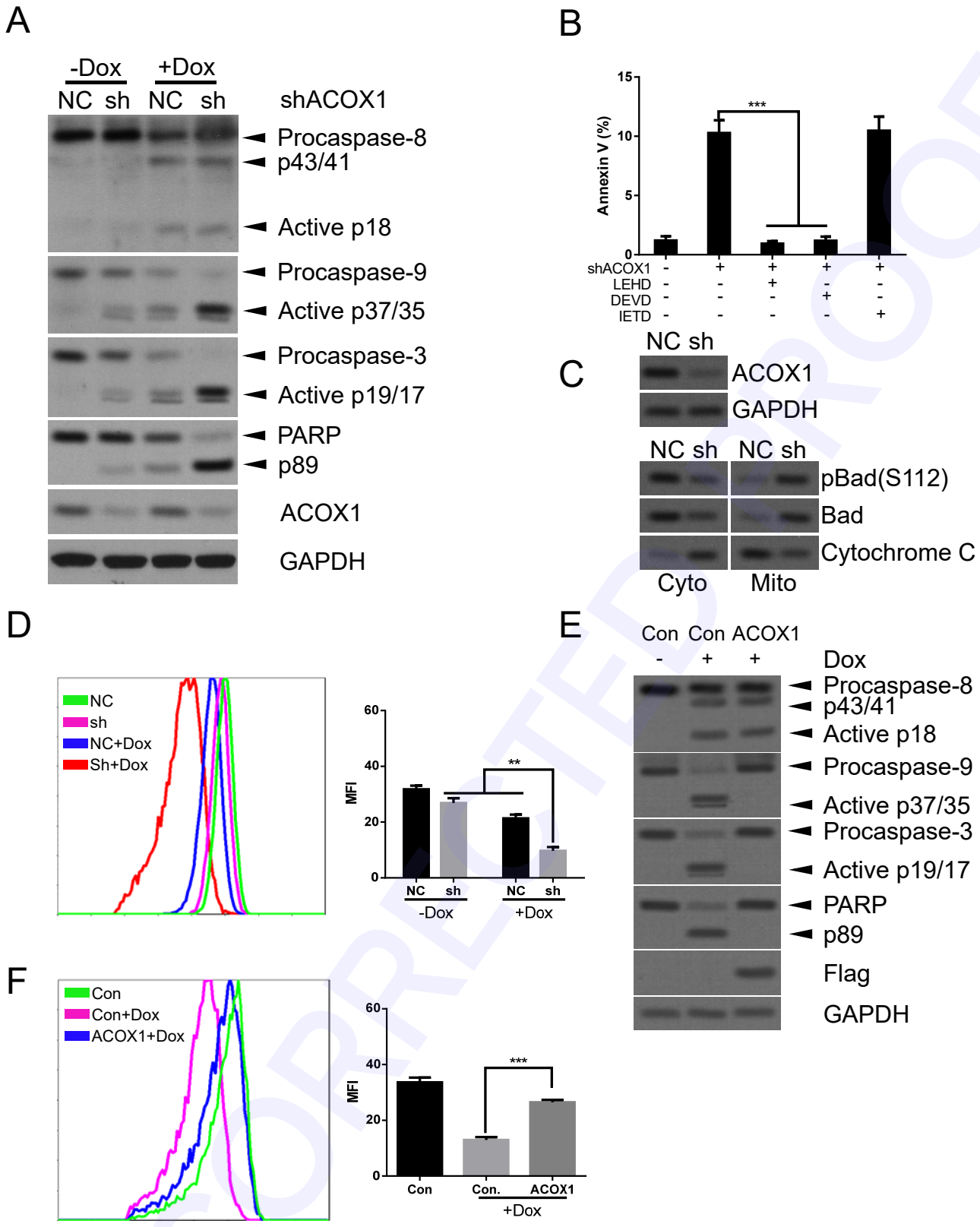


Fig. 3. Downregulation of ACOX1 induced apoptosis through the intrinsic pathway.

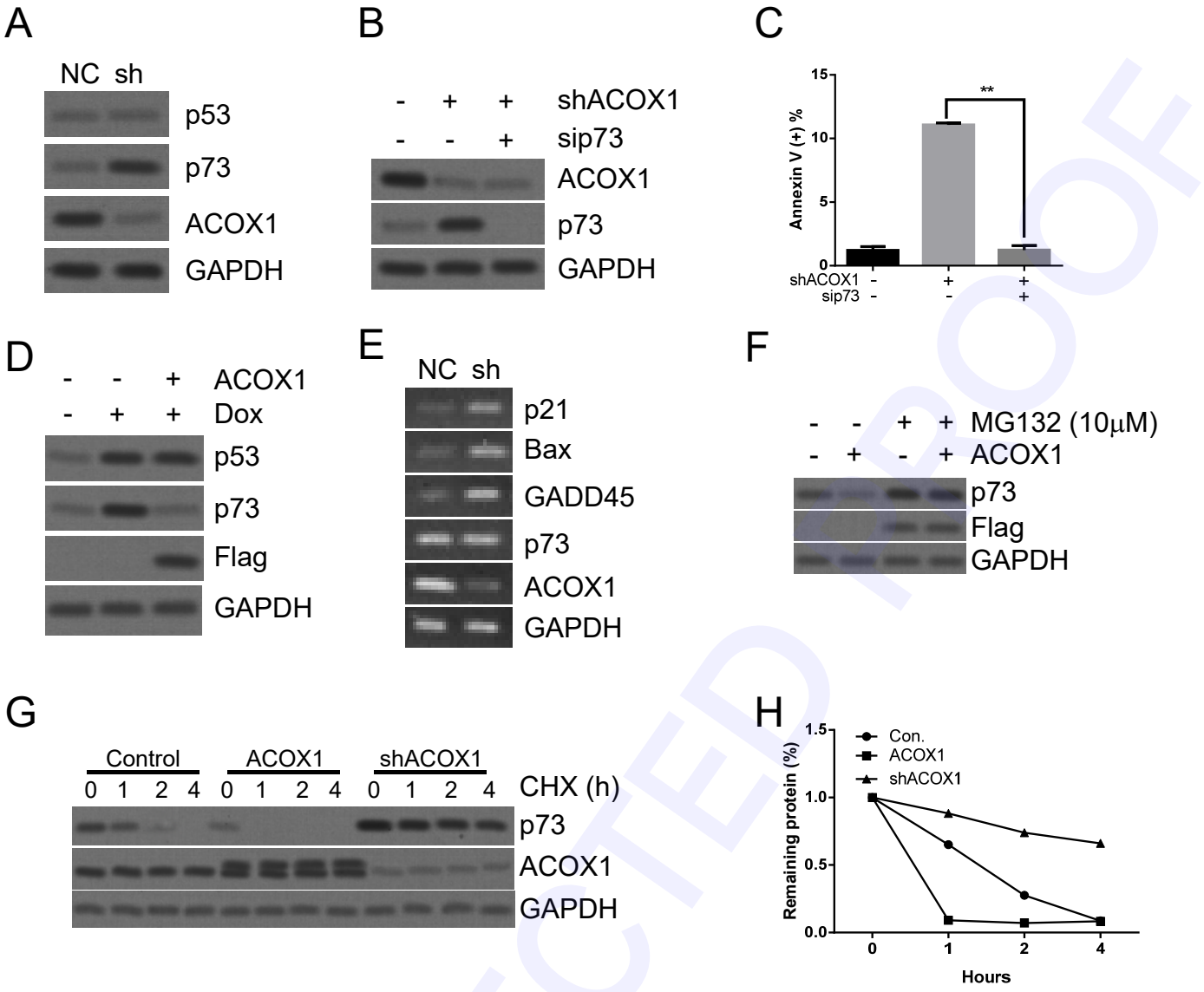


Fig. 4. ACOX1 destabilizes p73 to reduce apoptosis.

SUPPLEMENTARY MATERIALS AND METHODS

Annexin-V/PI staining and analysis

Cells were resuspended in binding buffer (500μl/sample) and were stained with Annexin-V-FITC (5μl/sample) followed by PI (1μl/sample). After incubation for 15 min, cells were subjected to flow cytometry analysis (BD Biosciences, CA, USA).

Cell proliferation assay

Cell proliferation was evaluated by MTT assays. 2×10^3 cells/well/100μl were exposed to different treatment for the indicated time. MTT solution (20μl, 5 mg/ml) were incubated with cells at 37°C for 1 h. Cells were precipitated by centrifuging at 1000rpm for 5min. The medium was carefully removed. DMSO (200μl) was used to solubilize the formazan. Finally, the absorbance was measured by the multiwell plate reader.

Plasmid

The shRNA sequences against ACOX1 (5'-CCGGGCAGCCAGATTAGTAGAAATTCTCGAGAATTTCTACTAATCTGGCTGCTTTTTG) were cloned into pLVTHM plasmid (MA, USA). ACOX1 (NM_004035.7) cDNA was amplified by PCR reaction and insert into a pLVX-DsRed-Monomer-N1 plasmid (Osaka, Japan) via XhoI and BamHI sites.

RNA interference

For small interfering RNA (siRNA)-mediated knockdown of p73, cells were transfected with 100 nM of either the targeting or control siRNA (Genepharma, Shanghai, China) using Lipofectamine 2000 (Thermo Fisher Scientific, USA) for 48 h. The targeting sequence in p73 mRNA was 5'-CTCTCCTTCCTGTGTGTCCAA.

TUNEL assay

Cells were analyzed by the In situ Direct DNA Fragmentation (TUNEL) Assay Kit (Abcam, Cambridge, U.K.) according to the manufacturer's instructions. In brief, cells

were washed with phosphate buffered saline buffer (PBS). For analysis, the cells were firstly fixed with 1% formaldehyde at 4 °C for 20 min. After fixation, cells were resuspended in 500 µl of PBS and incubated overnight in 70% (v/v) ethanol at 4 °C. On the next day, ethanol was washed off. Cells were resuspended in staining solution for 60 min at 37 °C. Cells were rinsed with rinse buffer. Cells were incubated with PI/RNase solution at 37 °C for 30 min. Cells were then analyzed by flow cytometry (BD Biosciences, CA, USA).

Mitochondrial membrane potential

The mitochondrial membrane potential was estimated using the NIR Mitochondrial Membrane Potential Assay Kit (Abcam, Cambridge, U.K.) and analyzed by flow cytometry.

Protein synthesis assay

Protein synthesis assay was reported previously (1). Cells were pretreated with or without CHX (60 µM) for 30 min at 37°C. Cells were then incubated for 30 min with puromycin (final concentration 1 µM) (Sigma-Aldrich, MA, USA). After puromycin pulse, cells were washed with PBS and replaced with fresh media (without puromycin, and with or without CHX). After indicated chase time, cells were washed with ice-cold PBS and lysed directly in lysis buffer. The lysates were subjected to immunoprecipitate with 1 µg of puromycine antibody for 1 h and then for 1 h with protein G-Sepharose beads (Sigma-Aldrich, MA, USA). Immunoprecipitates were washed with and resuspended in 2× Laemmli sample buffer. Samples were analyzed with 8% SDS-PAGE and transferred to nitrocellulose. Samples were analyzed by WB using the p73 or puromycine antibody.

Pulse-chase assay

Cells were cultured in growth medium lacking methionine for 30 min, in the same media containing 300 mCi/ml [35S]methionine (Perkin Elmer, USA) for 1 h. Cells were

washed with PBS and then cultured in growth medium supplemented with 0.15 mg/ml nonradioactive methionine for the indicated time. Cells were lysed in RIPA buffer. The lysates were subjected to immunoprecipitate with 1 μg of p73 antibody for 1 h and then for 1 h with protein G-Sepharose beads (Sigma-Aldrich, MA, USA). Immunoprecipitates were washed with and resuspended in 2× Laemmli sample buffer. Samples were analyzed with 8% SDS-PAGE and transferred to nitrocellulose. Autoradiography and immunoblotting were performed using the same membrane.

SUPPLEMENTARY REFERENCES

1. Schmidt EK, Clavarino G, Ceppi M and Pierre P (2009) SUnSET, a nonradioactive method to monitor protein synthesis. Nat Methods 6, 275-277

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Fig. 1. ACOX1 regulates proliferation and apoptosis in Daudi cells. A. ACOX1 was stably expressed in Daudi lymphoma cells via lentivirus-mediated gene transfer. Cells were subjected to WB analysis. B. Cells (1×10^5) stably expressed ACOX1 were culture for an indicated time. Then MTT assay was used to evaluate the proliferation. C. ACOX1 shRNA was stably expressed in Daudi lymphoma cells via lentivirus-mediated gene transfer. Cells were subjected to WB analysis. B. Cells (1×10^5) stably expressed ACOX1 shRNA were culture for an indicated time. Then MTT assay was used to evaluate the proliferation. E. and F. Cells (1×10^6) stably expressed ACOX1 were subjected to apoptosis analysis (E). The statistical results were shown (F). G. and H. Cells (1×10^6)stably expressed ACOX1 were subjected to apoptosis analysis (G). The statistical results were shown (H). The statistical results were shown (L). The bar represents mean ± SD of three independent experiments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, The ANOVA test, followed by Least Significant Difference test, were used to make statistical comparisons.).

Supplementary Fig. 2. ACOX1 modulates doxorubicin-induced cytotoxic effect in Daudi cells. A. and B. ACOX1 expressed Daudi cells and control cells (Con.) were treated with doxorubicin (0.6μM) or control solvent for 48 h. Cells were subjected to apoptosis analysis (A). The statistical results were shown (B). C. and D. shACOX1 expressed Daudi cells and control cells (NC) were treated with doxorubicin (0.1μM) or control solvent for 48 h. Cells were subjected to apoptosis analysis (C). The statistical results were shown (D). The bar represents mean ± SD of three independent experiments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, The ANOVA test, followed by Least Significant Difference test, were used to make statistical comparisons.).

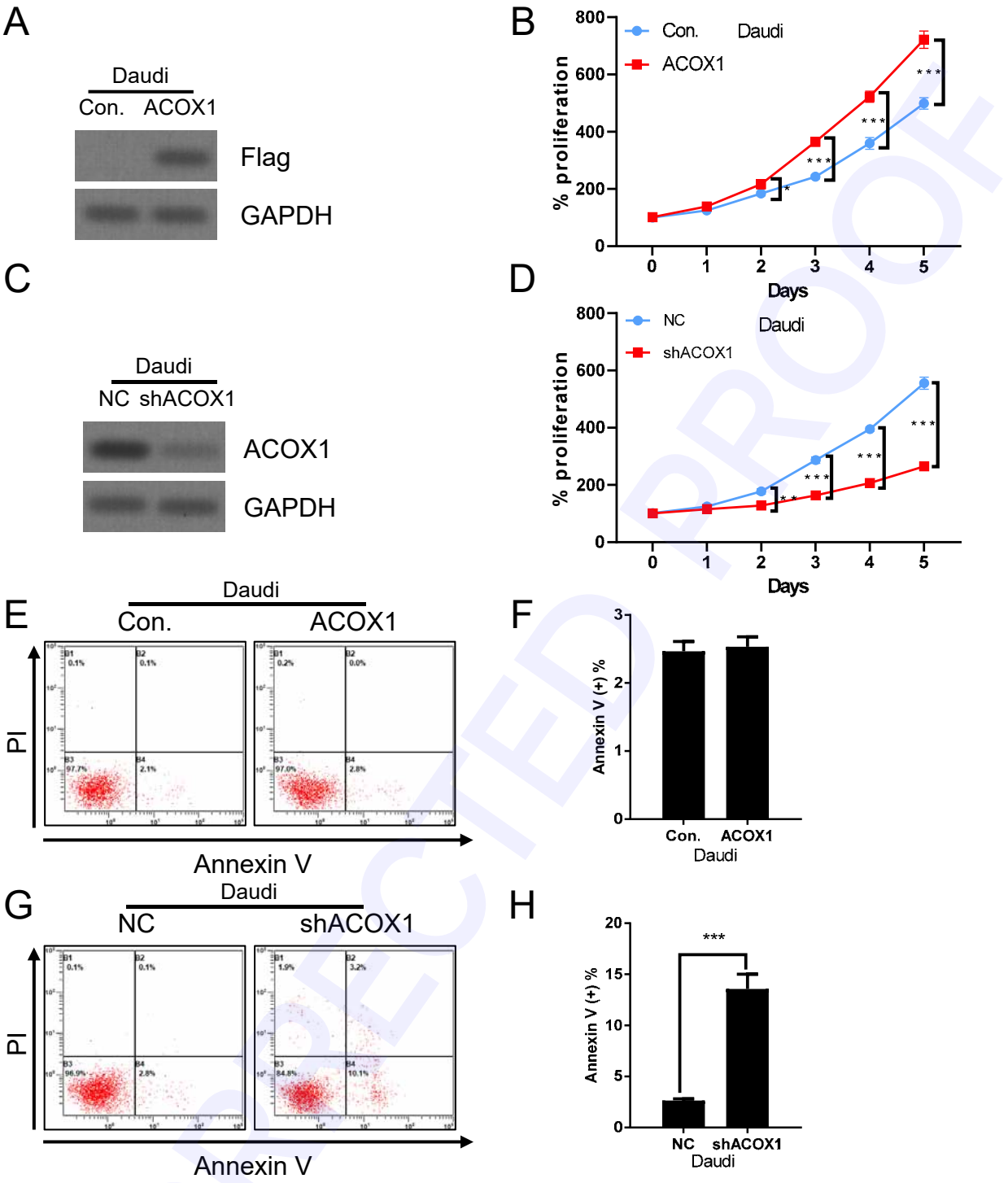
Supplementary Fig. 3. Downregulation of ACOX1 induced apoptosis through the

intrinsic pathway in Daudi cells. A. shACOX1 expressed Daudi cells (sh) and control cells (NC) were treated with doxorubicin (0.1μM) or control solvent for 48 h. Cells were subjected to WB analysis. B. shACOX1 expressed Daudi cells (sh) and control cells (NC) were subjected to total protein extraction or cytoplasmic/mitochondria protein extraction. These proteins were subjected to WB analysis. C. The treatment was similar to (A). Cells were subjected to mitochondrial membrane potential analysis. D. ACOX1 expressed Daudi cells and control cells (Con.) were treated with doxorubicin (0.6μM) or control solvent for 48 h. Cells were subjected to WB analysis. E. The treatment was similar to (D). Cells were subjected to mitochondrial membrane potential analysis. The bar represents mean ± SD of three independent experiments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, The ANOVA test, followed by Least Significant Difference test, were used to make statistical comparisons.).

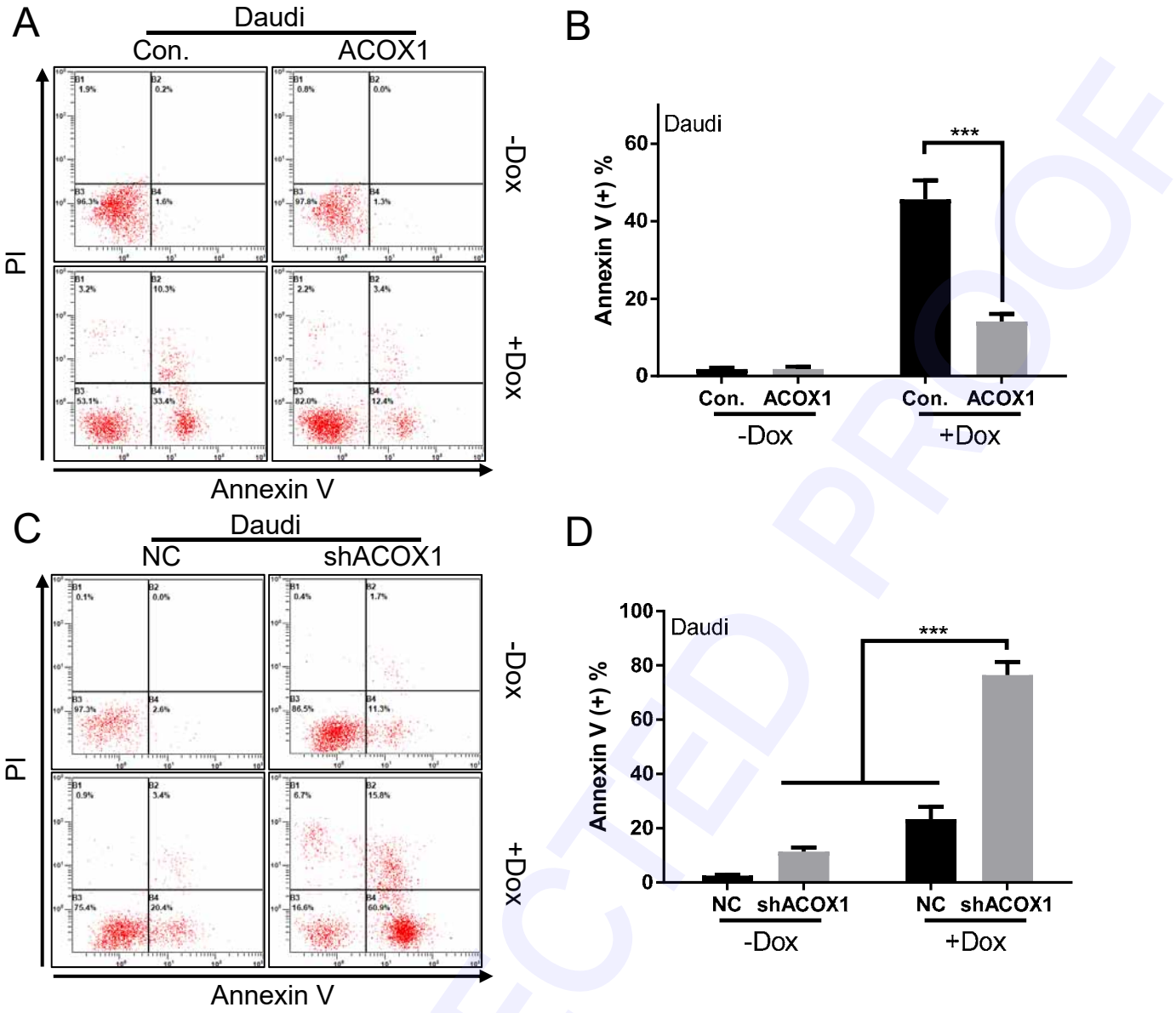
Supplementary Fig. 4. ACOX1 destabilizes p73 to reduce apoptosis in Daudi cells. A. shACOX1 expressed Daudi cells (sh) and control cells (NC) were subjected to WB analysis. B. shACOX1 expressed Daudi cells (sh) and control cells (NC) were subjected to WB analysis. Doxorubicin treatment was used as a positive control. C. and D. shACOX1 expressed Daudi cells (sh) and control cells (NC) were transfected with p73 siRNA for 48 h. Cells were subjected to WB analysis (C) or apoptosis analysis (D). E. ACOX1 expressed Daudi cells and control cells (Con.) were treated with doxorubicin (0.6μM) or control solvent for 48 h. Cells were subjected to WB analysis. F. shACOX1 expressed Daudi cells (sh) and control cells (NC) were subjected to RNA extraction and PCR analysis. G. Cells were pretreated with or without CHX and then incubated with puromycin. Cells were replaced with fresh media for indicated chase time. Cells were lysed and subjected to immunoprecipitated with puromycine antibody. Samples were analyzed by WB using the p73 or puromycine antibody. H. and I. ACOX1 expressed, shACOX1 expressed Daudi cells and control cells were treated with CHX for the indicated time. WB analysis of p73, ACOX1, and GAPDH was performed (H). The densitometric quantification of p73 normalized to GAPDH was plotted against

various time points to determine its half-life (I). J. and K. Pulse-chase assay was performed to evaluate p73 degradation when ACOX1 was overexpressed. Autoradiography and immunoblotting were performed (J). The densitometric quantification was shown in (K). L. Immunoprecipitation was performed to show the protein interaction between ACOX1 and p73.

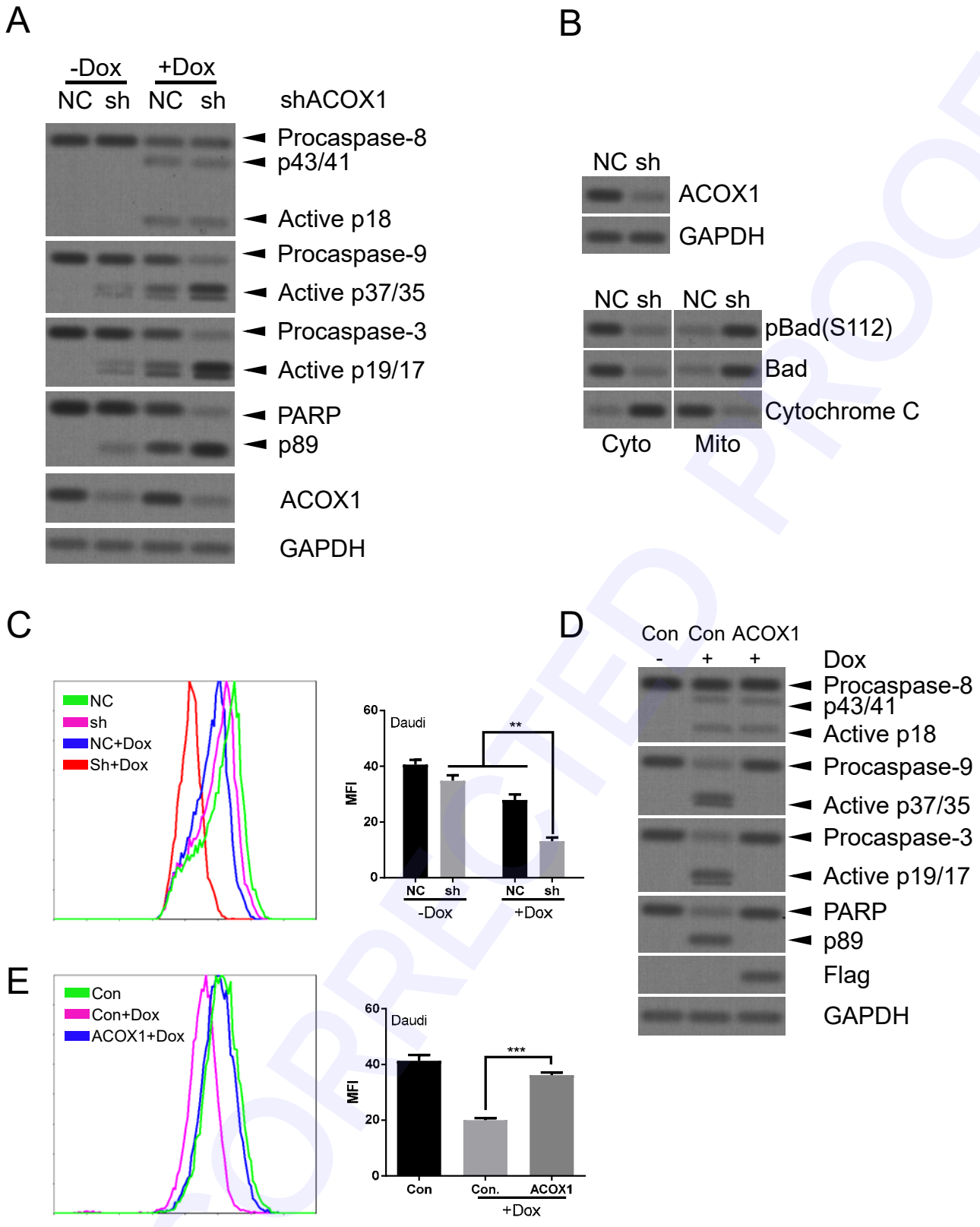
Supplementary Fig. 5. PPARα induced by ACOX1 inhibits apoptosis. A. shACOX1 expressed Raji cells (sh) and control cells (NC) were subjected to WB analysis. B. and C. Raji cells were treated with or without doxorubicin (0.6μM) or WY-14643 (PPARα agonist) for 48 h. Cells were subjected to WB analysis and apoptosis (B) analysis (C). The bar represents mean ± SD of three independent experiments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, The ANOVA test, followed by Least Significant Difference test, were used to make statistical comparisons.).



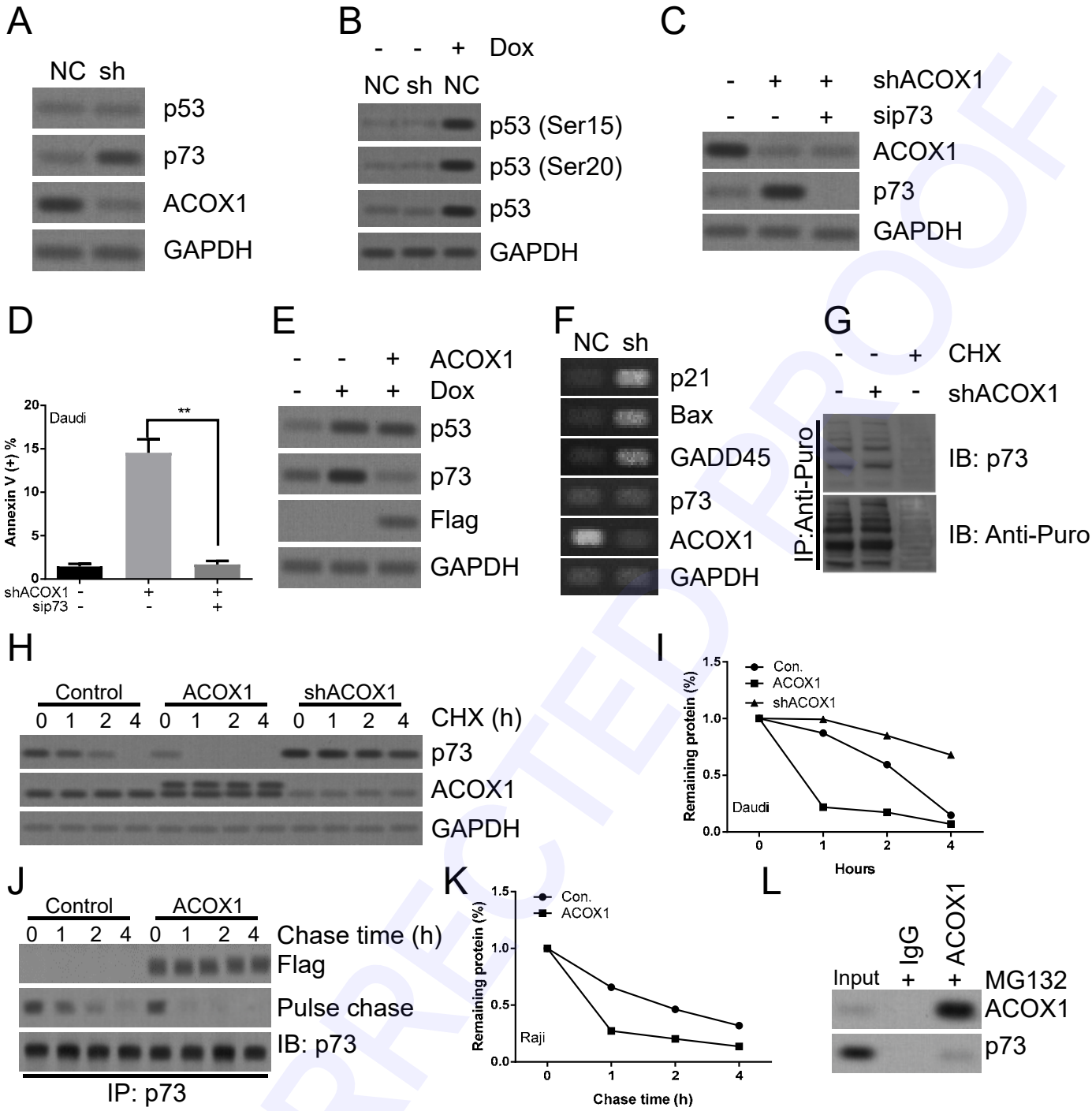
Supplementary Fig. 1. ACOX1 regulates proliferation and apoptosis in Daudi cells.



Supplementary Fig. 2. ACOX1 modulates doxorubicin-induced cytotoxic effect in Daudi cells.



Supplementary Fig. 3. Downregulation of ACOX1 induced apoptosis through the intrinsic pathway in Daudi cells.



Supplementary Fig. 4. ACOX1 destabilizes p73 to reduce apoptosis in Daudi cells.

Supplementary Fig. 5. PPARα induced by ACOX1 inhibits apoptosis.