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Original Paper

YAP Inhibits the Apoptosis and Migration of Human Rectal Cancer Cells via Suppression of JNK-Drp1-Mitochondrial Fission-Htra2/Omi Pathways

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Key Words
Yap • Drp1 • JNK • Mitochondrial fission • HtrA2/Omi • Apoptosis • Migration • F-actin • Cofilin • Lamellipodia

Abstract
Background/Aims: The Hippo-Yap pathway is associated with tumor development and progression. However, little evidence is available concerning its role in cancer cell apoptosis and migration via mitochondrial homeostasis. Here, we identify mitochondrial fission as a regulator of the Hippo–Yap pathway in human rectal cancer tumorigenesis and metastasis. ***Methods:*** In this study, we performed loss-of function assays concerning Yap in RCC via shRNA. Cellular viability and apoptosis were measured via MTT, the TUNEL assay and trypan blue staining. Mitochondrial function was assessed via JC1 staining, the mPTP opening assay, mitochondrial respiratory function analysis, electron microscopy and immunofluorescence analysis of HtrA2/Omi. Mitophagy and mitochondrial fission were assessed via western blots and immunofluorescence. Cell migration was evaluated via the Transwell assay, wound-healing assay and immunofluorescence analysis of F-actin. The interaction between JNK and Yap was detected via co-immunoprecipitation and Yap recombinant mutagenic plasmid transfection. Western blots were used to analyze signaling pathways in conjunction with JNK inhibitors or HtrA2/Omi siRNA. ***Results:*** Yap is upregulated in human rectal cancer cells, where its expression correlates positively with cell survival and migration. Functional studies established that silencing of Yap drove JNK phosphorylation, which induced Drp1 activation and translocation to the surface of mitochondria, initiating mitochondrial fission. Excessive mitochondrial fission mediated HtrA2/Omi leakage from the mitochondria into the cytoplasm, where HtrA2/Omi triggered cellular apoptosis via the mitochondrial apoptosis pathway. Moreover, released HtrA2/Omi also phosphorylated cofilin and inhibited cofilin-mediated F-actin polymerization. F-actin collapse perturbed lamellipodia formation and therefore impaired cellular migration

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and invasion. ***Conclusion:*** Collectively, our results demonstrate that Hippo-Yap can serve as a tumor promoter in human rectal cancer and acts by restricting JNK/Drp1/mitochondrial fission/ HtrA2/Omi, with potential implications for new approaches to human rectal cancer therapy.

Introduction

Rectal cancer (RC) has been the fourth leading cause of cancer mortality worldwide for several decades. The 5-year survival rate for localized RC is 90%; however, as RC spreads to regional lymph nodes or distant parts of the body, the 5-year survival rate drops to between 71% and 13% in the United States [1]. These malignancies are ascribed to accumulation of protein alterations, including dissemination of proto-oncogenes and loss or inactivation of tumor suppressor genes, which ultimately lead to tumor evolution and progression [2-6]. Furthermore, the increased survival and invasiveness of tumor cells are typical characteristics of RC [7, 8], suggesting a clinical need to abate excessive RC cell survival and migration.

Mitochondria are the energy metabolism centers of cell [9]. Previous studies [10, 11] have found that mitochondria also act as messengers to transmit or amplify damage signals via mitochondrial dynamics, a process that balances mitochondrial fusion and fission events to regulate the structure and function of the mitochondrion. Normally, mitochondrial fusion enables the exchange of proteins, lipids, and mitochondrial DNA and ATP across distances within the cell, which is essential to meet the metabolic demands of the cell [12]. However, mitochondrial fission (fragmentation) is essential for intrinsic apoptosis–it is necessary for cytochrome c (cyt-c) release and subsequent caspase activation [13]. Inhibition of dynamin-related protein 1 (Drp1)-dependent mitochondrial fission impairs and partially inhibits intrinsic apoptosis [10]. However, it is still unclear whether mitochondrial dynamics, especially fission, are vital for the RC phenotype related to RC survival and migration. If so, it is unknown what molecules govern fission to influence RC viability and mobilization.

Yes-associated protein (Yap), a downstream executor of the Hippo pathway, is implicated in the regulation of tissue regeneration [14, 15], proliferation of adult stem cells [16, 17] and cancer development, including liver cancer, prostate cancer, lung cancer, and ovarian cancer [18-20]. Moreover, Yap is particularly essential for tissue regeneration of the colon after DSS-mediated injury, and hyperactivation of Yap leads to intestinal cancer [21, 22]. These findings hint that Yap may be involved in RC development. Regulation of Yap phosphorylation in more aggressive cancers has been studied extensively, and increased levels of Yap (and TAZ) correlate with cancer progression [7, 23]. Moreover, much of the immunohistochemistry and microarray data from cancer specimens show elevated total Yap abundance rather than focused nuclear localization of Yap [22, 23], suggesting the important role of Yap in the cytoplasm. On the other hand, recent studies have indicated that the Hippo-pathway facilitates mitochondrial function via Bcl-xL [24], demonstrating a possible relationship between Yap and cytoplasmic mitochondria. Furthermore, accumulating evidence has described the regulatory action of Yap on JNK [25, 26]. Thus, together with the confirmed role of JNK in initiating mitochondrial fission [27], these findings raise the possibility that Yap may govern mitochondrial fission via JNK.

High-temperature requirement A2 (HtrA2)/Omi is a serine protease and chaperone protein [28] complex that resides in the mitochondrial intermembrane space (IMS). HtrA2/Omi maintains mitochondrial homeostasis including mitochondrial respiration; however, under stress conditions, HtrA2/Omi is released from the mitochondria into the cytoplasm, where it binds to and cleaves inhibitor of apoptosis proteins (IAPs), leading to cellular apoptosis [29]. However, whether mitochondrial fission can trigger HtrA2/Omi release remains unclear. As a consequence of HtrA2/Omi detachment from mitochondria, whether HtrA2/Omi has a role in controlling RC migration and invasion aside apoptosis remains unclear.

Tumor cell migration and invasion are primarily dependent on lamellipodia formation via cofilin-mediated F-actin rearrangement [30]. Phosphorylated cofilin fails to recycle and update cellular F-actin, leading to F-actin collapse and lamellipodia formation impairment. Given that HtrA2/Omi is a kinase that modifies proteins via post-transcriptional phosphorylation [31], we hypothesize that HtrA2/Omi released from mitochondria may impair RC migration by manipulating cofilin phosphorylation. In this study, using loss- and gain-of function experiments concerning Yap in the human rectal cancer cell line SW837, we demonstrated that Yap was increased in RC. High Yap expression was associated with dephosphorylated JNK, its inactive form, blocking Drp1 and mitochondrial fission. Deletion of YAP triggered JNK phosphorylation, signaling Drp1 activation and subsequent migration to the surface of mitochondria, and leading SW837 cells to undergo mitochondrial fission. Excessive mitochondrial fission released HtrA2/Omi into the cytoplasm, abating anti-apoptotic proteins. Moreover, cytoplasmic HtrA2/Omi also inactivated cofilin and elicited F-actin degradation into G-actin, ultimately leading to lamellipodia collapse and therefore impairing cellular migration and invasion.

Materials and Methods

Cell culture

All procedures described herein were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All experimental protocols were approved (Approval ID: 2015053) by the Ethics Committee of the first Affiliated Hospital of Zhengzhou University. The SW837, FHC and HEK293T human embryonic kidney cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), which performs routine cell line authentication testing with SNP and short tandem repeat analyses. These cells were cultured as described previously [32]. All cell lines were passaged in the laboratory for fewer than 4 months and were used at the fifth through tenth passage in culture for this study.

Western blotting and co-immunoprecipitation

Western blotting was performed as described previously [33]. The primary antibodies for the blots were as follows: pDrp1-s616 (1:1000, Cell Signaling Technology, Inc.), pDrp1-s637 (1:1000, Abcam plc), ATG5 (1:1000, Cell Signaling Technology, Inc.), Beclin1 (1:1000, Cell Signaling Technology, Inc.), LC3I (1:1000, Cell Signaling Technology, Inc.), LC3II (1:1000, Cell Signaling Technology, Inc.), YAP (1:1000, Cell Signaling Technology, Inc.), pYAP-s127 (1:1000, Cell Signaling Technology, Inc.), JNK (1:1000, Cell Signaling Technology, Inc.) and p-JNK (1:1000, Cell Signaling Technology, Inc.). Representative blots are shown from three independent experiments [34], and the images were obtained with an enhanced chemiluminescence (ECL) reagent.

Co-immunoprecipitation experiments were performed as previously described [11]. Briefly, cells were lysed by sonication in PBS with 1% Triton X-100 and incubated with the respective antibodies and protein A/G agarose beads. The beads were washed using RIPA lysis buffer at least three times, and then boiled in SDS loading buffer. Immunoprecipitated protein complexes were detected via western blotting.

qPCR and RNA interference

Total RNA was extracted from the cells using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and was reverse-transcribed into a total of 1 μl (60 ng/μl) cDNA using a One-Step RT-PCR kit (TransGen Biotech Co., Ltd., Beijing, China), according to the manufacturer's instructions [35]. Quantification of gene expression was performed using an ABI PRISM 7500 Sequence Detection system (Applied Biosystems Life Technologies, Foster City, CA) with SYBR® Green (TransGen Biotech Co., Ltd.) as previously described [33]. The primer sequences were as follows: E-cadherin, F: 5'-GTCACTGACACCAACGATAATCCT-3', R: 5'-TTTCAGTGTGGTGATTACGACGTTA-3'; Vimentin, F: 5'-CCTGAACCTGAGGGAAACTAA-3', R: 5'-GCAGAAAGGCACTTGAAAGC-3'. GAPDH, F: 5'-CACCAACTGGGACGACATG-3', R: 5'- GCACAGCCTGGATAGCAAC-3'

Scrambled shRNA control or shRNA specific to Yap was constructed using a lentiviral shRNA technique (GeneChem), as described previously [10, 11]. The shRNA was used to generate the stable cell line SW837

without expression of Yap. To suppress the HtrA2/Omi, siRNA was used as previously described [32]. The siRNA targeting HtrA2/Omi was purchased from Santa Cruz Biotechnology.

Wound-healing assays, transwell migration and matrigel invasion assays

For the wound-healing assay, 10^6 cells were seeded onto 36-mm plates and grown to 80–90% confluence. An artificial wound was created using a P200 pipette tip to scratch the confluent cell monolayer [36]. Photomicrographs were immediately obtained (0 h). The mobilization of the cells and closing of the scratch wound was observed 24 h later.

The migration assay and Matrigel invasion assays was performed using a 24-well transwell chamber with a pore size of 8 μm (Corning, USA) as previously described [33]. First, 10^5 SW837 cells were seeded into the upper chamber in serum-free medium. Stromal cell-derived factor-1α (SDF-1, Sigma-Aldrich, 50 ng/ml) was added to the lower chamber. After a 24-h incubation at 37°C, the cells that had traversed the membrane were fixed in methanol and stained with 0.05% crystal violet. For quantification, the number of migrated cells was calculated by counting at least five random separate fields for determination of the ratio of the experimental samples to the control samples × 100.

Immunofluorescence staining and electron microscopy

Immunofluorescence staining was performed as previously described [11]. The primary antibodies used were as follows: Yap (1:1500, Cell Signaling Technology, #8418), JNK (1:1000, Abcam, #ab124956), F-actin (1:500, Abcam, #ab205), tubulin (1:1000, Abcam, #ab7291), Drp1 (1:500, Cell Signaling Technology, #3455), HtrA2/Omi, (1:500, Abcam, #ab21307). DAPI (Sigma-Aldrich), lysosome stain, and a mitochondrion-selective MitoFluor ™ stain (Molecular Probes, Burlington, ONT, CA) were used to mark the nucleus, lysosome, and mitochondria, respectively [37].

Electron microscopy was used to observe the ultrastructure of the mitochondria. Samples were dehydrated using acetonitrile and graded methanol, embedded in epoxy resin (EMbed-812; Electron Microscopy Sciences, USA) and polymerized at 70°C overnight. A Hitachi H600 Electron Microscope (Hitachi, Japan) was used to capture the images.

Mitochondrial membrane potential (ΔΨm), mROS staining, mPTP opening, oxygen consumption rate (OCR) and respiratory chain complex activities Assays

The mitochondrial functions were evaluated by ΔΨm, mPTP opening, mitochondrial OCR and respiratory chain complex activities as previously described [10]. The mitochondrial transmembrane potential was analyzed using a JC-1 Kit (Beyotime, China). Images were captured using a fluorescence microscope (OLYMPUS DX51; Olympus, Tokyo, Japan) and were analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD) to obtain the mean densities of the regions of interest, which were normalized to those of the control group [38].

Mitochondrial reactive oxygen species (mROS) measurements were performed using a MitoSOX red mitochondrial superoxide indicator (Molecular Probes, USA). Staining with 10-N-nonyl acridine orange (NAO; 2 mmol/L, Molecular Probes) was used [39].

The opening of the mPTP was visualized as the rapid dissipation of tetramethylrhodamine ethyl ester fluorescence. Arbitrary mPTP opening time was determined as the time when tetramethylrhodamine ethyl ester fluorescence intensity decreased by half between the initial and residual fluorescence intensity as previously described. The cellular ATP levels were measured using a firefly luciferase-based ATP assay kit (Beyotime) based on a fluorescence technique (Genmed Scientifics Inc.) according to the manufacturer's protocol [40].

The mitochondria test compounds oligomycin, p-trifluoro-methoxy carbonyl cyanide phenylhydrazone (FCCP), and rotenone were preloaded in the reagent delivery ports A, B and C of the O2 sensor cartridge. OCR measurements were then performed according to the Seahorse assay protocol. Complex I, II, and V activity was measured as previously described [41].

Caspase3/9 activity, LDH release and TUNEL assay

Caspase 3/9 activity and LDH release were measured via a commercial kit (Beyotime) as previously described [32]. TUNEL assay was used to assess the apoptotic cells according to the manufacturer's protocol. The degree of apoptosis was calculated as the number of TUNEL-positive cells per 500 cell nuclei.

Yap-mutant vector construction and transfection

Transfection of plasmids was performed using Turbofect (Thermo Scientific, MA, USA) or Attractence transfection reagent (QIAGEN, Valencia, CA). Yap has 504 amino acids (aa), including an N-terminal TEAD-binding domain (TBD, 48-102 aa), a WW domains (WW, 171-263 aa) and a C-terminal transcriptional activation domain (TAD, 292-488 aa) [38]. Therefore, three types of YAP mutants were constructed via base deletion to generate *mutant-Yap* (102-504 aa), which lacks the TBD, *mutant-Yap* (263-504 aa), which lacks the TBD and WW, and *mutant-Yap (1-263 aa),* which lacks the TAD according to a previous study [8]. For lentiviral packaging, the three PCR-amplified mutant Yaps were cloned into pCMV vectors. Then, the above vectors were triple-transfected into 293T cells using Lipofectamine 2000. After transfection for 48 h, the supernatant fraction containing lentiviral particles was collected. Following amplification, the supernatant was acquired and filtered, and then applied to infect SW837 cells as previously described [11].

Statistical analysis

The data are described as the means ± standard deviation (SD) of at least three independent experiments and were analyzed by one-way analysis of variance (ANOVA). The limit of statistical significance between the treated and control groups was $P<0.05$.

Results

Deletion of Yap impairs cellular viability and mobilization

To investigate the role of Yap in the development of RC, we first used western blotting to examine Yap expression in the human rectal cancer cell line SW837. Our results showed that abundant Yap expression was observed in SW837 cells, but not in a normal human rectal mucosa cell line (FHC) (Fig. 1A-B). We thus evaluated whether Yap played a causal role in regulating rectal cancer cell phenotypes. We first stably knocked down Yap expression using a lentivirus vector–based shRNA technique in SW837 cells. The knockdown efficiency was confirmed by western blotting (Fig. 1A-B). Yap's ability to affect cell proliferation, migration and invasion was tested. MTT assays showed that silencing Yap in SW837 cells reduced cellular viability (Fig. 1C). Moreover, CCK8 assays indicated that Yap deficiency significantly limited cellular proliferation (Fig. 1D). In a transwell migration assay, Yap-depleted SW837 cells migrated approximately three times less than control cells (Fig. 1E and G), which is consistent with the observations that knocking down Yap potently suppresses SW837 cell invasiveness (Fig. 1F and H). These results suggested that Yap was upregulated in

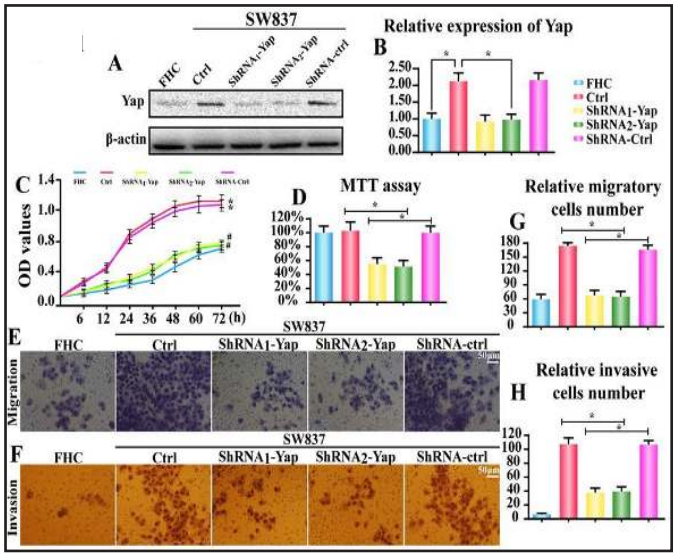

Fig. 1. Knockdown of Yap inhibits the cellular viability, migration and invasion of human rectal cancer cells. A-B. SW837 cells stably expressing the Yap-shRNA vector had lower Yap expression than the control-shRNA group and normal human rectal cells. C. The cellular viability was assessed by MTT assay. Loss of Yap suppressed cellular viability. D. Growth curves of SW837 cells with or without Yap deletion. Yap deficiency alleviated the proliferative capacity of SW837. E Representative photographs of SW837 migration via transwell assay are presented. F. Invasion assays in SW870 cells with or without Yap deletion. The relative numbers of migratory cells (G) and invasive cells (H) were counted. *P<0.05.

RC and that increased Yap promotes RC proliferation, migration and invasion *in vitro*.

Yap deletion induces mitochondrial fission via activating JNK/Drp1 pathways

Mitochondria underlie the development of cancer; however, limited evidence is available concerning the role of mitochondrial fission in RC. We asked whether Yap modulated RC viability and migration/invasion via fission. As shown in Fig. 3A-B, compared to the control group, silencing Yap in SW837 cells markedly caused increased mitochondrial debris with shorter lengths. Given that fission was activated by Drp1, which shuttles from the cytoplasm on the surface of mitochondria in the process of mitochondrial fission, we determined whether Yap could regulate Drp1 translocation on mitochondria. Through analysis of the localization of mitochondria and Drp1 by immunofluorescence (Fig. 2C), we found that loss of Yap stimulated colocalization of Drp1 and mitochondria. Moreover, fragmented mitochondria were labeled by more Drp1 in the amplification panel (Fig. 2C). These data indicate that Yap deficiency initiates fission. Drp1 is regulated via post-transcriptional modification through phosphorylation at Ser616 (the active form) and dephosphorylation at Ser637 (the inactive form). We evaluated the influence of Yap on Drp1 phosphorylation. As shown in Fig. 2D-F, compared to the control group, loss of Yap increased the level of p-Drp1^{S616} but diminished the level of p-Drp1^{S637}, indicative of Drp1 activation.

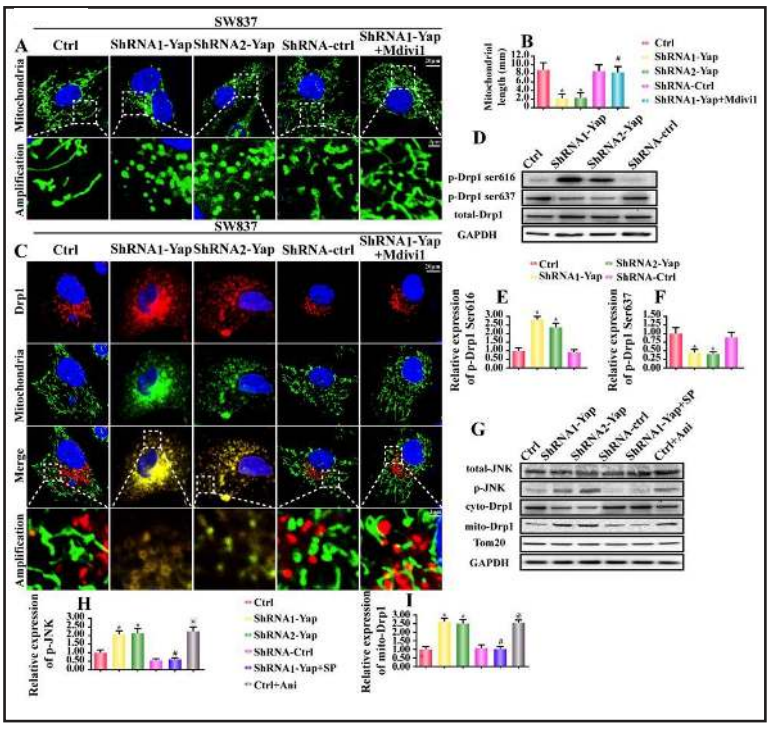

Fig. 2. Yap regulates mitochondrial fission via the JNK/Drp1 pathways. A. Mitochondrial morphology with Yap deletion. Many balloon or bulb-like structures were observed at the base of the mitochondrial tubules in the control group, but the mitochondria in Yap RNAi-treated cells had more free ends than those in control cells. Mdivi1 is an inhibitor of Drp1 that shuttles from the cytoplasm to the mitochondria, leading to inhibition of Drp1-mediated mitochondrial fission. B. The mitochondrial length was determined to quantify mitochondrial fission. C. Co-localization of Drp1 and mitochondria. In the control group, fewer Drp1 foci on the mitochondria were observed, and the mitochondria maintained almost a normal morphology with fewer fragments. However, Yap deletion caused Drp1 translocation to the fragmented mitochondria. The boxed areas under each micrograph represent an increased magnification of the white square. D-F. Western blot analysis of phosphorylated Drp1 (p-Drp1 at Ser 616 and Ser 637). G-I. The regulatory effects of JNK on Drp1 migration in mitochondria. SP600125 (SP) is an inhibitor of the JNK pathway, which not only reduced the expression of p-JNK but also impaired Drp1 migration from the cytoplasm to the mitochondria. Anisomycin (Ani) is an activator of the JNK pathway, which upregulated the p-JNK as well as mito-Drp1 levels. *P<0.05 vs. control group; #P<0.05 vs. shRNA-Yap group.

It has recently been hypothesized that phosphorylated JNK acts as an inducer of Drp1 activation [27] and that Yap has the ability to regulate JNK phosphorylation [25]. To

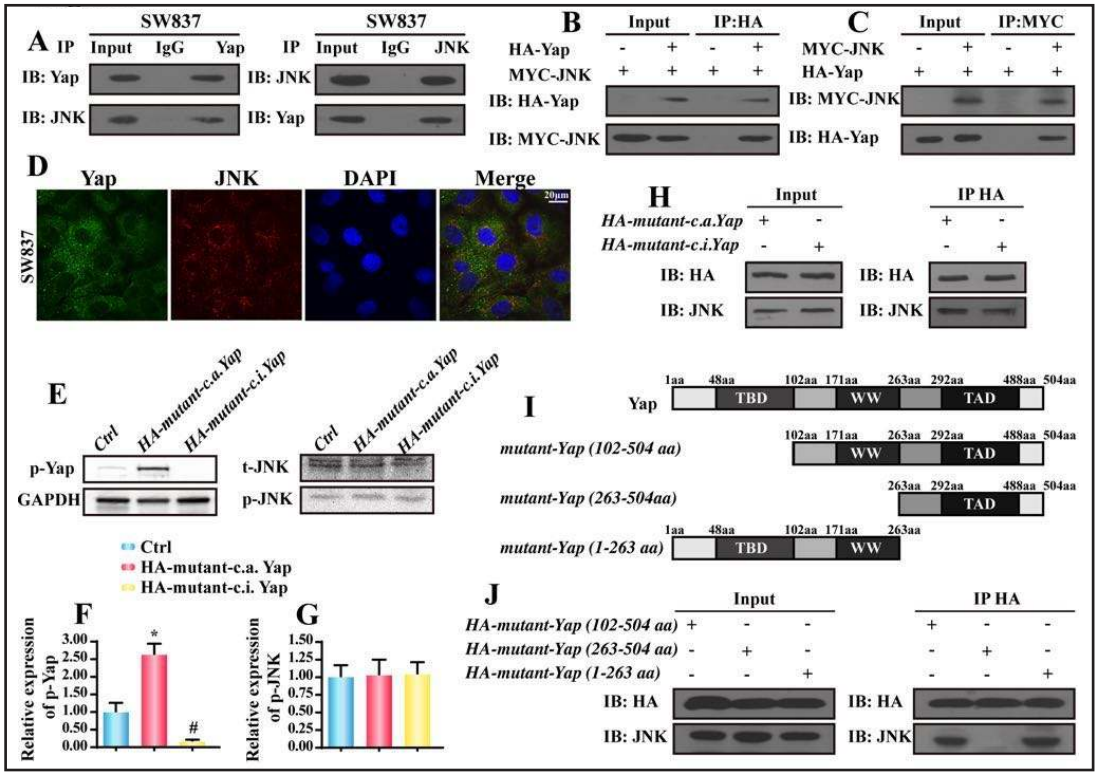

Fig. 3. Yap interacted with JNK via its WW domains independent of its phosphorylation. A. Cell lysates from SW837 cells were immunoprecipitated with anti-Yap or anti-JNK antibodies, followed by immunoblotting with anti-JNK or anti-Yap antibodies. IgG was used as a control. B-C. Immunoblotting analysis of lysates after immunoprecipitation from HEK293T cells transfected with Myc-JNK and HA-Yap. D. Cytoplasmic localization of JNK-Yap interaction was observed in SW837 cells. Confocal microscopy showed approximately 80% colocalization of JNK and Yap in the cytoplasm of SW837 cells. E-G. Two YAP mutant constructs, constitutively active HA-YAP (c.a.YAP), with serine 127 replaced with aspartic acid (permanent phosphorylation of residue 127), and constitutively inactive HA-Yap (c.i.Yap), with serine 127 replaced with alanine (cannot be phosphorylated), were used to transfect SW837 cells. c.a.Yap induced phosphorylation of Yap, but c.i.Yap limited Yap phosphorylation. However, both c.a.YAP and c.i.YAP had no effect on JNK phosphorylation compared with a control group. H. Co-IP assays also indicated that both c.a.YAP and c.i.YAP interacted with JNK. I. Map of the regions involved in Yap binding with JNK; structures of deletion mutants of Yap. YAP mutants were constructed via base deletion to generate mutant-Yap (102-504 aa), which lacks the TBDl mutant-Yap (263-504 aa), which lacks the TBD and WW domains; and mutant-Yap (1-263 aa), which lacks the TAD. J. Immunoprecipitation and immunoblot analysis of cell lysates from SW837 cells with deletion mutants of HA-Yap. The experiments were performed at least three times, and representative images of the blots are presented. *P<0.05 vs. control group; #P<0.05 vs. c.a.YAP group.

determine whether Yap influences Drp1-mediated fission via JNK, we first assessed changes of JNK. In the control group, little JNK phosphorylation was detected, whereas loss of Yap significantly increased the p-JNK levels (Fig. 2G-I), suggesting an inhibitory effect of Yap on JNK phosphorylation. Furthermore, to establish whether phosphorylated JNK had the ability to modify Drp1, an inhibitor (SP600125) and activator (Anisomycin) of JNK were used. After blockade of JNK via SP600125 in Yap-deleted SW837 cells, the active form (p-Drp1^{S616}) was reduced, while the inactive form (p-Drp1^{S637}) was increased (Fig. 2G-I), suggestive of Drp1 inhibition. By contrast, in normal SW837 cells, the agonist of JNK (Anisomycin) activated Drp1, as indicated by higher p-Drp1S^{616} but lower p-Drp1^{S637} levels (Fig. 2G-I). Taken together, these data illustrate that Yap acts to preserve mitochondrial fission homeostasis via inactivation of JNK and Drp1, leading to fission inhibition.

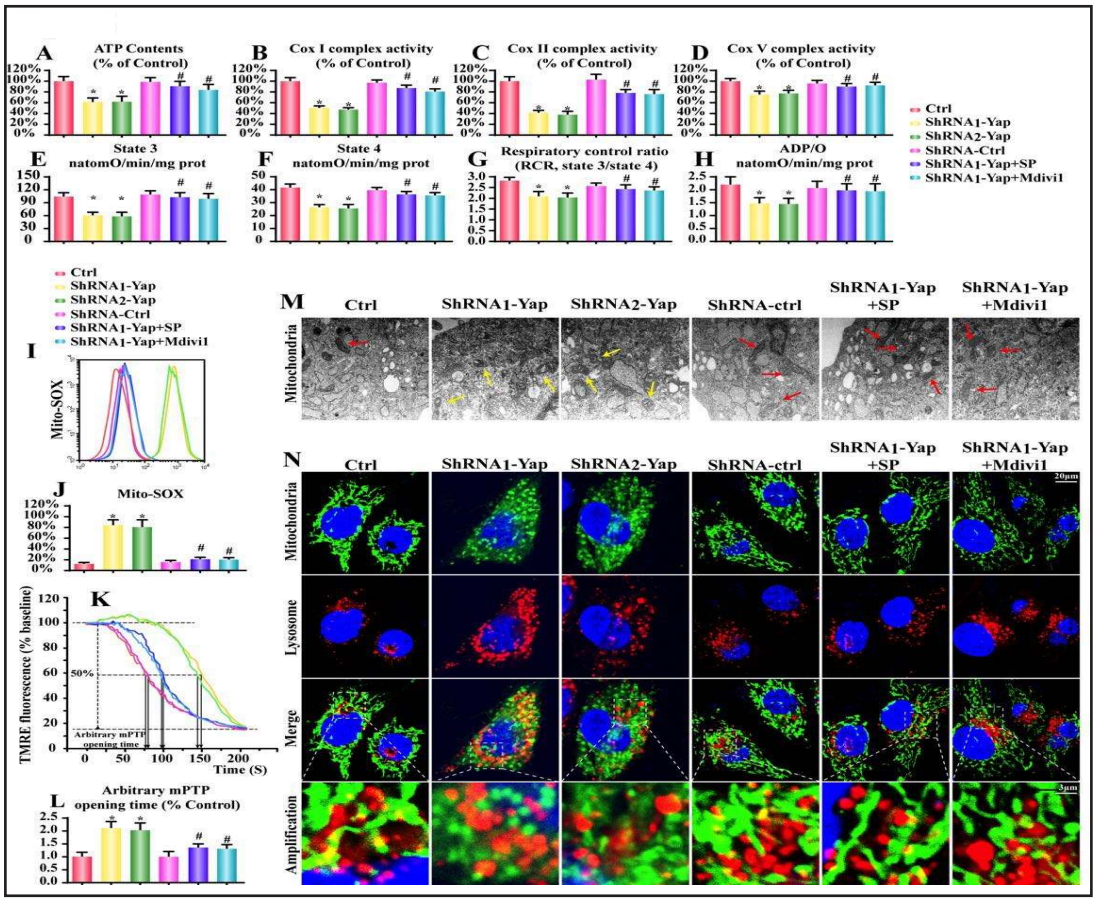

Fig. 4. Knockdown of Yap suppressed mitochondrial structure and function via the JNK/Drp1 pathway. A. Change in ATP levels. B-D. Changes in the ETC I, II, and V activities measured spectrophotometrically. E-H. Effects of Yap deletion on state 3 respiration, state 4 respiration, respiratory control ratio (RCR [state 3/state 4]), and nmol of ADP phosphorylated to atoms of oxygen consumed (ADP/O). I-J. Mitochondrial reactive oxygen species levels. The curve chart indicates the quantitative flow cytometry results. K-L. Arbitrary mPTP opening time assessed by tetramethylrhodamine ethyl ester (TMRE) fluorescence. The arbitrary mPTP opening time was determined as the time when the TMRE fluorescence intensity decreased by half between the initial and residual fluorescence intensities. M. Representative transmission electron microscopy (TEM) images of morphological changes in mitochondria with or without the Yap deficiency. Red arrow: normal mitochondria that exhibit a spindle shape. Yellow arrow: dysregulated mitochondria. N. Co-localization of mitochondria and lysosomes. Yap deficiency blunted the lysosome-mediated removal of fragmented mitochondria. Inhibition of JNK or fission reintroduced the overlap between fragmented mitochondria and lysosome. *P<0.05 vs. control group; #P<0.05 vs. shRNA-Yap group.

Yap interacts directly with JNK via its WW domains

To study the mechanism by which Yap inactivates JNK activity in RC, we determined whether Yap interacted with JNK. Surprisingly, coimmunoprecipitation (co-IP) assays showed a constitutive interaction between endogenous Yap and JNK in SW837 cells (Fig. 3A). Consistent with these results, an exogenous interaction between Yap and JNK was also observed in HEK293T cells co-transfected with Myc-JNK and HA-Yap (Fig. 3B-C). Considering that JNK often resides in the cytoplasm of cancer cells, we hypothesized that the Yap-JNK interaction occurs in the cytoplasm. Indeed, confocal microscopy showed that approximately 80% co-localization of Yap and JNK in the cytoplasm of SW837 cells (Fig. 3D). These results suggest that Yap can interact with JNK in the cytoplasm. As Yap can be phosphorylated at

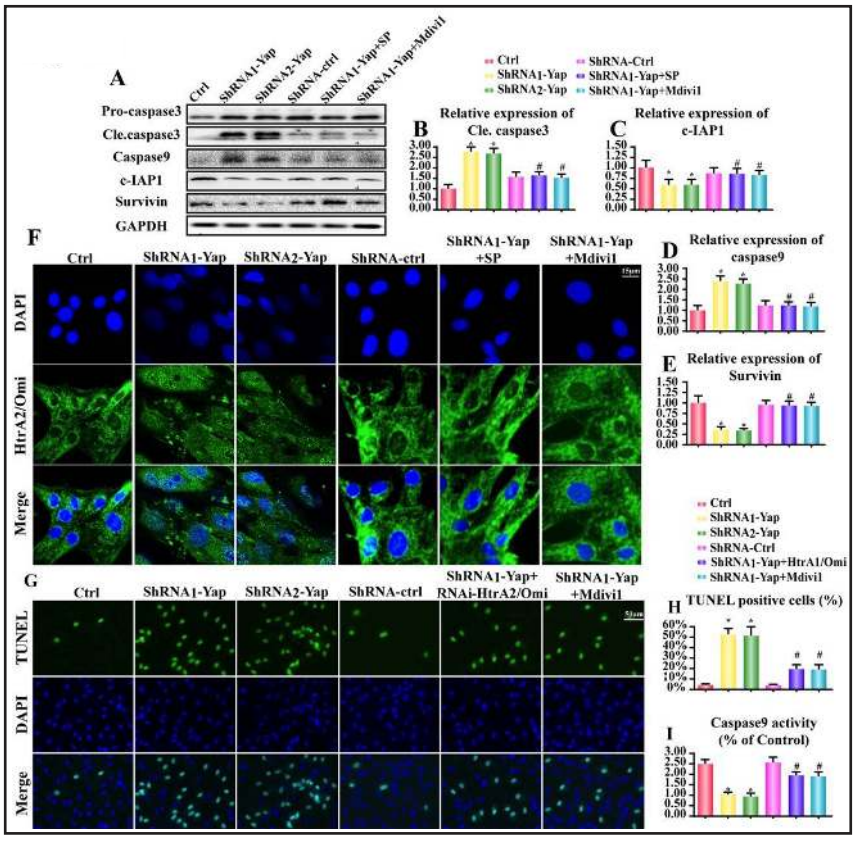

Fig. 5. Loss of Yap activated mitochondrial apoptosis by releasing HtrA2/Omi. A. Changes in mitochondria-related pro-apoptotic proteins. Inhibition of the JNK/Drp1 pathway alleviated Yap deficiency-mediated apoptosis. B-E. Quantification of the cleaved caspase 3, caspase 9, c-IAP and survivin levels. F. Yap deficiency caused more HtrA2/Omi leakage from the cytoplasm into the nucleus. G-H. In Yap-deleted SW837 cells, knockdown of HtrA2/Omi reduced the ratio of TUNEL-positive cells. I. Loss of HtrA2/Omi suppressed Yap deficiency-mediated caspase 9 elevation. *P<0.05 vs. control group; #P<0.05 vs. shRNA-Yap group.

Ser127, leading to its cytoplasmic sequestration, we asked whether phosphorylated Yap preferentially interacted with JNK. To evaluate this hypothesis, we constructed two Yap mutants: a constitutively active form HA-Yap (c.a.Yap), with the serine at residue 127 replaced with aspartic acid (mimicking permanent phosphorylation of Ser127), and a constitutively inactive form HA-Yap (c.i.YAP), with the serine at residue 127 replaced with alanine (unable to be phosphorylated at Ser127). After transfection with c.a.Yap and c.i.Yap in SW837 cells, we first examined Yap phosphorylation. The c.a.Yap significantly increased Yap phosphorylation at residue 127, whereas c.i.Yap suppressed p-YapS127 (Fig. 3E-F). Next, we measured the changes in JNK phosphorylation in the presence or absence of permanently phosphorylated Yap. The results shown in Fig. 3G indicated that both c.a.Yap and c.i.Yap had no influence on the level of JNK phosphorylation compared with normal SW837 cells, suggesting that Yap phosphorylation has no effect on JNK activation. Moreover, the co-IP assay also indicated the both c.a.YAP and c.i.YAP interacted with JNK (Fig. 3H), reconfirming that Yap phosphorylation was not the primary determinant of the YAP-JNK interaction.

To further explore the molecular basis of the interaction of Yap with JNK, we defined the regions of Yap that were required for their interaction. First, a series of Yap deletion mutants (Fig. 3J) were cotransfected with YAP into HEK293T cells. Co-IP assays revealed that the Yap mutant (102-504 aa) and Yap mutant (1-263 aa) interacted with JNK, whereas the Yap mutant (263–504 aa) abolished this particular interaction (Fig. 3J), indicating that the region (102–263 aa) covering the WW domains in Yap is indispensable for the interaction with JNK. Collectively, these data demonstrate that Yap interacts with JNK via its WW domains, independent of its phosphorylation status.

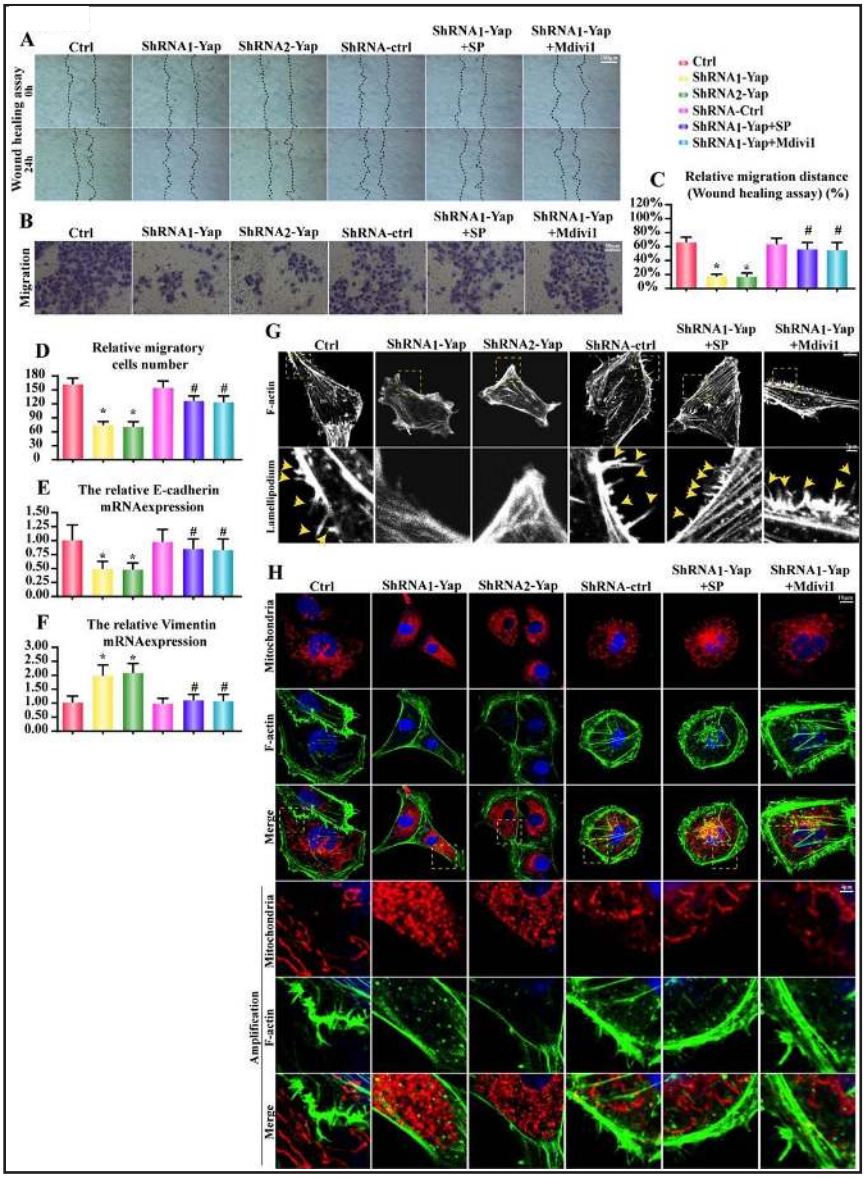

Fig. 6. Loss of Yap impaired human rectal cancer cell migration via the JNK/Drp1 pathways. The cellular mobilization capacity was examined via the wound-healing assay (A) and transwell assay (B). C. Quantification of the relative migration distance in wound-healing assays. Yap depletion resulted in an inability to mobilize in SW837 cells, while inhibition of JNK/Drp1 reversed the mobilization inhibition seen under Yap deficiency. D. Quantification of migrated cells in transwell assays. E-F. Changes in EMT markers. The levels of the endothelial marker E-cadherin and mesenchymal marker vimentin were analyzed using qPCR. YAP maintained the E-cadherin levels and suppressed the vimentin levels in a JNK/Drp1-dependent manner. G. Lamellipodia were observed by confocal microscopy via F-actin staining. Arrowheads indicate lamellipodia. Loss of Yap limited the formation of lamellipodia. However, inhibition of the JNK/Drp1 pathways restored the formation of lamellipodia in Yap-deleted SW837 cells. H. Co-staining of mitochondria and F-actin. Loss of Yap caused mitochondrial fragmentation and impaired F-actin homeostasis, as demonstrated by the disappearance of filiform F-actin in cytoplasm. The cytoplasmic F-actin collapse led to the failure of SW837 cells to generate lamellipodia around the cell membrane. The boxed areas under each micrograph represent the increased magnification of the white square. *P<0.05 vs. control group; #P<0.05 vs. shRNA-Yap group.

Loss of Yap damages mitochondrial structure and function

Because Yap activity played a role in the regulation of mitochondrial fission, based on the above results, we asked whether Yap also influenced mitochondrial structure and function. First, we evaluated the ability of Yap to sustain ATP production. Silencing of Yap suppressed the ATP levels in SW837 cells (Fig. 4A). Furthermore, the decline in ATP production in response to Yap deficiency was functionally attributed to the decreased activity of electron transport chain complexes (ETC) (Fig. 4B-D). Loss of Yap reduced the state 3/4 respiratory

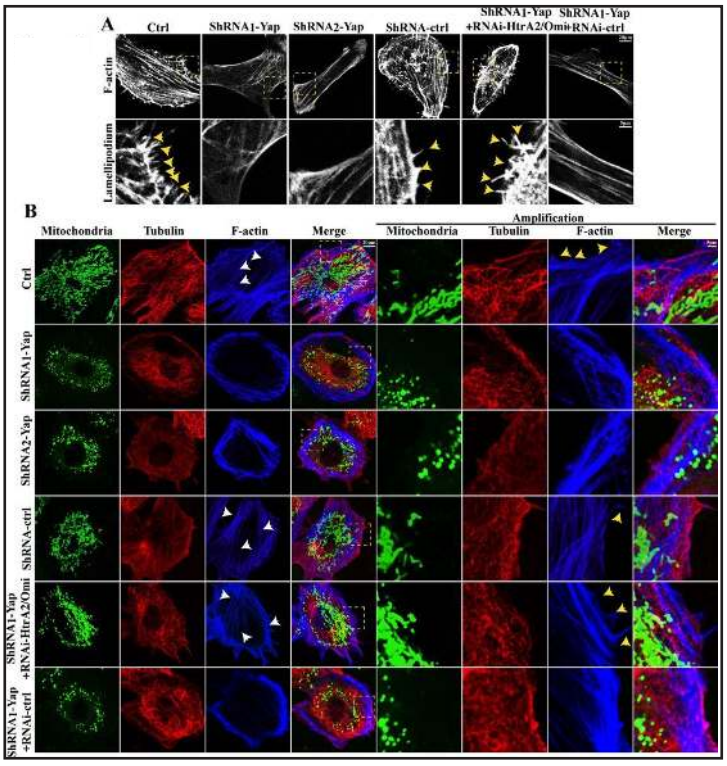

Fig. 7. Loss of YAP induced cytoskeletal degradation via HtrA2/Omi, which mediated cofilin phosphorylation. A. Knockdown of HtrA2/Omi restored lamellipodia formation in Yap-deleted SW837 cells. Arrowheads indicate lamellipodia. B. Co-staining of mitochondria, F-actin and α-tubulin. Yap deficiency primarily mediated F-actin degradation, as demonstrated by the disappearance of filiform F-actin in the cytoplasm. However, knockdown of HtrA2/Omi restored the F-actin structure in the cytoplasm. Notably, Yap and HtrA2/Omi had no influence on α-tubulin, suggesting that microfilaments (F-actin/G-actin) but not microtubules (α-tubulin/β-tubulin) were the target of the Yap/JNK/Drp1/fission/HtrA2/Omi axis. White arrowheads indicate F-actin collapse as indicated by the disappearance of clear and arranged F-actin. Yellow arrowheads indicate lamellipodia. C. HtrA2/Omi caused increased cofilin phosphorylation, the inactive form of cofilin, which was associated with F-actin degradation into G-actin. *P<0.05 vs. control group; #P<0.05 vs. shRNA-Yap group, @P<0.05 vs. shRNA-Yap+RNAi-HtrA2/Omi group.

rate (Fig. 4E-F), ADP phosphorylation (respiratory control ratio) (Fig. 4G) and efficiency of ATP synthesis (ADP/O) (Fig. 4H). Moreover, these changes were coupled with increased mROS accumulation (Fig. 4I-J). Additionally, loss of Yap also caused mPTP opening (Fig. 4K-L). Furthermore, we also used EM to observe the ultrastructural changes in the mitochondria associated with Yap deficiency [42]. Normal mitochondria exhibited a spindle shape (Fig. 4M, red arrows). However, in response to Yap silencing, the characteristic reticulo-tubular mitochondrial morphology disintegrated into numerous round fragments of varying sizes with abnormal appearances, such as swelling, disorganization and reduction, or complete loss of the cristae in several cells (bottom panel, Fig. 4M, yellow arrows). However, inhibition of fission via Mdivi1 or blockade of JNK via SP600125 alleviated the mitochondrial structural and functional damage in response to Yap deficiency, suggesting that Yap is a regulator of mitochondrial structure/function via inhibition of JNK/Drp1/fission. Finally, several studies have shown that selective mitochondrial autophagy (mitophagy) forms an essential axis of quality control of the mitochondria. Cells utilize mitophagy as a major strategy to eliminate dysfunctional mitochondria to prevent mitochondrial apoptosis. Based on this, we asked whether Yap had a regulatory role in mitophagy. Loss of Yap triggered increased levels of mitochondrial debris that could not be engulfed by lysosomes compared to the control group (Fig. 4N), suggestive of mitophagy inhibition in the context of the Yap deficiency.

Yap deletion activates apoptosis by releasing HtrA2/Omi by activation of the JNK/Drp1/fission pathways

Mitochondria serve as messengers to transmit apoptosis signals. To explore whether Yap regulates mitochondria-related apoptosis, we first examined proteins related to mitochondrial apoptosis. As shown in Fig. 5A-E, loss of Yap increased the levels of caspase 9 and caspase 3, suggesting that Yap inhibited mitochondrial apoptosis. Furthermore, pro-

apoptotic factors (such as cyt-c and HtrA2/Omi) were released by mitochondria into the cytoplasm, where they activate caspase 9 and trigger subsequent caspase 3 activation [43]. Notably, the majority of studies to date have focused on cyt-c, with scant attention focused on HtrA2/Omi. In our study, we found that the Yap deficiency promoted HtrA2/Omi leakage into the cytoplasm, as measured by immunofluorescent staining (Fig. 5F). Moreover, inhibition of fission or JNK limited HtrA2/Omi diffusion, suggesting that the JNK/Drp1/fission pathways were instrumental to HtrA2/Omi leakage. Furthermore, to determine whether HtrA2/Omi was the key element responsible for Yap deficiency-mediated apoptosis, we used siRNA to knockdown HtrA2/Omi. After ablation of HtrA2/Omi, caspase 9 activity was reduced in YAP-silenced SW837 cells (Fig. 5I). The TUNEL assay results were also consistent with these results (Fig. 5G-H). Collectively, these data suggest that the Yap deficiency mediates a pro-apoptotic effect in RC by inducing HtrA2/Omi detachment from mitochondria by activation of the JNK/Drp1/fission pathways.

Yap deficiency blunts migration via JNK/Drp1/fission pathways

In addition to cellular survival, cellular migration is vital for the development of RC. Therefore, we determined whether Yap played a role in the regulation of cellular mobilization via wound assays. As shown in Fig. 6A and C, loss of Yap impaired cellular mobilization. Consistently, transwell assays also revealed an inability of Yap-deleted SW837 cells to mobilize (Fig. 6B and D). However, inhibition of fission or JNK reversed the migration capacity of SW837 cells.

Given the evidence linking epithelial-to-mesenchymal transition (EMT) to cancer cell migration and invasion, we determined whether Yap was implicated in EMT via the JNK/Drp1/fission axis [44]. To explore this, expression of the epithelial marker E-cadherin and mesenchymal marker vimentin were analyzed using qPCR. The results revealed that silencing Yap reduced E-cadherin expression, but increased vimentin expression (Fig. 6E-F). By contrast, Mdivi1 and SP600125 treatment had the opposite effects as Yap deletion. These results indicated an important role for Yap in modulating EMT via the NK/Drp1/fission axis [45].

Importantly, apart from EMT, the formation of lamellipodia via F-actin is indispensable for cancer cell invasion [46, 47]. In our study, we found that loss of Yap hampered lamellipodia formation (Fig. 6G, yellow arrows). However, Mdivi1 and SP600125 treatment increased the numbers of lamellipodia (Fig. 6G, yellow arrows). Furthermore, we used mitochondria and F-actin co-staining to observe the role of mitochondrial fission in lamellipodia. As shown in Fig. 6H, control spindle mitochondria were surrounded by clear and regular F-actin with several lamellipodia outside of the cell. However, mitochondrial debris was encircled by disorganized F-actin with few lamellipodia. Furthermore, inhibition of fission or JNK reversed the mitochondria and F-actin morphological changes, as well as promoted the formation of lamellipodia.

Loss of Yap induces cytoskeleton degradation via HtrA2/Omi

Cellular membrane extension in lamellipodia is driven predominantly through F-actin polymerization. Several studies have hinted that F-actin polymerization could be regulated by HtrA2/Omi. Given that HtrA2/Omi was released into the cytoplasm with Yap deficiency via the JNK/Drp1/fission pathways as shown by our results, we wondered whether Yap loss played a causal role in F-actin collapse via HtrA2/Omi. We silenced HtrA2/Omi in Yap-deleted cells via siRNA. After knockdown of HtrA2/Omi, Yap-deleted cells displayed more clear and regular F-actin, with several lamellipodia outside of cells (Fig. 7A). Additionally, co-staining of F-actin and mitochondria further suggested that HtrA2/Omi had the ability to induce the collapse of F-actin (Fig. 7B). Malformed or absent F-actin appeared in Yap-deleted cells with mitochondrial debris. However, although deletion of HtrA2/Omi was able to maintain the structure of F-actin, it had no significant influence on mitochondrial debris. To further determine whether F-actin was specifically regulated by Yap and HtrA2/Omi, we also co-stained for tubulin, another element of the cytoskeleton that constitutes microtubules

and promotes intracellular transport but has no role in cellular migration. As shown in Fig. 7B, compared to normal cells, loss of Yap had no influence on the tubulin structure. Taken together, these data indicate that Yap controls F-actin homeostasis via HtrA2/Omi.

F-actin is composed of G-actin. Our study found that the loss of Yap caused increased F-actin disassembly into G-actin, as shown in Fig. 7C-F. However, knockdown of HtrA2/Omi reversed these conformational changes. A critical upstream factor regulating the balance of F-actin/G-actin is cofilin. Dephosphorylated cofilin promotes G-actin assembly into F-actin at the front of the cell, leading to the formation of lamellipodium, whereas phosphorylation of cofilin caused disturbances in F-actin synthesis, even inducing F-actin depolymerization into G-actin. As a consequence of F-actin collapse, lamellipodium-based migration was blunt. Given that HtrA2/Omi functions as a kinase and has the ability to induce protein phosphorylation, we determined whether HtrA2/Omi phosphorylated cofilin and subsequently induced F-actin depolymerization. As shown in Fig. 7C-F, loss of Yap elevated the level of phosphorylated cofilin, which was suppressed in response to HtrA2/Omi knockdown, suggesting that HtrA2/Omi induced cofilin phosphorylation-mediated inactivation. Collectively, out data illustrate that Yap loss is associated with HtrA2/Omi leakage, which disassembles F-actin to G-actin via induction of cofilin phosphorylation, ultimately leading to lamellipodium collapse and migration inhibition.

Discussion

Our results clearly show a tumor-promoting mechanism involving Yap in RC (Fig. 8). Overall, Yap expression is upregulated in RC. Elevated Yap has the ability to interact directly with JNK and induce JNK inactivation via dephosphorylation, blocking Drp1-mediated mitochondrial fission. Due to the inhibitory role of Yap on fission, Yap maintains the structure and function of mitochondria and alleviates the HtrA2/Omi leakage caused by the JNK/Drp1/fission pathways, limiting cellular apoptosis. Notably, HtrA2/Omi inhibition also perturbed RC migration and invasion. Reduced HtrA2/Omi leakage maintained the F-actin balance and lamellipodia formation via modification of cofilin. The key findings in the present study are that the JNK-Drp1-mitochondrial fission-HtrA2/Omi axis is a novel downstream molecular signaling mechanism of the Hippo-Yap pathway in RC tumorigenesis and metastasis, which highlights a new therapeutic targeting pathway for treating rectal cancer by targeting the Hippo–Yap/JNK/Drp1/mitochondrial fission/HtrA2/Omi axis.

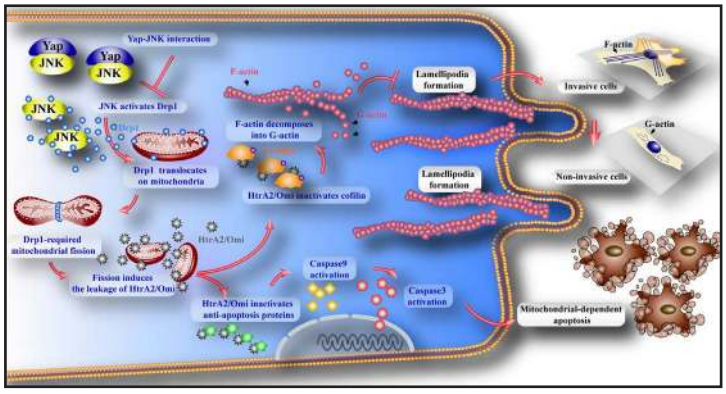

Fig. 8. The role of the Hippo–Yap/JNK/Drp1/mitochondrial fission/HtrA2/Omi axis in RC apoptosis and migration. Elevated Yap has the ability to interact directly with JNK and induce JNK dephosphorylated inactivation, blocking Drp1-mediated mitochondrial fission. Due to the inhibitory role of Yap on fission, Yap maintained the structure and function of mitochondria and alleviated the HtrA2/Omi leakage caused by the JNK/Drp1/fission pathways, inhibiting cellular apoptosis. Notably, HtrA2/Omi inhibition also maintained the F-actin balance and lamellipodia formation via modification of cofilin, ultimately promoting RC migration and invasion.

Yap has been implicated in various cancers [18, 19] and is often overexpressed in more aggressive cancers. Components of the Hippo–Yap pathway, such as Mst1/2, Lats1/2 and YAP, are deregulated in various human cancers, and their expression levels and activities correlate with tumor development and progression [21]. However, the molecular signals that

lie downstream of the Hippo-Yap pathway in cancer remain largely uncharacterized. Our present study found that mitochondrial fission was a novel sequential signal of the Hippo-Yap pathway in RC tumorigenesis and metastasis [48]. Yap functions through its direct interaction with JNK to suppress Drp1-mediated mitochondrial fission, sustaining RC growth and metastasis. Mechanistically, the interaction between Yap and JNK maintained JNK dephosphorylation, which prevented p-JNK accumulation. Furthermore, this inhibitory effect subsequently abolished Drp1 activation and mitochondrial fission. By contrast, disruption of the Yap-JNK interaction led to RC apoptosis and migration inhibition in a mitochondrial fission-dependent manner. These findings provide new information concerning the role of Yap in RC development, emphasizing the significant potential clinical applications regarding the Yap-mitochondrial fission pathway to treat RC. Moreover, we also determined that the Yap-JNK interaction depends on the Yap WW domains. Previous studies [49] have shown that Yap interacts with AMOT p130 via the Yap WW domains. Similarly, our findings suggest that the region (102–263 aa) covering the WW domains in Yap is indispensable for its interaction with JNK. Notably, Yap phosphorylation had no influence on its interaction with JNK, suggesting that Yap regulates RC survival and migration independent of its phosphorylation. The reported tumor-promoting effects of Yap have been attributed to its dephosphorylated nuclear localization, where it acts as a transcription co-activator to induce the expression of several oncogenes [50]. Our data substantiate the effects of the Yap level but not its localization in RC survival and migration. In fact, a growing body of research has suggested that Yap nuclear translocation is unlikely to fully explain the role of Yap in cancer development [50, 51] and that Yap nuclear localization accounts for only a small portion of cancer progression [22]. Additionally, several studies have confirmed that the mRNA levels of Yap (and TAZ) sufficiently correlate with target gene expression and are significantly associated with cancer progression [7, 23]. Meanwhile, an increasing number of studies have also shown that Yap has the ability to interact with tumor suppressors in the cytoplasm [52], supporting the idea that excessive expression of Yap is actually a more important factor in tumorigenesis regardless of Yap nuclear localization or cytoplasmic retention [7, 8].

We hypothesized that the JNK/Drp1/fission pathways were the primary damage signals abated by Yap to preserve RC survival and migration. In previous studies, we have demonstrated that excessive fission is the main pro-apoptotic mechanism mediating cellular programmed death [10, 11]. Under normal conditions, mitochondria undergo continual fusion and fission to maintain the cellular energetic demands. A shift to excessive fission causes an increase in non-functional mitochondria debris [48], and this mitochondrial debris contains pro-apoptotic factors, such as cyt-c, Smac and HtrA2/Omi, which disrupt the balance between cellular survival and apoptosis [53]. In this study, we found that loss of Yap triggered the leakage of HtrA2/Omi from mitochondria into the cytoplasm via activation of mitochondrial fission, leading to a marked drop in anti-apoptotic proteins. The previous studies have determined the mechanism by which fission promoted cyt-c release [10]. They found that mPTP opening and cardiolipin oxidation were key events evoking cyt-c leakage [54]. In the present study, we determined that HtrA2/Omi is a novel factor leaking from mitochondria. Unlike cyt-c, which is detained by cardiolipin but liberated by oxidized cardiolipin, HtrA2/Omi freely resides in the mitochondrial intermembrane space [29]. Thus, compared to cyt-c, HtrA2/Omi is more prone to leakage into the cytoplasm in response to mitochondrial damage. After detachment from mitochondria, the cytoplasmic HtrA2/Omi cleaves and neutralizes the anti-apoptotic proteins [55], leading to down-regulation of c-IAP and survivin. However, knockdown of HtrA2/Omi restored the level of anti-apoptotic factors. These data validate that mitochondrial fission is vital for RC homeostasis involving mitochondrial apoptosis.

Apart from the established role of fission in cellular apoptosis, we also found that Yap-mediated fission manipulated RC migration and invasion via HtrA2/Omi [4]. Loss of HtrA2/Omi augmented the numbers of lamellipodia and promoted RC mobilization and migration. Mechanistically, HtrA2/Omi had the ability to depolymerize F-actin into G-actin via phosphorylation of cofilin, leading to F-actin collapse and lamellipodia formation

impairment. Notably, previous studies have reported that the actin cytoskeleton is regulated by p53 via activation of HtrA2/Omi [56], which hampers cell invasion. However, our studies further characterized the mechanism by which HtrA2/Omi disturbs actin homeostasis [57]. HtrA2/Omi induced cofilin phosphorylation-mediated inactivation, leading to inhibition of actin recycling and renewal [58]. However, although HtrA2/Omi is a serine protease, we still did not know the molecular basis of the interaction between HtrA2/Omi and cofilin. Notably, we uncovered an indispensable role of Yap in RC migration and invasion, which enriched the tumor-promoting effects of Yap [59]. More importantly, we confirmed that fission is the main target of Yap required to sustain RC mobilization, suggesting that the role of mitochondrial signaling goes beyond their capacity to induce cellular apoptosis and may also be associated with cancer metastasis via F-actin homeostasis regulation by HtrA2/Omi.

In summary, the results of our report illustrate that Yap functions as a novel tumor promoter in rectal cancer development and progression. We identified a critical mechanism for Yap in the regulation of rectal cancer tumorigenesis and metastasis that operates through inhibition of the JNK/Drp1/mitochondrial fission/HtrA2/Omi pathways. Thus, novel therapeutic strategies to regulate the balance of the Hippo-Yap pathway and mitochondrial fission could improve the prognosis of rectal cancer in clinical practice.

Disclosure Statement

The authors have declared that they have no conflicts of interest.

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