



Original article

Heterozygous knockout insulin-like growth factor-1 receptor (IGF-1R) regulates mitochondrial functions and prevents colitis and colorectal cancer

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ABSTRACT

Although insulin-like growth factor-1 receptor (IGF-1R) has been accepted as a major determinant of cancers, its biological roles and corresponding mechanisms in tumorigenesis have remained elusive. Herein, we demonstrate that IGF-1R plays pivotal roles in the regulation of mitochondrial respiratory chain and functions during colitis and tumorigenesis. Heterozygous knockout IGF-1R attenuated azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced colitis and colitis associated cancer (CAC) in *Igf1r*^{+/-} mice. Heterozygous knockout IGF-1R confers resistance to oxidative stress-induced damage on colorectal epithelial cells by protecting mitochondrial dynamics and structures. IGF-1R low expression improves the biological function of mitochondrial fusion under oxidative stress. Mechanically, an increase in respiratory coupling index (RCI) and oxidative phosphorylation index (ADP/O) was seen in colorectal epithelial cells of *Igf1r*^{+/-} mice. Seahorse XF^e-24 analyzer analysis of mitochondrial bioenergetics demonstrated an increase in oxygen consumption rate (OCR) and a decrease of extracellular acidification rate (ECAR) in *Igf1r*^{+/-} cells. Further analysis suggests the protection mechanisms of *Igf1r*^{+/-} cells from oxidative stress through the activation of the mitochondrial respiratory chain and LKB1/AMPK pathways. These results highlight the biological roles of IGF-1R at the nexus between oxidative damage and mitochondrial function and a connection between colitis and colorectal cancer.

1. Introduction

Inflammatory bowel disease (IBD) *i.e.* ulcerative colitis (UC) has been identified as one of the important risk factors leading to colorectal cancer (CRC). Patients with IBD have a three-fold higher risk of CRC. Many reports have shown that an increased degree of inflammation and duration of disease largely increases the risk of CRC. Chronic IBD is characterized by colonic mucosal damage and ulceration, involving the rectum and colon [1]. Although the relationship between IBD and CRC has been established over time, their pathogenesis remains poorly understood. It is assumed that both genetic and environmental factors such as genetic instability, epigenetic alteration, and immune response by mucosal inflammatory mediators, oxidative stress, and intestinal

microbiota may contribute to the progression of this pathogenesis [2]. Among these risk factors, one of the major causes of genetic instability and cancer-associated protein post-translational modifications is oxidative stress provoked by reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidative stress occurs as a result of an imbalance of ROS and RNS produced by inflammatory and epithelial cells [3,4]. Even though in particular conditions ROS and RNS are capable of incorporating themselves as part of several signaling pathways, their upregulation is frequently harmful to colorectal epithelial cells which modify several antioxidant mechanisms to regulate ROS and RNS homeostasis. IBD has thus been considered as an “oxyradical overload” disease.

The insulin/insulin-like growth factor (IGF) system is a

Abbreviations: IGF-1R, insulin-like growth factor-1 receptor; IGF, insulin/insulin-like growth factor; IBD, inflammatory bowel disease; UC, ulcerative colitis; CRC, colorectal cancer; ROS, reactive oxygen species; RNS, reactive nitrogen species; CAC, colitis associated cancer; MDA, malondialdehyde; SOD, superoxide dismutase; TAC, antioxidant capacity; GSH, glutathione; GSSG, GSH/glutathione; GST, glutathione S-transferase; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; AOM, azoxymethane; DSS, dextran sulfate sodium; NC, negative cells; SiRNA, IGF-1R silenced cells; WT mice, wild type mice; *Igf1r*^{+/-} mice, heterozygous knockout IGF-1R mice

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multifactorial network that regulates energy metabolism, cell growth, and cancer. In colonic mucosal cells, IGF-1R triggers a number of intracellular signaling cascades that enhance cell cycle progression and inhibits apoptosis. IGFs/IGF-1R signaling plays an important role in the maintenance of inflammation and stricture formation in UC and Crohn's disease (CD) [5]. IGF-1R is considered to be a major determinant in CRC. Although previous studies have provided largely compelling preclinical evidence; to date, this has failed to translate into positive results that can benefit patients. Thus, the pathophysiological roles of IGF-1R need to be further investigated.

In this work, we demonstrated that heterozygous knockout IGF-1R confers resistance to oxidative stress-induced ulcerative colitis and tumorigenesis in *Igf1r*^{+/-} mice. The mechanisms underpinning the roles of IGF-1R are associated with the regulatory activity of the mitochondrial respiratory chain, dynamics and structures. Under oxidative stress, heterozygous knockout IGF-1R could protect the ability of ATP generation from oxidative damage by regulating the mitochondrial respiratory chain and activating the LKB1/AMPK pathways in *Igf1r*^{+/-} cells. Heterozygous knockout IGF-1R activates the mitochondrial fission/fusion activity which results in normal mitochondrial structures and physiological functions of colorectal epithelial cells. Our study shows the biological roles of IGF-1R in colitis and tumorigenesis and its mechanisms through the regulation of mitochondrial functions and structures under oxidative stress.

2. Materials and methods

2.1. Animal care and diet

All experiments related to mice were approved by the Animal Welfare Committee of the Capital Medical University (permit no. AEEI-2016–043). Mice were caged under controlled room temperature, humidity and light (12/12 h light/dark cycle) and fed standard mouse chow (containing 52% carbohydrate, 12% fat, 23% protein, 4% fiber, 6% ash, and 3% moisture) and tap water *ad libitum* [6].

2.2. Heterozygous knockout IGF-1 receptor (*Igf1r*^{+/-}) mice

Igf1r^{+/-} mice were generated at Cyagen Biosciences Inc. (Guangzhou, China) according to the description of *Igf1r*^{+/-} mice [7]. The information for *Igf1r*^{+/-} mice is presented in [Supplementary data S1](#). *Igf1r*^{+/-} colorectal epithelial cells showed a significant reduction in IGF-1R as determined by Western blotting assay ([Supplementary Fig. S1](#)). Heterozygous knockout of IGF-1R might lead to the upregulation of serum IGF-1 level [7]. However, biochemical parameters such as bilirubin, cholesterol, creatinine, glucose, lactate, total protein, triglycerides, urea and uric acid were all normal in adult *Igf1r*^{+/-} mice [7].

2.3. Colitis and colitis-associated cancer (CAC) models

For the colitis model, sex- and age-matched *Igf1r*^{+/-} mice and their wild type (WT) littermates (5–6 weeks old) were exposed to DSS (molecular weight 36,000–50,000; MP Biomedicals, Santa Ana, CA) in drinking water. A low dose of DSS was used to induce colitis. Mice were fed with water containing 1.0% DSS for 1 week followed by regular water for 14 days. This cycle was repeated four times, and then the mice were sacrificed after 10 weeks. In order to investigate the roles of IGF-1R in regulation lifespan and resistance to oxidative stress, further dose of 2% DSS were provided to induce ulcerative colitis. Mice were given 2% DSS in drinking water daily for 6 consecutive days, and disease activity and survival curves were observed. For the CAC model, mice were injected intraperitoneally with AOM (10 mg/kg; Sigma-Aldrich) and after 5 days, DSS (1%) was given in drinking water for 7 consecutive days followed by regular water for 14 days. This cycle was repeated twice. During colitis and tumorigenesis, signs of disease such as reduced body weight, rectal bleeding and diarrhea were monitored

daily. The severity of colitis was scored according to the disease activity index (DAI) as described by [8]. Mice were sacrificed at 20 weeks-old for analyses of colitis and colorectal tumorigenesis.

2.4. Reverse transcriptase and qPCR assay

RNA was isolated from colorectal mucosal cells and then qPCR assay was performed according to the conventional method. PCR primer pairs are listed in [Supplementary data S2, Table S1](#).

2.5. Western blot analysis

Western blotting assay was performed using antibodies against PKM2 (ab150377/58 kDa), ULK1 (ab128859/120 kDa), Oct-4 (ab19857/43 kDa), Mitofusin 1 (ab57602/84 kDa), Mitofusin 2 (ab56889/86 kDa), OPA1 (ab42364/86, 92 kDa; Abcam), Non-phospho (active) β -catenin (8814/92 kDa), GSK3 β (12456/46 kDa), E-cadherin (3195/135 kDa), Akt (9272/60 kDa), p-Akt (9271/60 kDa), Survivin (2802/16 kDa), NF κ B (8242/65 kDa), p-NF κ B (3033/65 kDa), Cox2 (12282/74 kDa), Sox2 (23064/35 kDa), Nanog (8822/29–42 kDa), Erk (4695/42, 44 kDa), p-Erk (4370/42, 44 kDa), Cyclin D1 (2978/36 kDa), Beclin-1 (3738/60 kDa), LKB1 (3050/54 kDa), AMPK α (2793/62 kDa), p-AMPK α 1 (Ser485, 2537/62 kDa), p-AMPK α (Thr172, 2535/62 kDa), IKK α (11930/85 kDa), IKK β (8943/87 kDa), I κ B α (4814/39 kDa), p-I κ B α (2859/40 kDa), LC3B (3868/14, 16 kDa), mTOR (2983/289 kDa), p-mTOR (5536/289 kDa), Bax (14796/20 kDa), caspase3 (9662/17, 19, 35 kDa) (Cell Signaling). Densitometric analyses of bands were adjusted with β -actin (Sigma).

2.6. ELISA assay

Levels of TNF- α (CSB-E04741m), IL-6 (CSB-E04639m) and PGE2 (CSB-E07966m) in supernatant of colorectal mucosa isolated from mice were quantified by using ELISA kits according to the manufacturer's protocols (Cusabio).

2.7. Determination of malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant capacity (TAC) and ATP levels

MDA was measured with chemical chromatometry kit (KGT004-1, KeyGEN, China). Total antioxidant capacity (TAC) was determined using the total antioxidant assay kit (S0121, Beyotime, China). SOD was determined using total superoxide dismutase assay kit with WST-8 (S0101, Beyotime, China) and cellular ATP level was determined using ATP assay kit (S0026, Beyotime; China).

2.8. Measurement of ROS generation

Reactive oxygen species (ROS) generation, including superoxide anion (O₂^{•-}), hydroxyl, alkoxyl, and peroxy were detected using ROS fluorescent probe-DHE kit. Colorectal mucosal cells were isolated and prepared for frozen sections. Then the sections were labeled with DHE probe (5 μ M) in PBS for 20 min at 37 °C. Slides were photographed with a fluorescence microscope (Olympus, Japan) at 488 nm excitation and 590 nm emission.

2.9. Nitrite and nitrate determination

Samples were deproteinized with ice-cold 6% sulfosalicylic acid at room temperature for 30 min and then centrifuged at 10,000 g for 15 min. 50 μ l supernatant were incubated with 4 μ l 1.25% NaOH, 36 μ l of 14 mM phosphate dehydrogenase, 750 μ M glucose-6-phosphate, 30 mU nitrate reductases, and 10 μ l of 3 μ M NADPH for 60 min at room temperature. Nitrite level (expressed in nmol/ml) was determined with the Griess reaction which converts nitrite into a colored azo compound spectrophotometrically detected at 550 nm [9].

2.10. Reduced glutathione (GSH) and oxidized glutathione (GSSG) assay

GSH and GSSG levels were measured using GSH and GSSG assay kit (S0053, Beyotime, China) according to the manufacturer's instruction. Fresh colorectal mucosal cells were maintained at 4 °C to calculate total GST activity, following the procedure as described by [10,11], using 30 mM 1-chloro-2,4-dinitrobenzene as substrate. GST activity was spectrophotometrically measured for 3 min at 340 nm, and expressed as mmol/g tissue.

2.11. Mitochondrial protein isolation

Following the manufacturer's instruction, mitochondria isolation kit (Beyotime, China) was used for the isolation of mitochondrial protein. Colorectal tissues ($n = 6$, per group) were washed twice with ice-cold PBS, resuspended in lysis buffer, and then homogenized by a homogenizer in ice-water. After removing nuclei and cell debris by centrifugation at 1000 g for 10 min at 4 °C, the supernatants were further centrifuged at 10,000 g for another 20 min at 4 °C. Protein level was determined by the Bicinchoninic acid (BCA) assay kit (Beyotime, China). Mitochondrial pellets were resuspended in lysis buffer on ice within 4 h before use.

2.12. Mitochondrial high-resolution respirometry

Mitochondrial respiration was measured using a Clark-type electrode (Oxygraph-2k, Oroboros Instruments). State 3 respiration was obtained after the addition of ADP to a final concentration of 300 μ M, and State 4 respiration was measured after the depletion of the added ADP. The respiratory control ratio (RCR; state 3/state 4) was calculated as described by [12]. The ADP to oxygen (ADP/O) ratio was determined based on the addition of a known amount of ADP and the associated O_2 consumption according to the method described by [13]. Citrate synthase specific activity was determined spectrophotometrically.

2.13. Cell line, cell culture, gene silencing and treatment

Human colonic adenocarcinoma cell line HT-29 was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells cultured DMEM medium were transfected with siRNA IGF-1R (10 μ M). The information of IGF-1R siRNA and determination of IGF-1R by Western blot assay are shown in [Supplementary data S3](#) and [Supplementary Fig. S2](#). For the analysis of mitochondrial protein, HT-29 cells or IGF-1R silenced cells (siRNA) were treated with IGF-1 (Peptide, 1 μ M) or Compound C (AMPK inhibitor, 20 μ M) for 24 h and then exposed to H_2O_2 (10 μ M) for 2 h.

2.14. Seahorse analysis of mitochondrial respirometry

Cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were detected using XF Cell Mito Stress Test and XF Glycolysis Stress Test (Seahorse Bioscience, XFp) [14]. HT-29 cells or IGF-1R silenced cells (siRNA) were cultured on XFp culture miniplates. The sensor cartridge for XFp analyzer was hydrated in a 37 °C non- CO_2 incubator a day before the experiment. For OCR assay, the injection port A on the sensor cartridge was loaded with 2.0 μ M oligomycin (complex V inhibitor) while 0.5 μ M FCCP was loaded to port B and 5 μ M rotenone/antimycin A (inhibitors of complex I and III) was loaded to port C. During sensor calibration, cells were incubated in a 37 °C non- CO_2 incubator in 180 μ l assay medium (XF base medium, 10 mM glucose, 10 mM pyruvate and 2 mM L-glutamine; pH 7.4). The plate was immediately placed onto the calibrated XFp Extracellular Flux Analyzer for Mito Stress Test. OCR was normalized for total protein/well.

2.15. JC-1 mitochondrial staining analysis

HT-29 cells or IGF-1R silenced cells (siRNA) seeded on slides were washed three times in PBS. Cells were then stained with 5 μ M JC-1 dye by incubating at 37 °C for 15 min. The dye was changed reversibly from red to green as mitochondrial membrane became depolarized. Fluorescence was analyzed under a fluorescence microscope using ImagePro Plus software.

2.16. Mitochondrial membrane potential staining assay

Mitochondrial morphology was analyzed after Mito Tracker Red staining (Beyotime, China) under a fluorescence microscope (Olympus, XSZ-D2, Japan) as described by [15]. Continuous mitochondrial structures were counted, and the number was normalized to the total mitochondrial area to determine mitochondrial fragmentation count. Cells with greater fragmentation were associated with higher mitochondrial fragmentation. Lengths of mitochondria were analyzed using NIS-Elements software.

2.17. Transmission electron microscopy

For mitochondrial structure analysis, fresh colorectal tissues were fixed with 2.5% glutaraldehyde (Sigma) in a phosphate buffer (pH 7.4). Samples were implemented as described previously. Obtained sections were stained and observed under a transmission electron microscope (HT7700, Japan).

2.18. Statistical analysis

Data are expressed as mean $\pm$ SD. P values were calculated by independent t -test, one-way and two-way ANOVA. $P < 0.05$ was considered significant.

3. Results

3.1. Heterozygous knockout IGF-1R attenuated DSS-induced colitis in *Igf1r*^{+/-} mice

The application of the DSS model has been critical in unraveling the pathogenesis of colitis in humans. To induce colitis, *Igf1r*^{+/-} mice and their WT littermates were exposed to 1%. WT mice exhibited the clinical signs of chronic ulcerative colitis such as diarrhea, bloody stools, and hair bristling. Body weight loss started to appear at day 7 ([Fig. 1A](#)). DAI reached its highest level in the third DSS cycle. In contrast, *Igf1r*^{+/-} mice had less signs of colitis, and less DAI than their WT littermates ([Fig. 1B](#)).

Histopathological analysis of the distal colonic tissues demonstrated that massive tissue damages such as crypts loss, infiltration of chronic and acute inflammatory cells in lamina propria and patchy re-epithelialization were frequently observed in WT mice unlike in *Igf1r*^{+/-} mice. In consistency with clinical signs, *Igf1r*^{+/-} mice exhibited essentially normal crypt architecture with slight inflammatory cells in the intraepithelial and lamina propria of colonic tissues ([Fig. 1C](#)).

IGF-1R regulates lifespan by developing resistance to oxidative stress. In particular, continuous 2% DSS induced acute inflammation with frequent ulceration in colorectal tissues of WT mice. In addition to body weight loss, other serious clinical signs such as bloody stools and rectal bleeding were frequently observed thus leading to a decrease in the lifespan of WT mice. In contrast, *Igf1r*^{+/-} mice had a mean survival rate of 27% more than their WT littermates ([Fig. 1D](#), * $P < 0.05$, ** $P < 0.01$; Cox's test). Histopathological analysis showed crypts loss, serious erosion from surface to lamina propria with exuberant inflammatory exudates, acute inflammatory cells and bleeding in WT mice compared to *Igf1r*^{+/-} mice with less acute inflammation ([Fig. 1E](#)).

The COX-2/PGE₂ pathway is crucial in colitis and CAC by

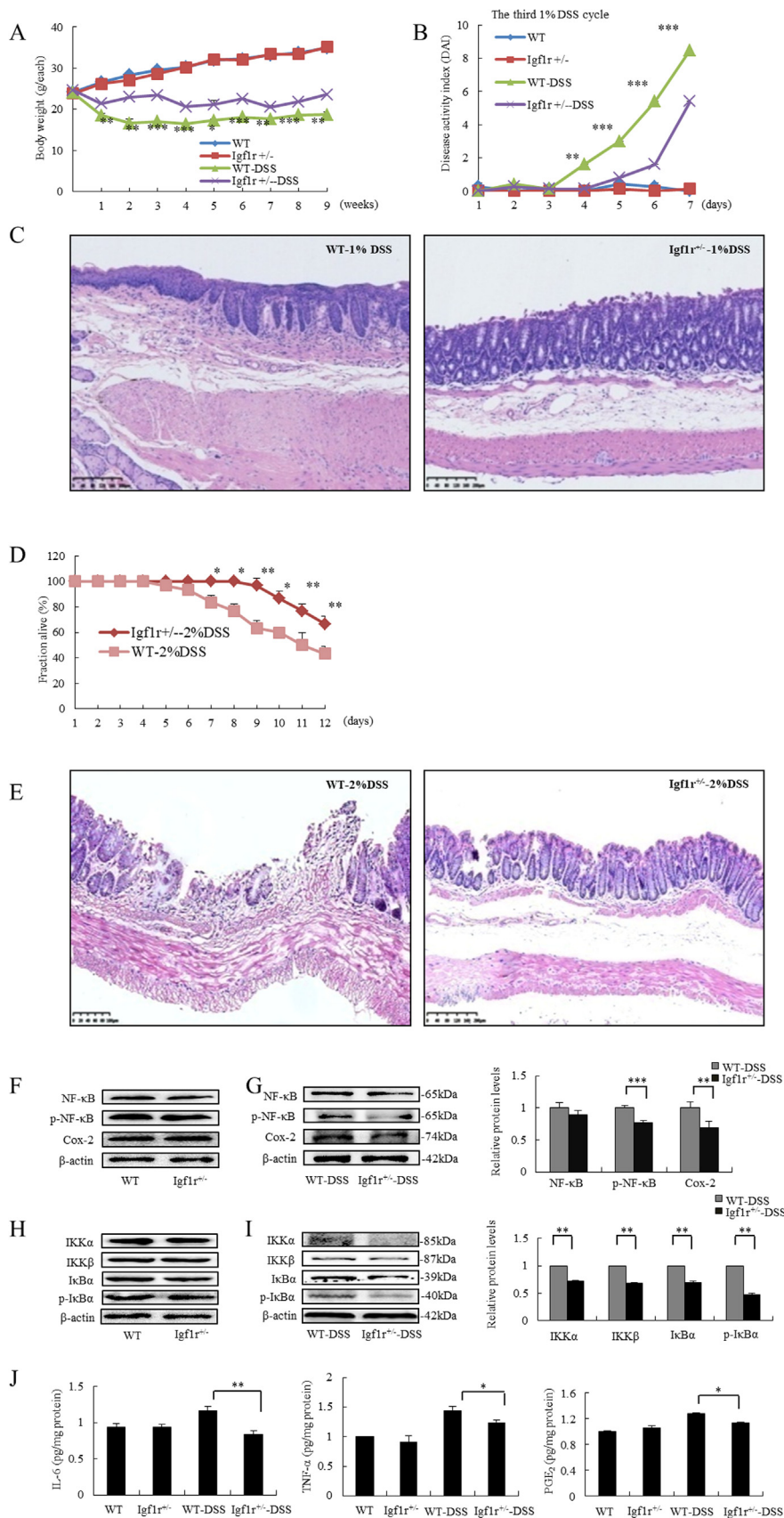


Fig. 1. Heterozygous knockout insulin-like growth factor-1 receptor (IGF-1R) attenuated dextran sulfate sodium (DSS)-induced colitis in mice. (A) Body weight changes during colitis. (B) *Igflr*^{+/-} mice scored less disease activity index (DAI) than their wild type (WT) littermates in the third 1% DSS cycle. (C) Representative of H&E-stained sections from WT and *Igflr*^{+/-} mice exposed to 1% DSS. (D) *Igflr*^{+/-} mice lived longer than WT mice under the challenge of continuous 2% DSS. (E) Representative of H&E-stained sections from WT and *Igflr*^{+/-} mice exposed to 2% DSS. (F) *Igflr*^{+/-} mice exhibit normal levels of NF-κB-COX-2 cascades as WT mice had. (G) Heterozygous knockout IGF-1R prevented the DSS-induced activation of the NFκB pathway. (H) *Igflr*^{+/-} mice exhibit normal levels of the IKK cascades as WT mice had. (I) Heterozygous knockout IGF-1R prevented the DSS-induced activation of the IKK cascades as WT mice had. (J) ELISA assay showed decreased IL-6 (left), TNF-α (middle) and PGE₂ (right) in colorectal tissues of *Igflr*^{+/-} mice. Each dot represents six mice (n = 6). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 between *Igflr*^{+/-} and WT mice exposed to DSS.

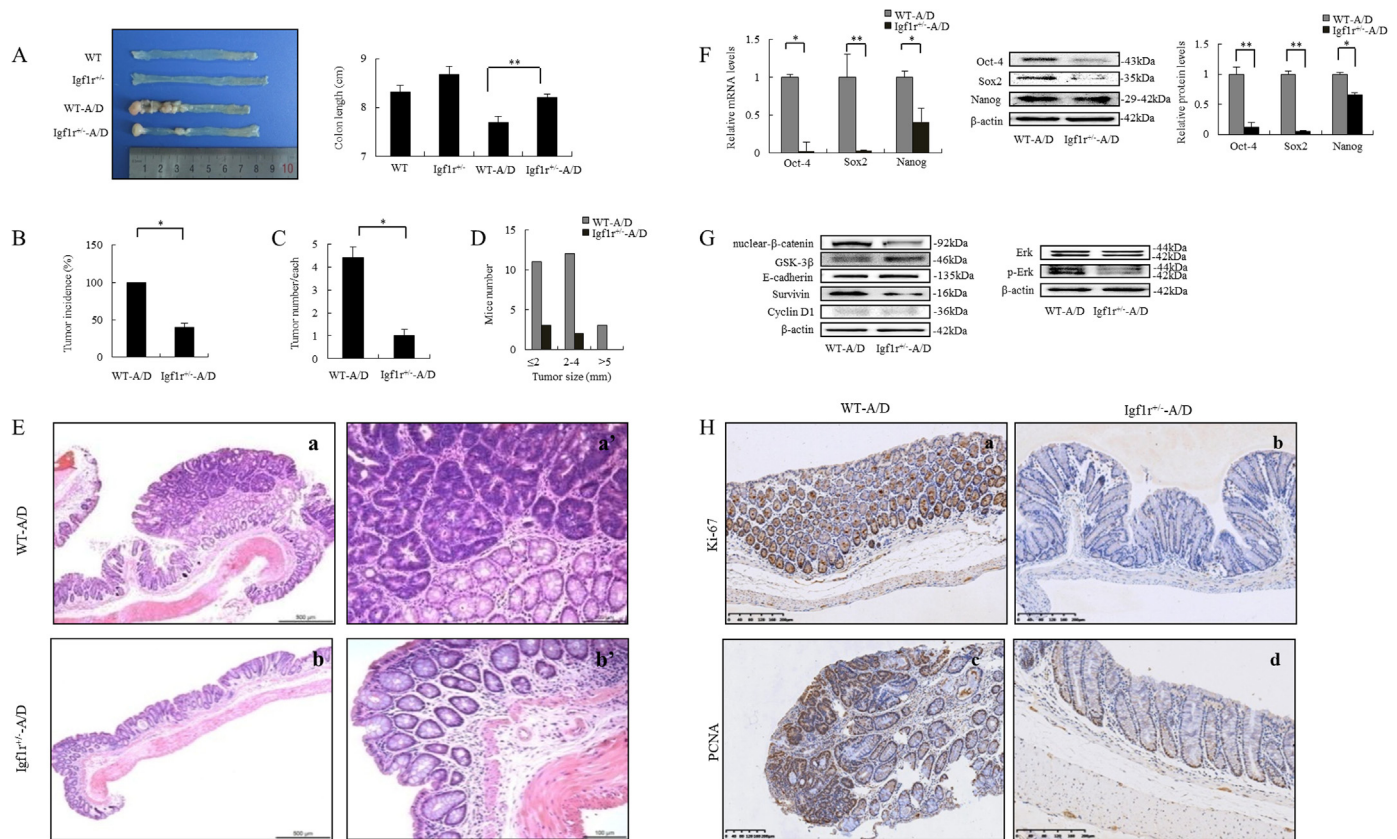


Fig. 2. Heterozygous knockout IGF-1R prevented azoxymethane (AOM)/DSS-induced colorectal cancer. (A) Gross view of colorectal tumor in WT and *Igf1r*^{+/−} mice. (B) Tumor incidence. (C) Total tumor number and (D) size distribution in WT and *Igf1r*^{+/−} mice. (E) Representative of H&E-stained sections from WT (Fig. 2E-a × 50, Fig. 2E-a' × 400) and *Igf1r*^{+/−} mice (Fig. 2E-b × 50, Fig. 2E-b' × 400). (F) Heterozygous knockout IGF-1R reduced pluripotent potential in mRNA level of colorectal mucosal cells (left column) and protein level (right column). (G) Heterozygous knockout IGF-1R inhibited nuclear β-catenin pathway. (H) IHC analyzed Ki67 and PCNA levels in colorectal tissues. Each dot represents six mice (n = 6). **P* < 0.05, ***P* < 0.01 between *Igf1r*^{+/−} and WT mice. A/D = AOM/DSS.

maintaining the inflammatory microenvironment. In such microenvironment, tumor-associated macrophages express proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. These cytokines further activate nuclear factor (NF)-κB transcription factors in colorectal epithelial cells. Western blotting assay showed that heterozygous knockout IGF-1R did not affect the expressions of the NF-κB-COX-2 pathway and the upstream IKK in the epithelial cells of *Igf1r*^{+/−} mice. Further, in the presence of DSS, NF-κB-COX-2 and IKK were significantly upregulated in WT mice but not in *Igf1r*^{+/−} mice (Fig. 1F-I). ELISA analysis of IL-6, TNF-α and PGE₂ in the intraepithelial and lamina propria showed significant differences between WT and *Igf1r*^{+/−} mice (Fig. 1J).

3.2. Heterozygous knockout IGF-1R prevented AOM/DSS-induced colitis associated cancer (CAC)

AOM/DSS induced CAC, resulting in shortening of colorectal length (Fig. 2A). Heterozygous knockout IGF-1R prevented AOM/DSS-induced shortening of colorectal length (***P* < 0.01 vs. WT). AOM/DSS induced colorectal tumor by 100% in WT mice, compared to 40% in *Igf1r*^{+/−} mice (Fig. 2B, **P* < 0.05). *Igf1r*^{+/−} mice developed less tumors as compared to WT mice (Fig. 2C, **P* < 0.05). Larger sized tumors (> 5 mm) were frequently occurred in WT mice, but not in *Igf1r*^{+/−} mice (Fig. 2D).

Histopathological analysis of colorectal tumors showed less malignancy in *Igf1r*^{+/−} mice than WT mice. WT mice expressed advanced tubular adenoma with focal high-grade dysplasia, loss of nuclear polarity and architectural distortion. Gross ulceration was seen in large-sized polyps (Fig. 2E-a, b). *Igf1r*^{+/−} mice exhibited a significant

reduction in number and size of adenomatous polyps with reduced dysplastic cells and dysplasia. Interdigitated normal appearing glands containing goblet cells were seen in *Igf1r*^{+/−} mice (Fig. 2E-a', b').

AOM induced high levels of Oct-4, Sox2, and Nanog in colorectal mucosal cells of WT mice but not in *Igf1r*^{+/−} mice (Fig. 2F, **P* < 0.05, ***P* < 0.01). These data suggest that heterozygous knockout IGF-1R reduced the pluripotent potential in mucosal cells.

AOM induced β-catenin nuclear translocation, a direct target for phosphorylation by glycogen synthase kinase-3β, leading to downstream signal accumulation [16,17]. Heterozygous knockout IGF-1R prevented AOM-induced β-catenin nuclear translocation and its downstream signals Survivin, Cyclin D1, p-Erk (Fig. 2G), Ki67 and PCNA (Fig. 2H). In addition, GSK-3β and E-cadherin levels were increased in colorectal mucosal cells.

3.3. Heterozygous knockout IGF-1R prevented oxidative damage in colorectal mucosal cells in *Igf1r*^{+/−} mice

AOM/DSS-induced oxidative stresses damaged mitochondrial structures thus leading to massive intracellular malondialdehyde (MDA), ROS and NO_x accumulation in colorectal mucosal cells. Simultaneously, increased oxidative stress reduced superoxide dismutase (SOD) and total cellular antioxidant capacity (TAC) (Fig. 3A). Heterozygous knockout IGF-1R prevented AOM/DSS-induced MDA (***P* < 0.001 vs. WT), ROS (***P* < 0.01 vs. WT) and NO_x (**P* < 0.05 vs. WT), while SOD and TAC levels were significantly increased in colorectal mucosal cells of *Igf1r*^{+/−} mice (**P* < 0.01, ****P* < 0.001 vs. WT mice).

In AOM/DSS model, MDA was time-dependently elevated in WT

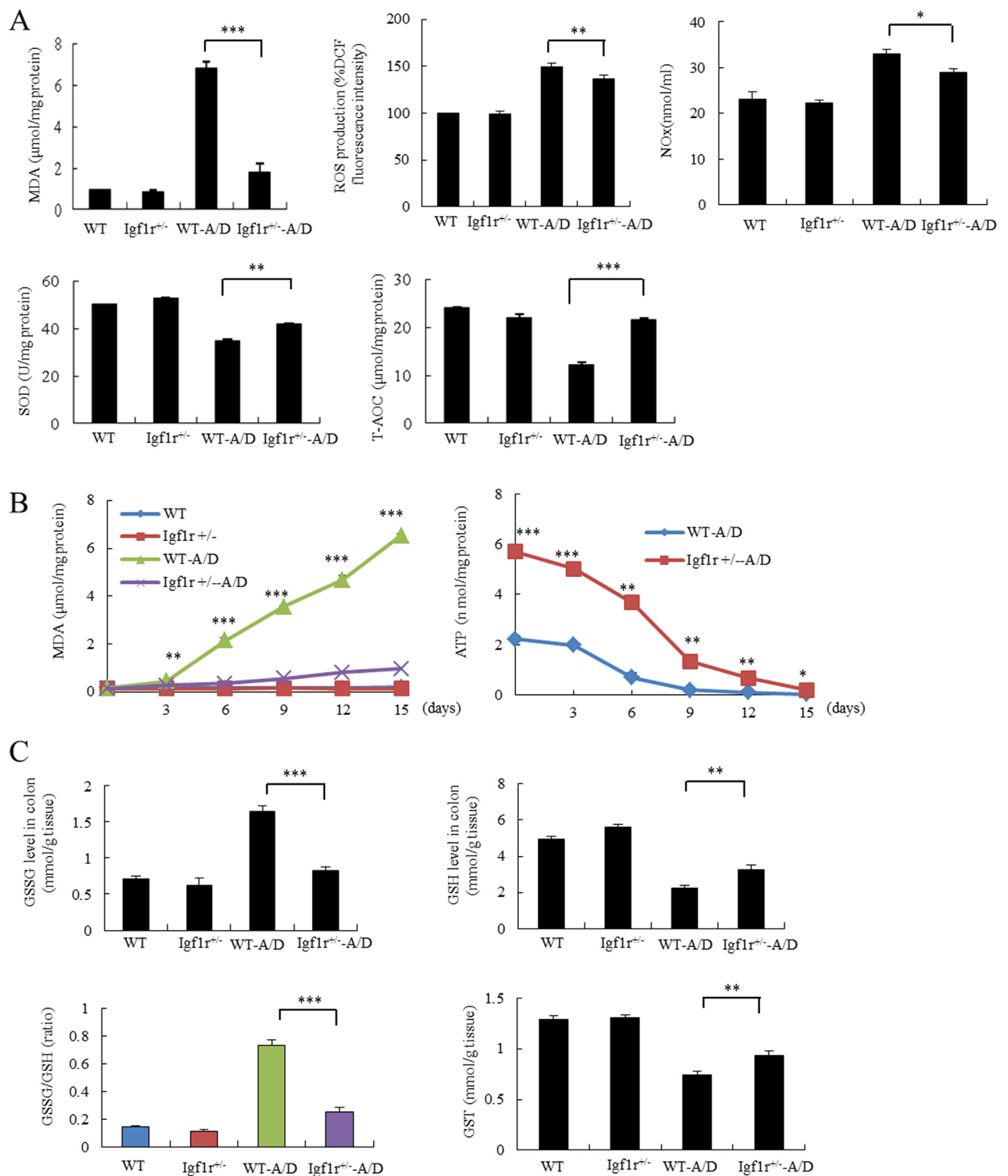
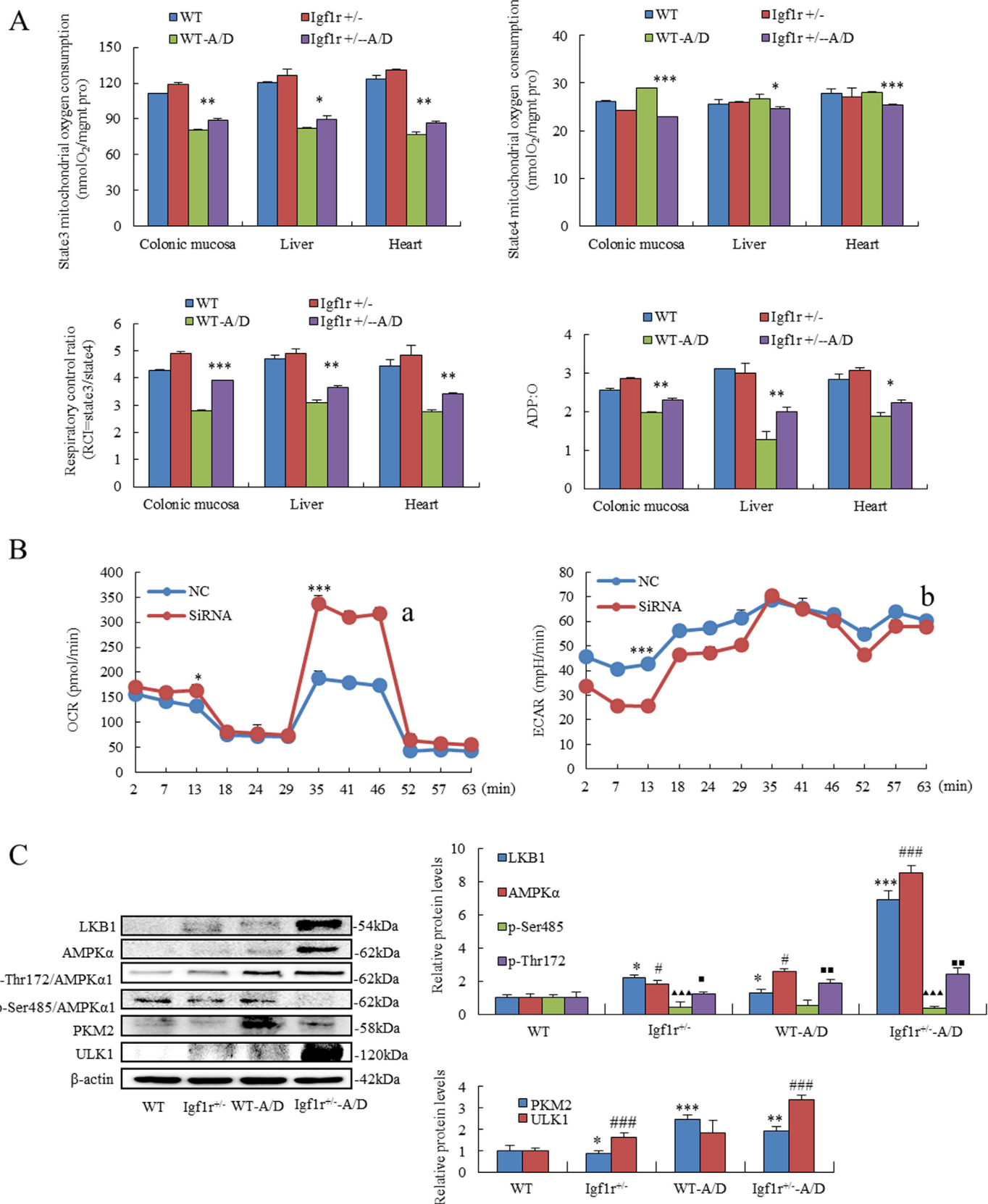


Fig. 3. Heterozygous knockout IGF-1R confers the resistance to oxidative stresses and protects mitochondrial dynamics from oxidative stress. (A) ELISA assay analyzed the levels of malondialdehyde (MDA), reactive oxygen species (ROS), NOx, superoxide dismutase (SOD) and antioxidant capacity (T-AOC) in colorectal mucosal cells of mice. (B) In the AOM/DSS model, MDA levels were time-dependently increased in WT mice but not in *Igf1r*^{+/-} mice. Luminance ATP assay determined a higher level of ATP in *Igf1r*^{+/-} mice than that in WT mice. (C) WT mice showed a higher GSH/glutathione (GSSG) and GSSG/GSH ratio, and a lower glutathione (GSH) and glutathione S-transferase (GST); whereas a lower GSSG and GSSG/GSH ratio, and a higher GSH and GST in *Igf1r*^{+/-} mice. Each dot represents six mice (n = 6). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 between *Igf1r*^{+/-}-A/D and WT-A/D mice.



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Fig. 4. Heterozygous knockout IGF-1R activates mitochondrial respiratory chain and mitochondrial dynamics in *Igf1r*^{+/-} mice. (A) Under AOD/DSS challenge, state 3 respirations presented a stronger increase in *Igf1r*^{+/-} mice than WT mice, while state 4 respirations had still maintained a lower level in colorectal mucosal cells, liver cells and heart muscle cells in *Igf1r*^{+/-} mice as compared to WT mice. Mitochondrial coupling index determined by respiratory control ratio (RCR) and oxidative phosphorylation index determined by ADP/O were both increased in *Igf1r*^{+/-} mice as compared to WT mice. Each dot represents six mice (n = 6). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 between WT-A/D and *Igf1r*^{+/-}-A/D. (B) Seahorse analysis of mitochondrial respirometry in human colonic cancer HT29 cells. Silenced IGF-1R cells had a higher oxygen consumption rate (OCR) (left) and a lower extracellular acidification rate (ECAR) (right) as compared to negative control cells (NC). **P* < 0.05, ****P* < 0.001 between NC and siRNA. (C) Western blotting assayed the LKB1/AMPK pathways in colorectal mucosal cells from WT and *Igf1r*^{+/-} mice. Each dot represents six mice (n = 6).

mice but not in *Igf1r*^{+/-} mice (Fig. 3B, ***P* < 0.01, ****P* < 0.001). Simultaneously, *Igf1r*^{+/-} mice maintained higher levels of intracellular ATP in comparison to WT mice which showed a significant reduction in ATP (Fig. 3B, **P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Glutathione (GSH) plays pivotal roles in exerting antioxidant functions. However, GSH reduced GSH/glutathione disulphide (GSSG) ratio induces an increased susceptibility to oxidative stress, while elevated GSH ratio promotes antioxidant capacity against oxidative stress [18]. In AOM/DSS model, heterozygous knockout IGF-1R decreased GSSG (Fig. 3C, ****P* < 0.001) and increased GSH in *Igf1r*^{+/-} mice as compared to WT mice (***P* < 0.01). The GSH/GSSG ratio decreased in *Igf1r*^{+/-} mice (****P* < 0.001 vs. WT mice). In addition, glutathione S-transferase (GST) was activated in the colorectal mucosal cells of *Igf1r*^{+/-} mice (**P* < 0.01 vs. WT mice). These results suggest that heterozygous knockout IGF-1R could prevent oxidative stress-induced inflammatory metabolism in *Igf1r*^{+/-} mice.

3.4. Heterozygous knockout IGF-1R protects the mitochondrial respiratory chain from oxidative stress through the activation of LKB1/AMPK pathways

Mitochondrial coupling and oxidative phosphorylation in colorectal mucosal cells, liver cells and heart muscle cells under oxidative stress (Fig. 4A) were analyzed. Heterozygous knockout IGF-1R significantly increased State 3 respiration and decreased State 4 respiration in *Igf1r*^{+/-} mice. Mitochondrial coupling index RCI and oxidative phosphorylation index ADP/O were both increased in *Igf1r*^{+/-} mice as compared to WT mice (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, between *Igf1r*^{+/-}-A/D mice and WT-A/D mice). These results suggest that IGF-1R low expression could prevent mitochondrial respiratory chain from oxidative damage in *Igf1r*^{+/-} mice.

Mitochondrial respirometry was analyzed by measuring cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in human colonic cancer HT-29 cells. As compared to negative cells (NC), IGF-1R silenced cells (siRNA) showed a higher OCR basal (132.10 ± 10.26 vs. 163.90 ± 12.61 pmole/min/10⁵ cells, **P* < 0.05) and maximal levels (187.43 ± 15.58 vs. 337.73 ± 15.96 pmole/min/10⁵ cells, ****P* < 0.001) (Fig. 4B-a), and a lower level of ECAR (glycolysis) (42.73 ± 2.00 vs. 25.56 ± 2.25 mpH/min/10⁵ cells, ****P* < 0.001) (Fig. 4B-b).

Liver kinase B1 (LKB1) acts as a metabolic energy sensor to activate AMP-activated protein kinase (AMPK) and is thus responsible for sensing cellular ATP availability [19]. The roles of IGF-1R in regulation of LKB1/AMPK pathways (Fig. 4C) were investigated. WT mice had a lower basal LKB1 level which was only weakly activated when mice were exposed to AOM/DSS (**P* < 0.05). In contrast, *Igf1r*^{+/-} mice demonstrated a higher basal LKB1 level (**P* < 0.05 vs. WT). Importantly, basal LKB1 was greatly activated with AOM/DSS (****P* < 0.001 vs. WT-A/D). Analysis of AMPKα showed the same profile as in LKB1 (****P* < 0.001 between *Igf1r*^{+/-}-A/D and WT-A/D). In addition, activated LKB1 phosphorylated AMPKα on threonine-172 (p-Thr172-AMPKα) (■*P* < 0.05 between *Igf1r*^{+/-} and WT, ■■*P* < 0.01 between *Igf1r*^{+/-}-A/D and WT, ■■■*P* < 0.01 between WT-A/D and WT), while phosphorylation of serine-485 (p-Ser485/491-AMPKα) was significantly decreased (▲▲▲*P* < 0.001 between *Igf1r*^{+/-} and WT, ▲▲▲*P* < 0.001 between *Igf1r*^{+/-}-A/D and WT-A/D).

Numerous studies have shown that activated AMPK down-regulates

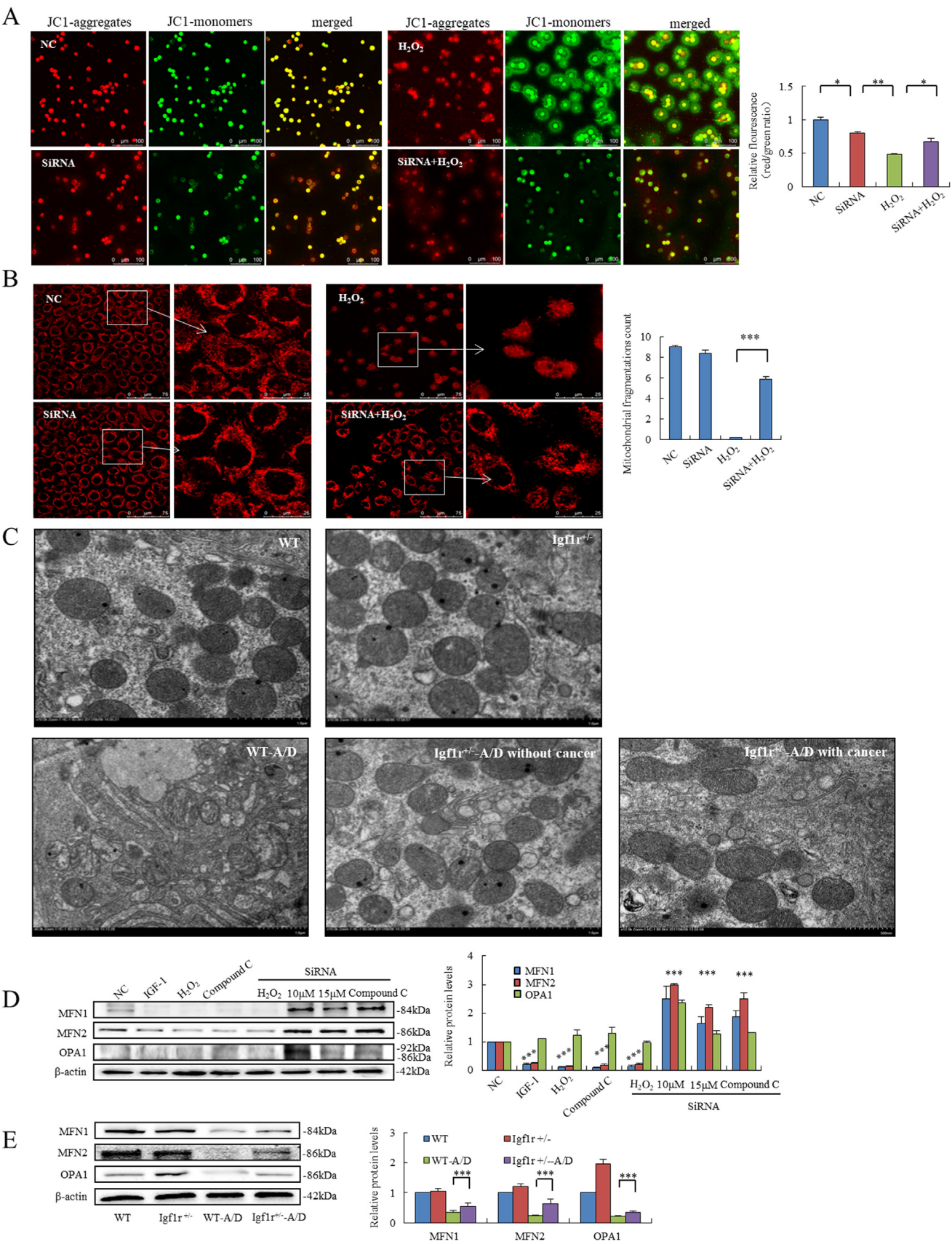
pyruvate kinase M2 (PKM2), an essential regulator of aerobic glycolysis [20]. Activated AMPK directly phosphorylates ULK1, an important autophagy-initiating kinase under glucose starvation [21]. Heterozygous knockout IGF-1R significantly reduces PKM2 expression (**P* < 0.05 vs. WT). In AOM/DSS model, WT mice demonstrated a significant increase in PKM2 (****P* < 0.001 vs. WT without AOM/DSS), with only a slight increase in *Igf1r*^{+/-} mice as compared to WT-A/D (***P* < 0.01). Contrary to PKM2, heterozygous knockout IGF-1R elevated ULK1 expression (****P* < 0.001 vs. WT) and the expression was further increased in *Igf1r*^{+/-} mice in the AOM/DSS model (****P* < 0.001 vs. WT; ****P* < 0.001 vs. WT-A/D). These results suggest that heterozygous knockout IGF-1R activates mitochondrial functions by the activation of the LKB1/AMPK pathways.

3.5. Heterozygous knockout IGF-1R protected mitochondrial structures from oxidative damage

It has been shown that at low levels hydrogen peroxide elicits reversible mitochondrial swelling, fragmentation, and fine structural alterations by modulating fission/fusion proteins [22]. Firstly, we measured ΔΨ_m level, an index of mitochondrial integrity, in human colonic cancer cells by JC-1 fluorescence mitochondrial staining assay. HT-29 cells exposed to a low concentration of H₂O₂ resulted in the dissipation of ΔΨ_m thus showing a decrease in the red/green ratio (Fig. 5A, first panel). In contrast, IGF-1R silenced cells (siRNA) maintained an essential mitochondrial integrity by showing a significant increase in the red/green ratio (Fig. 5A, second panel).

The filamentous mitochondria were further analyzed by MitoTracker Red staining assay (Fig. 5B). Compared to negative cells (NC), the silenced IGF-1R cells (siRNA) retained the normal filamentous shape of the mitochondria. Oxidative stress was then performed on cells using H₂O₂. H₂O₂ significantly induced mitochondrial fusion by showing its filamentous shape and perimeter of fusion. H₂O₂ increased the fragmentation of mitochondria into granules and loss of staining with MitoTracker Red (Fig. 5B, first panel). In contrast, IGF-1R silenced cells (siRNA + H₂O₂) had less altered mitochondrial shapes and essentially maintained normal filamentous shape. IGF-1R knock-down by siRNA prevented H₂O₂-induced mitochondrial fission thus demonstrating an increase in mitochondrial fragmentations and a decrease in its perimeter (Fig. 5B, ****P* < 0.001 between H₂O₂ and siRNA + H₂O₂).

We finally analyzed mitochondrial structures using transmission electron microscopy which showed that heterozygous knockout IGF-1R did not affect mitochondrial structures (Fig. 5C, right, first row). In the AOM/DSS model, the mitochondrial structures were markedly altered in WT mice, resulting in swollen mitochondria with cristae fracture, double-membrane decomposition and bound vacuoles with remnants. These changes in mitochondrial structures were observed in 100% WT mice (Fig. 5C, left, second row). In contrast to WT mice, the mitochondria in colorectal mucosal cells of *Igf1r*^{+/-} mice without tumor exhibited fewer changes in their structures (Fig. 5C, middle, second row). An analysis of mitochondria in *Igf1r*^{+/-} mice with developed colorectal tumor showed middle-grade swollen mitochondria with cristae fracture (Fig. 5C, right, second row). These results suggest that heterozygous knockout IGF-1R could protect mitochondrial structure from oxidative damage.



(caption on next page)

Fig. 5. Heterozygous knockout IGF-1R prevented oxidative stress-induced changes of mitochondrial functions and structures. (A) 5, 5', 6, 6'-Tetrachloro-1, 1', 3, 3'-tetraethyl-imidazocarbocyanine iodide (JC-1) assay analyzed the integrity of mitochondria in human colonic cancer HT-29 cells. HT-29 cells silenced IGF-1R presented a decrease of green fluorescence, indicating the severity of H₂O₂-induced damage on mitochondria was attenuated. ^{*}*P* < 0.05, ^{**}*P* < 0.01. (B) MitoTracker Red staining analyzed mitochondria filamentous shape in HT-29 cells. H₂O₂ induced mitochondrial fusion, fragmentation of mitochondria into granules shape and even loss of staining with MitoTracker Red. In contrast, silenced IGF-1R (siRNA) cells treated with H₂O₂ had lower level of mitochondrial damages as compared to the cells only treated with H₂O₂ (^{***}*P* < 0.001 between H₂O₂ and siRNA + H₂O₂). (C) Transmission electron microscope analyzed the mitochondrial structures from WT and *Igf1r*^{+/-} mice. Mitochondria counts were decreased and mitochondrial structure was damaged in AOM/DSS in WT mice. Heterozygous knockout IGF-1R prevented oxidative damage on mitochondrial counts and structure. The representatives of mitochondria from WT and *Igf1r*^{+/-} mice (first row). Mitochondria counts were decreased and mitochondrial structures were altered in AOM/DSS in WT mice (left, first row). The mitochondria in *Igf1r*^{+/-} mice without colorectal tumor exhibited fewer changes in their structures and accounts (middle, second row). The mitochondria in *Igf1r*^{+/-} mice with colorectal tumor showed middle-grade swollen mitochondria with cristae fracture (right, second row). (D) Western blotting assay analyzed levels of mitochondrial fusion protein mitofusin-1 (MFN1), mitochondrial fusion protein mitofusin-2 (MFN2), and OPA1 in HT-29 cells. The basal levels of MFN1 and MFN2 were determined, and these basal levels were reduced after addition of IGF-1, H₂O₂ and Compound C. IGF-1R knockdown by silencing IGF-1R promoted levels of MFN1, MFN2 and OPA1. ^{***}*P* < 0.001 between NC cells and siRNA cells. (E) Analysis of mitochondrial protein isolated from colonic mucosal cells of mice. Heterozygous knockout IGF-1R prevented AOM/DSS-induced reduction of MFN1, MFN2 and OPA1 in *Igf1r*^{+/-} mice. Each dot represents six mice (*n* = 6). ^{***}*P* < 0.001 between WT-A/D and *Igf1r*^{+/-}-A/D.

Mitochondrial membranes undergo frequent fusion to maintain a ribbon-like morphology. However, the balance in mitochondrial fission/fusion might be offset by oxidative stress, leading to further amplification in ROS generation [23]. Our results suggest that heterozygous knockout IGF-1R maintains the function of mitochondrial fission/fusion under oxidative stress. Western blot analysis showed a basal level of mitofusin 1 (MFN1), mitofusin 2 (MFN2) and OPA1 (Fig. 5D). With the addition of IGF-1 or Compound C, MFN1 and MFN2 levels were significantly reduced (^{***}*P* < 0.001 vs. NC), and further reduced under H₂O₂ (^{***}*P* < 0.001 vs. NC). However, significant changes in OPA1 were not observed without H₂O₂ stress. In contrast, MFN1, MFN2, and OPA1 levels were both elevated in silencing IGF-1R siRNA (^{***}*P* < 0.001 vs. NC). We further analyzed mitochondrial proteins isolated from colorectal mucosal cells of mice (Fig. 5E). There were no observable differences in MFN1, MFN2 and OPA1 expressions in WT and *Igf1r*^{+/-} mice without AOM/DSS. In the AOM/DSS model, although MFN1, MFN2 and OPA1 were all reduced in both WT and *Igf1r*^{+/-} mice, the decreased levels in the mitochondrial fission/fusion proteins were significantly reduced in *Igf1r*^{+/-} mice than in WT mice (^{***}*P* < 0.001). These results suggest that heterozygous knockout IGF-1R could activate the functions of mitochondrial fusion under oxidative stress.

4. Discussion

Presently, altered metabolic programming is considered as a major feature in the pathophysiology of colitis and CAC. Currently, oxidative stress is identified as a major risk factor in the initiation of colitis and tumorigenesis [24]. In our study, we used the AOM/DSS model which reveals that the clinicopathological features in rat are similar to those found in humans [25–27], to investigate the pathophysiological roles of IGF-1R in the regulation of metabolic programming in the pathogenesis of colitis and CAC. Heterozygous knockout IGF-1R confers resistance to DSS-induced ulcerative colitis in *Igf1r*^{+/-} mice. Particularly, *Igf1r*^{+/-} mice developed resistance to high dose DSS damage in its colonic tissues, resulting in a longer lifespan for *Igf1r*^{+/-} mice than WT mice. These data highlight the previous observation that IGF-1R regulates lifespan and resistance to paraquat-induced oxidative stress [7]. During colitis and tumorigenesis, COX-2/PGE₂ and NF-κB/IL6/STAT3 amplification cascades have been known to play pivotal roles in sustaining tumor-associated microenvironment. Activation of COX-2/PGE₂ cascades in intestinal epithelial cells promotes survival pathways that are required for premalignant cell growth and subsequent tumorigenesis. Increased IL-6 stimulates both the initiation and further progression of CAC and the activation of STAT3 downstream of IL-6 play an essential role in the regeneration of the intestinal mucosa exposed to oxidative stress. Orchestral expressions of TNF-α and IL-6 and the activation of NF-κB and STAT3 have been further studied in patients with colitis and in those who progressed to cancer [25,27]. Our results showed that heterozygous knockout IGF-1R attenuated colitis and tumorigenesis by

preventing TNF-α and IL-6, and NF-κB and STAT3 cascades in colonic epithelial cells. Importantly, we investigated the mechanisms underpinning IGF-1R regulation of colitis and tumorigenesis in *Igf1r*^{+/-} mice.

How does oxidative stress contribute to tumorigenesis? During colitis, in addition to regulating DNA, RNA, proteins, and lipids [28], enhanced MDA, ROS and RNS are (in turn) able to modify either directly or indirectly several mitochondria shaping proteins. This creates a positive feedback loop which leads to mitochondrial dysfunction [29]. Thus, mitochondria are less functional in inflamed colonic epithelial cells. Decreased mitochondrial function is considered to be tumorigenic, mainly because of enhanced ROS. ROS accumulation in inflamed epithelial cells is thus a potent driver of initial tumorigenesis. Several studies have revealed that ROS activates several oncogenic signaling pathways, such as Ras/MAPK/ERK, PI3K/Akt and IKK/NFκB, to promote tumorigenesis through the activation of β-catenin nuclear translocation and its downstream signals [2–4]. A previous study suggests that heterozygous knockout of IGF-1R results in the reduction of p66 isoforms of Shc. Shc and IRS-1 binding to Grb2 on phosphorylation and the activation of mitogenactivated protein (MAP) kinase pathway involved in mitogenic response. IGF-1R is thus considered to regulate lifespan by reducing p66 Shc and producing resistance to oxidative stress [7].

Accumulating studies have shown that decreased mitochondrial function confers tumorigenesis, mainly because of enhanced ROS [29]. It is known that tumor cells are characterized by a decrease in mitochondrial respiration and oxidative phosphorylation [30]. Although multiple reasons have been given for this, however, the ROS alteration of mitochondrial respiratory complex is considered to be the major reason for decreased mitochondrial function [31]. However, the mechanism of ROS alteration of mitochondrial respiratory complex has not been investigated. Recent studies suggest that any lowering of the electron transfer chain activity is either caused by mtDNA mutations and hence, structural lesions of the respiratory complexes or depending directly or indirectly on hypoxia, enhanced ROS generation in state 4 mitochondrial oxygen consumption [32]. Herein, we analyzed the effects of heterozygous knockout IGF-1R on mitochondrial respiratory chain in colorectal epithelial cells. Mitochondria are highly sensitive to oxidative stimuli, which cause substantial damage to the mitochondrial electron transport chain and disrupts ATP resulting in a shift towards anaerobic respiration. Heterozygous knockout IGF-1R inhibits State 4 mitochondrial oxygen consumption, leading to the prevention of ROS generation. The protection of mitochondrial respiratory chain from oxidative stress through heterozygous knockout IGF-1R also occurs in other tissues such as liver and heart muscle cells in *Igf1r*^{+/-} mice. Furthermore, heterozygous knockout IGF1R demonstrated a higher oxygen consumption rate (OCR) and a lower extracellular acidification rate (ECAR) as compared to WT cells. These data suggest that heterozygous knockout IGF-1R prevents mitochondrial metabolism towards anaerobic glycolysis under hypoxic conditions.

Heterozygous knockout IGF-1R also regulates mitochondrial

dynamics by activating the LKB1/AMPK pathways. It is widely accepted that AMPK plays a predominant role as the master regulator of cellular energy in homeostasis [33]. In normal cells, LKB1 phosphorylates AMPK α at Thr172 in the activation loop. AMPK α is also phosphorylated at Thr258 and Ser485. However, LKB1 phosphorylation of AMPK α at serine-485/491 (p-Ser485/491-AMPK α) might reduce accessibility to Thr172 phosphorylation site, thereby inhibiting its catalytic activity. The activation of AMPK results in the stimulation of bioenergetic pathways and an inhibition of ATP-consuming processes such as biosynthesis and cell proliferation. However, the loss of LKB1 could lead to dysfunctional mitochondria and metabolic dysregulation that renders LKB1-deficient cells hypersensitive to oxidative stresses [34]. In our study, heterozygous knockout IGF-1R activated the mitochondrial dynamics, LKB1/AMPK, and PKM2 and ULK1 pathways. Activated AMPK directly phosphorylated ULK1, an important autophagy-initiating kinase under glucose starvation. As AMPK is a negative regulator of Warburg effect [35], AMPK-regulated PKM2 is hence the central regulator in mitochondrial metabolism. We thus suggest that heterozygous knockout IGF-1R protects mitochondrial respiratory chain from oxidative stress by activating the LKB1/AMPK pathways.

Mitochondrial cycling and culling are crucial for maintaining cellular homeostasis and are conducted through mitochondrial fusion, fission, and mitophagy [36]. During colitis, defects in mitochondrial dynamics might cause the accumulation of damaged mitochondrial components due to inefficient elimination through mitophagy coupled with fission [23]. The mitochondrial fusion/fission balance is susceptible to oxidative stress, which could further amplify ROS generation [37]. We suggest that heterozygous knockout IGF-1R could prevent ROS generation by activating the mitochondrial fission/fusion proteins, leading to protection of mitochondria from oxidative damage. Mitochondrial fusion in mammalian cells is controlled by the large GTPases mitofusin (MFN) 1, MFN2, and OPA 1. OPA1-driven cristae remodeling is strictly required for regulating ATP production [38]. The loss of any of these proteins might lead to the accumulation of mitochondrial fragmentation. Cells lacking MFNs or OPA1 lose mitochondrial membrane potential ($\Delta\psi$), reduce their oxygen consumption rate (OCR), and increase ROS production [39,40]. Our study indicates that heterozygous knockout IGF-1R activated the mitochondrial dynamics and protected mitochondrial structures from oxidative damage through the activation of these mitochondrial fission/fusion proteins.

In conclusion, IGF-1R regulation is critical in the initiation of colitis and tumorigenesis. During colitis, heterozygous knockout IGF-1R confers resistance to oxidative damage on mitochondrial structures, therefore resulting in attenuate chronic inflammation and tumorigenesis. The results of this study indicate the pivotal role of IGF-1R at the nexus between oxidative stress and mitochondrial dynamics during colitis and tumorigenesis.

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A conflict of interest disclosure statement

Authors declare that there is no potential conflict of interest or financial dependence regarding this work.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.freeradbiomed.2018.12.035](https://doi.org/10.1016/j.freeradbiomed.2018.12.035).

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