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# Wuho/WDR4 deficiency inhibits cell proliferation and induces apoptosis via DNA damage in mouse embryonic fibroblasts

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ABSTRACT

Wuho known as WDR4 encodes a highly conserved WD40-repeat protein, which has known homologues of WDR4 in human and mouse. Wuho-FEN1 interaction may have a critical role in the growth and development, and in the maintenance of genome stability. However, how *Wuho* gene deletion contributes to cell growth inhibition and apoptosis is still unknown. We utilized CAGGCre-ER transgenic mice have a tamoxifen-inducible cre-mediated recombination cassette to prepare primary mouse embryonic fibroblasts (MEFs) with *Wuho* deficiency. We have demonstrated that Wuho deficiency would induces γH2AX protein level elevation, heterochromatin relaxation and DNA damage down-stream sequences, including p53 activation, caspase-mediated apoptotic pathway, and p21-mediated G2/M cell cycle arrest.

## 1. Introduction

Wuho belongs to the evolutionarily conserved family of WD repeat proteins and is encoded by *wuho* in *Drosophila*, *TRM82* in yeast and *WDR4* (hereafter called *Wuho*) in humans and mice [1]. We previously identified the gene in a *Drosophila* mutant strain that has a sterile phenotype (wuho means no progeny in Chinese) [2], and in a subsequent study, we found that Wuho functions as a guardian of genome stability at DNA replication forks in human and mouse cells [1]. The protein contains multiple WD40 repeats, and in yeast, it is known to form a disc-like structure with seven β-propeller blades [3].

The WD40 repeat domain is a common structural motif in eukaryotes that was first identified in the β-subunit of heterotrimeric G-proteins [4,5]. WD40 domains usually contain four to eight repeating sequences, which are separated by approximately 40 amino acids. Each repeat consists of two sites, including a poorly conserved site with a pair of glycine-histidine residues (GH) and a well-conserved site with a pair of tryptophan-aspartate residues (WD). Together, the WD40 repeats join to form circular β-propeller structures. These β-propellers act as scaffolds for protein-protein interactions and contribute to a wide range of functions [6,7], such as signal transduction, cell cycle regulation, RNA splicing, and transcription [8-10].

Flap endonuclease 1 (FEN1) functions at the replication fork in eukaryotes to remove 5′-RNA primers from the lagging strand. This protein has both slap and gap endonuclease activity, of which gap endonuclease activity can potentially introduce strand breaks at DNA replication sites [11]. A previous report indicated that Wuho guards mammalian genome stability by inhibiting the gap cleavage activity of FEN1 at the replication fork. This inhibition was shown to be essential for cell survival, as downregulation of Wuho resulted in accumulation of double-strand DNA breaks (DSBs) and programmed cell death [1].

The cell cycle and apoptosis are tightly controlled through interlinked cell signaling pathways. Among the factors that control the cell cycle, checkpoint machineries are the most important, and by coordinating with apoptotic and DNA repair mechanisms, these proteins form a circuitry that orchestrates the cellular response to DNA damage [12]. Checkpoints that arrest cell cycle progression can be activated in response to DNA damage and contribute to the maintenance of genome stability by allowing the cell time to repair DNA damage. By this mechanism, the cell can prevent the use of damaged or incompletely replicated chromosomes as material for genetic transmission [13,14]. Genomic stability, cell cycle and programmed cell death pathways are linked by the tumor suppressor gene, *TP53* (encodes p53), and its downstream target gene *CDKN1A* (encodes $p21^{WAF1/CIP1}$). Both p53 and p21 are mutifunctional proteins that can regulate apoptosis and cell cycle arrest through various actions [15–17].

A previous report showed that knockdown of *Wuho* expression by siRNA led to accumulation of DNA damage and apoptosis through the ATM/Chk2/p53 signaling pathway in both mouse and human cells [1]. Mouse embryonic fibroblasts (MEFs) are often used as a tool to analyze tissue-nonspecific effects of genetic modifications, since the cells are easily derived and maintained in a relatively low-maintenance

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anchorage-dependent cell culture system. This primary cell culture system is especially suited for evaluating the effects on cell proliferation caused by dysregulated *Wuho* gene function [18]. In this study, we established a *Wuho*-knockout system with inducible Cre-loxP recombination. Because knocking out *Wuho* is early embryonic lethal in mice [1], we used *Wuho*-knockout MEFs to examine the effects of *Wuho* deficiency on DNA damage, apoptosis, cell cycle distribution and cell signaling.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Caspase-3/CPP32 inhibitor, *Z*-DEVD-FMK (1009-20C), Caspase-9/Mch6 inhibitor, Z-LEHD-FMK (1074-20C), and Apo-BrdU-Red In Situ DNA Fragmentation Assay Kit (K404-60) were purchased from BioVision (USA). 4-Hydroxytamoxifen (H7904) and propidium iodide (PI) were purchased from Sigma-Aldrich (USA). Penicillin streptomycin (10,000 U/ml), Lipofectamine™ 3000, JC-1 Mitochondrial Potential Sensor (T3168) and ProLong Gold antifade reagent with DAPI (P36935) were purchased from Invitrogen (USA). Comet Assay kit (4250–050-K) was purchased from Trevigen. Annexin V-FITC Apoptosis Detection Kit (ab14085) was purchased from Abcam.

### 2.2. Antibodies

The peptide LKKKRQRSPFPGSPEQTK based on the protein sequence of mouse Wuho. The antibodies were purified by affinity chromatography with peptide antigens before being used for Western blot.

The following antibodies were obtained from Cell Signaling: PCNA mouse mAb (2586), p53 mouse mAb (2524), phospho-p53 (Ser15) rabbit mAb (12571), Cleaved PARP antibody (9544), Caspase-3 antibody (9662), phospho-Histone H2A.X (Ser139) rabbit mAb (9718), HP1α antibody (2616), HP1β rabbit mAb (8676), HP1γ antibody (2619), phospho-cdc2 (Tyr15) (10A11) Rabbit mAb (4539), cdc2 Antibody (77055), Cyclin B1 Antibody (4138), Caspase-9 (C9) Mouse mAb (9508). The following antibodies were obtained from EMD Millipore: Anti-GAPDH Antibody (AB2302), Anti-Cre Antibody (69050). The following antibodies were obtained from Abcam: Anti-p21 antibody (ab109199).

### 2.3. Mouse Wuho gene modification

Knockout of the mouse *Wuho* gene was carried out by the Cre-loxP system with a targeting construct to delete exons 2 and 3. The $Wuho^{LoxP/LoxP}$ and *Wuho* heterozygous (+/−) mice were generated as described in a previous report [1].

We first generated a tamoxifen-inducible Cre mouse model of $Wuho^{LoxP/+}$ ($Wuho^{LoxP/+}$,Cre) by crossing $Wuho^{LoxP/LoxP}$ C57BL/6 mice with CAGG-Cre-ER C57BL/6 mice (purchased from Jackson Lab). Following this cross, tamoxifen-inducible Cre mouse embryos of $Wuho^{LoxP/LoxP}$ or $Wuho^{LoxP/-}$ ($Wuho^{LoxP/LoxP}$,Cre or $Wuho^{LoxP/-}$,Cre) were generated by by crossing $Wuho^{LoxP/+}$,Cre mice with $Wuho^{LoxP/-}$ mice.

Genotypes of mice and embryos were assessed by genomic DNA PCR using the following primers: 5′-AAGGAGGGTTTATTCTGGCTGGTCG-3′ and 5′-TCCATGGCAGCTGAGAATATTGTAG-3′ to identify the floxed allele by amplifying a 0.9 kb fragment; 5′-TGGAGCTCACGGGGGCAG GTGAGAC-3′ and 5′-TCCATGGTTATAAATCGCCATGTAG-3′ to identify the floxed allele by amplifying a 0.2 kb fragment; 5′-ACCACGAGCCTA GAGGATCAGTGGC-3′ and 5′-TCCATGGCAGCTGAGAATATTGTAG-3′ to identify the *Wuho* knockout allele by amplifying a 0.4 kb fragment; 5′-GCGGTCTGGCAGTAAAAACTATC-3′ and 5′-GTGAAACAGCATTGCT GTCACTT-3′ to identify the *Cre* allele by amplifying a 0.1 kb fragment.

### 2.4. MEF culture

MEFs were isolated from $Wuho^{LoxP/+}$ and $Wuho^{LoxP/-}$, Cre mouse embryos at 14.5 days post coitum. After the head, liver tissue and blood were removed, embryos were homogenized and typsinized at 37 °C for 30 min. Dissociated cells were resuspended in complete medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml of penicillin streptomycin] at 37 °C with 5% $CO_2$. The MEFs were trypsinized and incubated with complete medium. Cells were passaged consecutively, until a stable cell growth rate was achieved. MEFs were then cultured for an additional 15–20 passages before use in experiments.

### 2.5. Tamoxifen induction of Cre recombinase

We used a tamoxifen-inducible Cre-*lox*P system with Cre-Estrogen receptor (ER) fusion protein to delete *Wuho*. Cells with $Wuho^{LoxP/LoxP}$-Cre or $Wuho^{LoxP/-}$- Cre genotypes are expected to eliminate any existing copies of the *Wuho* gene after tamoxifen treatment. Induction of Cre recombination in MEFs was performed by adding 4-hydroxytamoxifen (TM) methanol solution to the complete medium. MEFs that were cultured in complete medium at about 70% confluence (d0) were treated with 0.1 μM TM for 3 days. The culture medium was replaced with fresh MEF medium for further incubation.

$Wuho^{LoxP/+}$ MEFs were used as wild-type controls and $Wuho^{LoxP/-}$,Cre without TM treatment were used as *Wuho* heterozygous controls. Methanol was used as the 0 μM TM vehicle control. *Wuho* deletion was confirmed by western blot and quantitative RT-PCR.

### 2.6. Protein extraction and immunoblotting

Whole cell extracts were prepared with RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF). Protein concentration of the cell lysates was determined by the Bradford assay. The lysates were separated on 10% polyacrylamide gels and then transferred to PVDF membrane. The membrane was blocked for 1 h with 5% silk milk. The membrane was then incubated for overnight with primary antibodies. Detection was carried out with the appropriate horseradish peroxidase-conjugated secondary antibodies (Millipore).

### 2.7. Quantitative RT-PCR (qRT-PCR)

Total RNA from MEFs was extracted using TRIzol Reagent (Invitrogen), according to the manufacturer instructions. Synthesis of complementary DNA (cDNA) from total RNA was performed using SuperScript III First-Strand Synthesis System kit (Invitrogen). The first-strand cDNA served as template and was amplified with gene-specific primers for mouse *Wuho* (5′- CCTCTGAGGCTGTGAAGGTC -3′ and 5′-AAGCGTCTGATTCTTTTCCG -3′). The relative gene expression levels were normalized to mouse *GAPDH* with gene-specific primers (5′-TGA TGACATCAAGAAGGTGGTGAAG-3′ and 5′-TTCTTGGAGGCCATGTAG GCCAT-3′).

### 2.8. RNA interference (RNAi)

Gene-specific ON-TARGET small interference RNA (siRNA) and control non-targeting pool siRNA were purchased from Dharmacon (Chicago, IL, US), and used for knockdown experiments. The sequences for all the siRNA are provided in Table 1. In brief, the $Wuho^{LoxP/LoxP}$,Cre MEFs ($3 \times 10^5$) were seeded in 60-mm dishes and transfected with 50 nM siRNA using Lipofectamine 3000 in medium with or without TM (0.1 μM) at day-7.