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# Hispolon promotes MDM2 downregulation through chaperone-mediated autophagy

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ABSTRACT

Amplification and overexpression of murine double minute (MDM2) has been observed in several human cancers. Some chemotherapeutic agents cause MDM2 ubiquitination and degradation in a proteasome-dependent system. In addition to the proteasome system, chaperone-mediated autophagy (CMA) is a lysosomal pathway for selective misfolded protein degradation. Molecular chaperone heat shock cognate 70 protein (Hsc70) recognizes the misfolded proteins, which are then delivered to lysosome-associated membrane protein type 2A (LAMP2A) for lysosomal degradation. Our previous study reported that hispolon was able to induce cell apoptosis and downregulate MDM2 expression. In this study, our results showed that the proteasome inhibitor, MG132, could not inhibit hispolon-induced MDM2 downregulation. In contrast, both inhibition of lysosomes with $NH_4Cl$ and inhibition of LAMP2A using siRNA partially attenuated hispolon-induced MDM2 downregulation. To determine whether Hsc70 recognizes MDM2 on amino acids 135–141, SMP14 antibody was used to compete with Hsc70 for interaction with MDM2. After Hsc70 knockdown, SMP14 antibody immunoprecipitated increased MDM2. We also found that hispolon induced increased association of Hsp70, Hsc70, Hsp90 and LAMP2A with MDM2. This association was inhibited in cells pretreated with geldanamycin (GA), an Hsp90 inhibitor. GA also attenuated hispolon-induced MDM2 downregulation. Meanwhile, inhibition of Hsc70 using siRNA attenuated hispolon-induced MDM2 downregulation. Our study provides the first example of the ability of hispolon to mediate MDM2 downregulation in lysosomes through the CMA pathway.

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## 1. Introduction

The MDM2 protein plays a major role in maintaining p53 at a low level under most physiological conditions. Through direct binding of p53, MDM2 inhibits p53 transcriptional activity and initiates p53 ubiquitination and degradation [1]. Although MDM2 has a half-life of approximately 20–90 min in cells [2–4], the MDM2 protein is overexpressed in many human cancers, and high MDM2 levels are associated with a poor prognosis. Both *mdm2* gene amplification and enhanced translation of MDM2 contribute to overproduction of the MDM2 protein [5]. MYCN, a transcription factor, directly modulates baseline MDM2 levels in neuroblastomas [6]. Ectopic expression of mutant p53 increases MDM2 stabilization and expression levels, indicating that mutant p53 can prevent MDM2 degradation [3]. Stabilization and downregulation of MDM2 is a complex process that involves multiple proteins. Zinc induces conformational change in MDM2 that makes it susceptible to proteolytic cleavage [7]. Hausp, a deubiquitinase, and death domain-associated protein dissociate from MDM2, resulting in MDM2 auto-ubiquitination [8]. In response to chemotherapeutic agents, the RING finger domain of MDM2 is required for MDM2 auto-ubiquitination, which targets MDM2 for subsequent degradation in the ubiquitin–proteasome system [9].

Autophagy is the other cellular pathway involved in the clearance of ubiquitinated proteins in lysosomes during nutrient deprivation or cellular stress. Macroautophagy delivers cytosolic components to a double membrane bound vesicle, which fuses with the lysosome to form an autolysosome. In microautophagy, cytosolic components are directly invaginated by the lysosome. The materials inside the large vesicles that are fused with lyso-

*Abbreviations:* CMA, chaperone-mediated autophagy; CHIP, C-terminus of Hsc70-interacting protein; GA, geldanamycin; Hsc, heat shock cognate protein; Hsp, heat shock protein; IP, immunoprecipitation; LAMP2A, lysosome-associated membrane protein type 2A; 3-MA, 3-methyladenine; MDM2, murine double minute.

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somes are digested by hydrolases in the lysosomes. In contrast, CMA degrades misfolded cytosolic proteins through chaperone recognition [10]. ATP-bound Hsp90 recruits one set of co-chaperones to exhibit its chaperone properties, resulting in substrate protein folding and stabilization [11]. When folding to the correct structure is impossible, the substrate protein associates with ADP-bound Hsp90 and other co-chaperones, including Hsc70 and the C-terminus of Hsc70-interacting protein (CHIP) [12]. CHIP, a specific E3 ligase, binds to Hsc70 and Hsp90, resulting in substrate protein ubiquitination and degradation [13,14].

Hsc70 plays an important role in CMA through recognition of the KFERQ sequence and its related amino acids in substrate proteins [15]. The related amino acids consist of a Q flanked on either side by four amino acids, which contain only a basic amino acid (K and R), an acidic amino acid (D and E), a bulky hydrophobic amino acid (F, I, L and V) and a repeated basic or bulky hydrophobic amino acid (K, R, F, I, L and V) [16]. The misfolded protein recognized by Hsc70 and other chaperones, including Hsp90, is delivered to the lysosomal receptor, LAMP2A, and then translocated into lysosomes for degradation [17,18]. The MDM2 sequence contains a hexapeptide ($_{135}$QKDLVQ$_{141}$) that is consistent with an Hsc70 recognition motif. In addition, MDM2 has been reported to associate with Hsp90 and mutant p53 to form a stable complex. Dissociation of Hsp90 from MDM2 by GA increases MDM2 degradation, indicating that Hsp90 and mutant p53 stabilize MDM2 [19]. Whether Hsp90 recruits other chaperones to regulate MDM2 stability is not clear.

Hispolon, isolated from many *Phellinus* species, is able to downregulate MDM2 [20]. Interestingly, hispolon-mediated MDM2 downregulation is not inhibited by proteasome inhibitors, indicating that an alternative protein degradation system is involved in MDM2 degradation. Therefore, we investigated whether autophagy is involved in hispolon-mediated MDM2 downregulation. We first examined whether the lysosome was involved in hispolon-induced MDM2 downregulation using the lysosome inhibitor, $NH_4Cl$. We then investigated whether macroautophagy or CMA was involved in hispolon-induced MDM2 downregulation using their respective inhibitors, 3-MA or GA. GA, but not 3-MA, partially attenuated hispolon-induced MDM2 downregulation. In addition, increased associations of Hsp90, Hsp70, Hsc70 and LAMP2A with MDM2 were found in hispolon-treated cells. These results indicate that chaperones are involved in hispolon-induced MDM2 downregulation. To determine whether amino acids 135–141 of MDM2 were recognized by Hsc70, an MDM2 antibody, SMP14, competing with Hsc70 for a binding site was used. The MDM2 immunoprecipitated by SMP14 antibody was compared before and after Hsc70 knockdown. Finally, siRNA knockdowns of Hsc70 and LAMP2A were used to determine whether these proteins are involved in hispolon-induced MDM2 downregulation. We herein report that in response to hispolon treatment, MDM2 associates with Hsc70, Hsp90 and Hsp70, which directs MDM2 to LAMP2A for lysosomal degradation.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Hispolon was purchased from BJYM Pharm. & Chem. Co., Ltd. (Beijing, China). Etoposide, 3-MA and GA were obtained from Sigma (St. Louis, MO). MG132 was from Calbiochem (La Jolla, CA). Antibodies for β-actin, LAMP2A, MDM2 (SMP14) and Hsc70 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for MDM2 (clone IF2) was from Calbiochem. Antibody for Hsp90 was from Thermo Fisher Scientific Inc. (Fremont, CA). Antibody for Hsp70 was from BD Biosciences Pharmingen (San Jose, CA). Anti-rabbit and anti-mouse IgG–horseradish peroxidases were from Jackson Immunoresearch (West Grove, PA).

### 2.2. Cell culture, cDNA, transfection and fluorescence analysis

HepG2 and MCF7 cells were cultured in MEM Earle's medium with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and incubated at 37 °C with 5% CO2. LC3–GFP was a generous gift of Dr. Ming-Jer Tang [21]. HepG2 cells at 90% confluence were transiently transfected with the LC3–GFP plasmid using Lipofectamine 2000 manufacturer's according to the instructions (Invitrogen). After cells were split and attached to coverslips, the cells were treated with hispolon. Slides were mounted using a SlowFade Antifade kit (Invitrogen) and examined using a Nikon Eclipse TE2000-S microscope. The final composite image was created using Adobe Photoshop 7.0.

### 2.3. Western blot analysis

Western immunoblotting was performed as previously described [20].

### 2.4. Immunoprecipitation

Cells were treated with or without hispolon and then collected with the lysis buffer. The protein concentration was assayed using the BCA protein assay kit. Briefly, 1 mg of cell lysate was incubated with 4 μg MDM2 antibody (clone IF2 or SMP14). MDM2 was immunoprecipitated using the catch and release reversible immunoprecipitation system (Upstate, Temecula, CA). Unbound proteins were washed away with wash buffer. The bound MDM2 was eluted by 70 μL denaturing buffer containing 5% β-mercaptoethanol at 90 °C for 10 min. The equal amount of elutes was resolved on SDS–PAGE for Western blot analysis.

### 2.5. Small interfering RNA (siRNA) and transfection

HepG2 cells at 50% confluence in six-well dishes were transfected with 80 pmol siRNA (Santa Cruz Biotechnology) containing a pool of 3 target-specific 20–25 nucleotide siRNAs directed against respective target genes, or non-targeting siRNA as a control. The sequences for non-targeting siRNA were sense UUCUCCGAACGUGUCACGUtt and antisense ACGUGACACGUUCGGAGAAtt. The sequences for siRNA duplexes targeting *Hsc70* were sense GAUCGAUUCUCUCUAUGAAtt, CCUUCGAGAUGCCAAACUAtt and CUGGACAAGUGUAAUGAAAtt; antisense UUCAUAGAGAGAAUCGAUCtt, UAGUUUGGCAUCUCGAAGGtt and UUUCAUUACACUUGUCCAGtt. The sequences for siRNA duplexes targeting *LAMP2A* were: sense CCAUCAGAAUUCCAUUGAAtt, GUUGCUUCAGUUAUUAACAtt and CAGCACCAUUAAGUAUCUAtt; antisense UUCAAUGGAAUUCUGAUGGtt, UGUUAAUAACUGAAGCAACtt and UAGAUACUUAAUGGUGCUGtt.

## 3. Results

### 3.1. The proteasome is not involved in hispolon-induced MDM2 downregulation

DNA damage has been reported to accelerate MDM2 ubiquitination and degradation in the proteasome [9]. Our previous study showed that hispolon was able to downregulate MDM2 levels [20]. In this study, when cells were treated with hispolon for 15 or 60 min, MDM2 was degraded after hispolon treatment for 60 min (Fig. 1A and B, lane 3). To determine whether the proteasome was involved in hispolon-induced MDM2 downregulation, cells were pretreated with the proteasome inhibitor, MG132, following hispolon treatment. When cells were pretreated with MG132 only, MDM2 level was elevated (Fig. 1A and B, lane 4 compared to lane 1). These results indicate that MDM2 is constitutively degraded in proteasome. Interestingly, MDM2 was rapidly de-

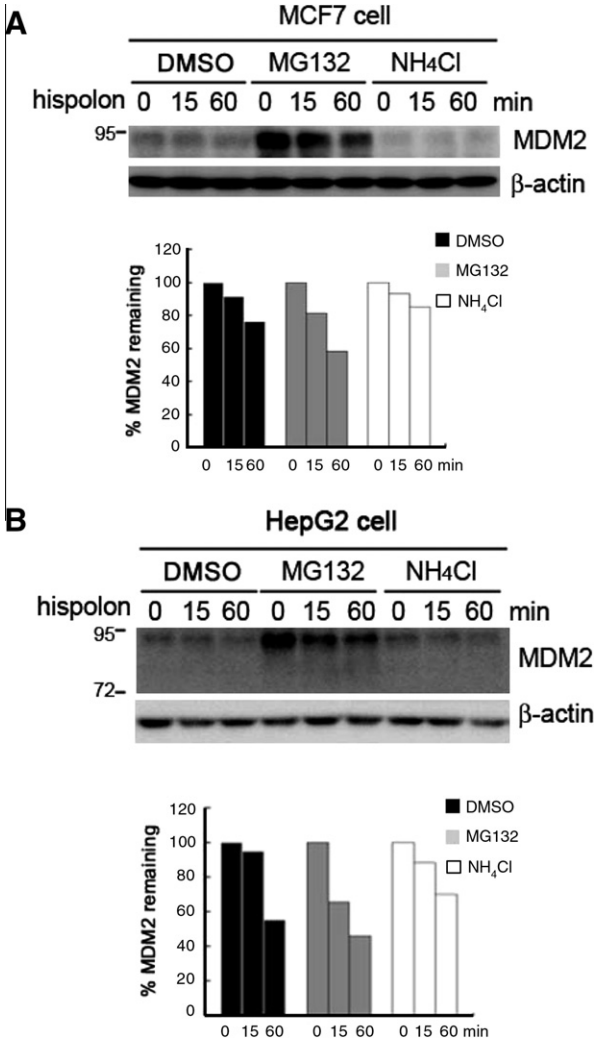


**Fig. 1.** The proteasome is not involved in hispolon-induced MDM2 downregulation. (A) MCF7 and (B) HepG2 cells were preincubated in DMSO, 10 μM MG132 or 20 mM $NH_4Cl$ for 6 h and were then treated with or without 10 μg/mL hispolon for 15 or 60 min. Cells were lysed, and an equivalent amount of protein was used for Western blotting with antibodies to MDM2 and β-actin (as a loading control). Quantification of the amount of MDM2 was determined from densitometric analysis and is shown in the lower panel. The amounts of MDM2 with only DMSO (■), MG132 (▩) or $NH_4Cl$ (□) pretreatment are defined as 100% of the value for each condition. The amount of MDM2 remaining at each time point after hispolon treatment is expressed as a percentage of the MDM2 with hispolon addition. Similar results were obtained in three independent experiments.

graded with MG132 pretreatment following hispolon treatment for 15 min (Fig. 1A and B, lane 5 compared to lane 4) and MDM2 was further degraded after hispolon treatment for 60 min (Fig. 1A and B, lane 6). These results indicate that the proteasome is not involved in hispolon-induced MDM2 downregulation. However, the lysosome inhibitor, $NH_4Cl$, partially inhibited hispolon-induced MDM2 downregulation after hispolon treatment for 60 min (Fig. 1A and B, lane 9 compared to lane 7). These results indicate that proteasome is not involved in hispolon-induced MDM2 downregulation, but the lysosome is involved.

### 3.2. Hispolon induces autophagy

Lysosomes are important in the digestion of cellular organelles and cytosolic proteins during autophagy. To determine the effect of hispolon on autophagy, we examined the localization of LC3–GFP, which was widely used for monitoring autophagy, by fluorescence microscopy [21]. After transfection with the LC3–GFP plasmid, LC3–GFP was seen as a diffuse background in cells. Following hispolon treatment, dot formation of LC3–GFP was significantly elevated in MCF7 and HepG2 cells (Fig. 2). These results show that hispolon induces autophagy.

### 3.3. CMA is involved in hispolon-induced MDM2 downregulation

Three types of autophagy have been identified in mammals, macroautophagy, microautophagy and CMA. We examined whether macroautophagy was involved in hispolon-induced MDM2 downregulation using the macroautophagy inhibitor, 3-MA [22]. MDM2 was still degraded when cells were pretreated with 3-MA followed by hispolon treatment (Fig. 3A), indicating that macroautophagy may not be involved in hispolon-induced MDM2 downregulation.

Hsp90, with other co-chaperones mediates CMA by interacting with substrate proteins and then translocating them across lysosomal membrane [18,23]. Next, we examined whether CMA was involved in hispolon-induced MDM2 downregulation using an Hsp90 inhibitor, GA. GA-pretreatment-induced MDM2 degradation, indicating that Hsp90 regulates MDM2 stability (Fig. 3B, lanes 3 and 7). Pretreatment with GA followed by hispolon treatment partially attenuated hispolon-induced MDM2 downregulation (Fig. 3B, lanes 4 and 8). These results indicate that Hsp90 may be involved in hispolon-induced MDM2 downregulation. To determined whether Hsp90, Hsp70 and Hsc70 directly interact with MDM2 by immunoprecipitating MDM2. Following hispolon treatment, increased Hsp90, Hsp70 and Hsc70 were co-precipitated with MDM2 (Fig. 3C and D, lane 6). GA has been reported to bind to the ATP-binding site of Hsp90, thus abrogating the formation of Hsp90 and the substrate–protein complex [24]. When cells were pretreated with GA only, the co-precipitation of Hsp90 with MDM2 was inhibited (Fig. 3C and D, lane 7). Although more Hsp70 and Hsc70 co-precipitated with MDM2 in GA-pretreated cells (Fig. 3C and D, lane 7), the further association of Hsp70 and Hsc70 with MDM2 was inhibited after hispolon treatment (Fig. 3C and D, lane 8). These results indicate that the chaperones Hsp90, Hsp70 and Hsc70 are involved in hispolon-induced MDM2 downregulation.

Cytosolic Hsc70 also plays an important role in CMA by recognizing the KFERQ sequence and related motifs in substrate proteins [15]. MDM2 contains a hexapeptide sequence ($_{135}$QKDLVQ$_{141}$) that is consistent with an Hsc70 recognition motif. To determine whether amino acids 135–141 were a target sequence for Hsc70, monoclonal antibody, SMP14, which recognizes amino acids 154–167 of MDM2 was used to immunoprecipitate MDM2. We hypothesized that if SMP14 antibody recognized a region of MDM2 close to a target sequence of Hsc70, SMP14 antibody could immunoprecipitate more MDM2 after Hsc70 knockdown. Due to the low transfection efficiency in MCF7 cells, Hsc70 siRNA was transiently transfected into HepG2 cells. We found that Hsc70 co-precipitated with MDM2 in MDM2 immunoprecipitates using SMP14 antibody (Fig. 4A, lane 3). When cells were transiently transfected with Hsc70 siRNA for 24 h, less Hsc70 co-precipitated with MDM2. Meanwhile, SMP14 antibody was able to immunoprecipitate more MDM2 in Hsc70 knockdown cells (Fig. 4A, lane 4). In addition, hispolon-induced MDM2 downregulation was inhibited in Hsc70 knockdown cells (Fig. 4B). These results indicate that Hsc70 is involved in hispolon-induced MDM2 downregulation.

### 3.4. Lysosomes are involved in hispolon-induced MDM2 downregulation

LAMP2A is an integral membrane protein with a highly glycosylated region, resulting in a variable electrophoresis mobility from molecular weight 90–110 kDa. LAMP2A serves as a receptor for the

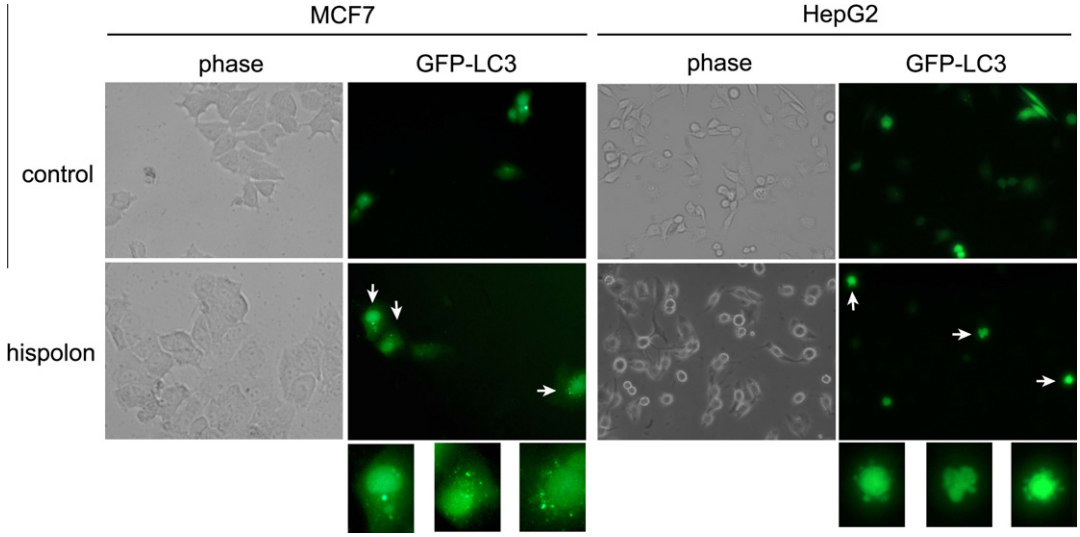


**Fig. 2.** Hispolon induces autophagy. MCF7 and HepG2 cells were transiently transfected with GFP–LC3 plasmid for 24 h and then treated with or without (control) 10 μg/mL hispolon for 1 h. Images were captured by light and fluorescence microscopy. Arrows indicate the cells with GFP–LC3 dot formation. Enlarged images of the cells (arrows) are shown at the bottom. Similar results were obtained in three independent experiments.

selective uptake and degradation of proteins by lysosomes [17]. To determine whether hispolon induced the association of LAMP2A with MDM2, we examined the interaction of LAMP2A with MDM2 by immunoprecipitating MDM2. More LAMP2A co-precipitated with MDM2 after hispolon treatment (Fig. 4C). To determine whether LAMP2A is involved in hispolon-induced MDM2 downregulation, LAMP2A was knocked down by siRNA in HepG2 cells. After transfection with LAMP2A siRNA, hispolon-induced MDM2 downregulation was partially inhibited (Fig. 4D). These results indicate that the lysosome is involved in hispolon-induced MDM2 downregulation.

## 4. Discussion

CMA plays an important role in digesting misfolded proteins to mediate cellular and organ homeostasis. Misfolded proteins containing the CMA motif are recognized by Hsc70, resulting in their delivery to the lysosome for degradation [15]. Defective CMA has been proposed to contribute to aging and cancers; hence, activation of CMA may act as a suppressor of cancer [25]. MDM2 contains a KFEQR-like motif at residues 135–141, which may be recognized by Hsc70. We found that more Hsc70, Hsp70 and Hsp90 associate with MDM2 after hispolon treatment. However, pretreatment with an Hsp90 inhibitor attenuates this association, resulting in the inhibition of hispolon-induced MDM2 downregulation. Hispolon also induces the association of LAMP2A with MDM2. Inhibition of lysosomes with the lysosome inhibitor, $NH_4Cl$, and inhibition of LAMP2A suing siRNA partially inhibited hispolon-induced MDM2 downregulation. In addition, inhibition of Hsc70 using siRNA attenuated hispolon-induced MDM2 downregulation. We report that hispolon induces MDM2 downregulation through the CMA pathway.

The autophagy–lysosome system and the ubiquitin–proteasome system (UPS) are the two systems that function to degrade misfolded proteins in mammalian cells [26]. Several proteins are able to be degraded by proteasome, lysosome or CMA pathways. For example, accumulation of α-synuclein, which is implicated in the pathogenesis of Parkinson's disease, can be degraded by the proteasome or CMA [27]. Both proteasomes and lysosomes are involved in epidermal growth factor receptor (EGFR) degradation [28]. A recent report shows that cyclodepsipeptide toxin, which induces cytotoxic activity against several cancer cells, promotes EGFR degradation by CMA [29]. When the proteasome is inhibited by a proteasome inhibitor, autophagy may be induced as a compensatory system to digest polyubiquitinated proteins [26]. Our results show that the MDM2 expression level is elevated in only MG132 treated cells (Fig. 1A and B, lane 4). These results are consistent with MDM2 being degraded in the proteasome. However, MDM2 downregulation is accelerated and more MDM2 is downregulated in cells that have been pretreated with MG132 followed by hispolon treatment (Fig. 1A and B, lanes 5 and 6). These results suggest that when the proteasome is inhibited, the lysosome may be a compensatory system that contributes to hispolon-induced MDM2 downregulation. In addition, both the lysosome inhibitor (Fig. 1A and B, lane 9), and inhibition of LAMP2A with siRNA partially reduce hispolon-induced MDM2 downregulation (Fig. 4D). These results indicate that hispolon-induced MDM2 downregulation is via the autophagy–lysosome system.

Microautophagy and macroautophagy have the capacity to engulf large structures and then fuse with lysosomes. Upon induction of autophagy, LC3 is conjugated to autophagic membranes, which then fuse with lysosomes. Changes in LC3 localization have been used to measure macroautophagy [21]. Our study showed that hispolon significantly induced LC3–GFP dot formation in LC3–GFP transiently transfected cells (Fig. 2B). Interestingly, the macroautophagy inhibitor, 3-MA, does not inhibit hispolon-induced MDM2 downregulation (Fig. 3A). LC3 has been reported to associate with BAG1 (Bcl-2-associated athanogene 1), which interacts with Hsc70 to participate in protein folding and degradation [30]. Based on these results, we investigated whether CMA is involved in hispolon-induced MDM2 downregulation.

Substrates containing KFERQ or KFEQR-like amino acids are recognized by Hsc70 and then delivered to the lysosomal membrane [15,31]. Approximately 30% of cytosolic proteins contain the KFEQR-like motif [32]. Our results showed that more Hsc70 and Hsp70 interact with MDM2 after hispolon treatment (Fig. 3C and D). To determine whether Hsc70 recognizes KFEQR-like motif of MDM2 on residues 135 to ($_{135}$QKDLVQ$_{141}$), SMP14 antibody was used to immunoprecipitate MDM2. SMP14 antibody recognizes an epitope located on MDM2 residues 154–167, which is close to the putative Hsc70 recognition motif. In Hsc70 knockdown cells, both Hsc70 and Hsp70 are knocked down and less Hsc70 co-precipitated with MDM2. However, more MDM2 is immunoprecipi-

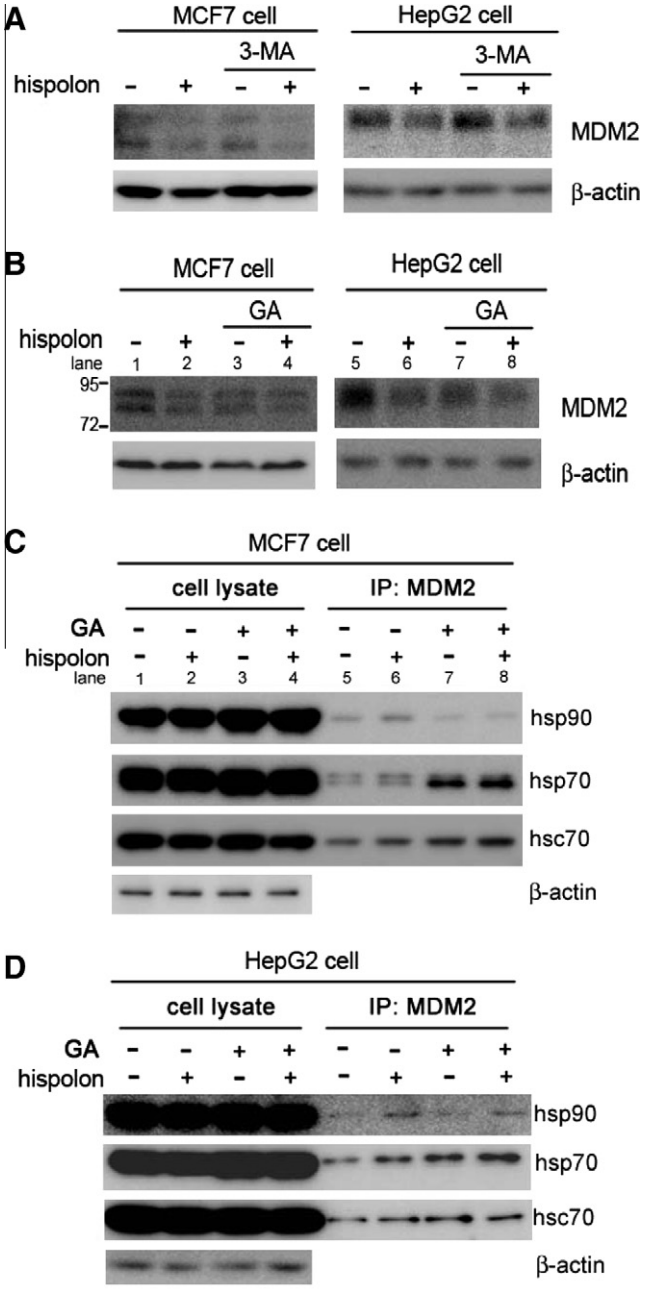


**Fig. 3.** CMA is involved in hispolon-induced MDM2 downregulation. MCF7 and HepG2 cells were preincubated in the presence or absence of (A) 50 μM 3-MA or (B–D) 2 μM GA for 6 h and then were treated without or with 10 μg/mL hispolon for an additional 1 h. MDM2 immunoprecipitation (IP:MDM2) was performed as described in Section 2 with MDM2 antibody (clone IF2). Equivalent amount of protein from whole cell lysates (cell lysate) and the immunoprecipitates were immunoblotted with anti-MDM2, Hsp90, Hsp70 and Hsc70 antibodies. β-Actin was used as a loading control. Similar results were obtained in three independent experiments.

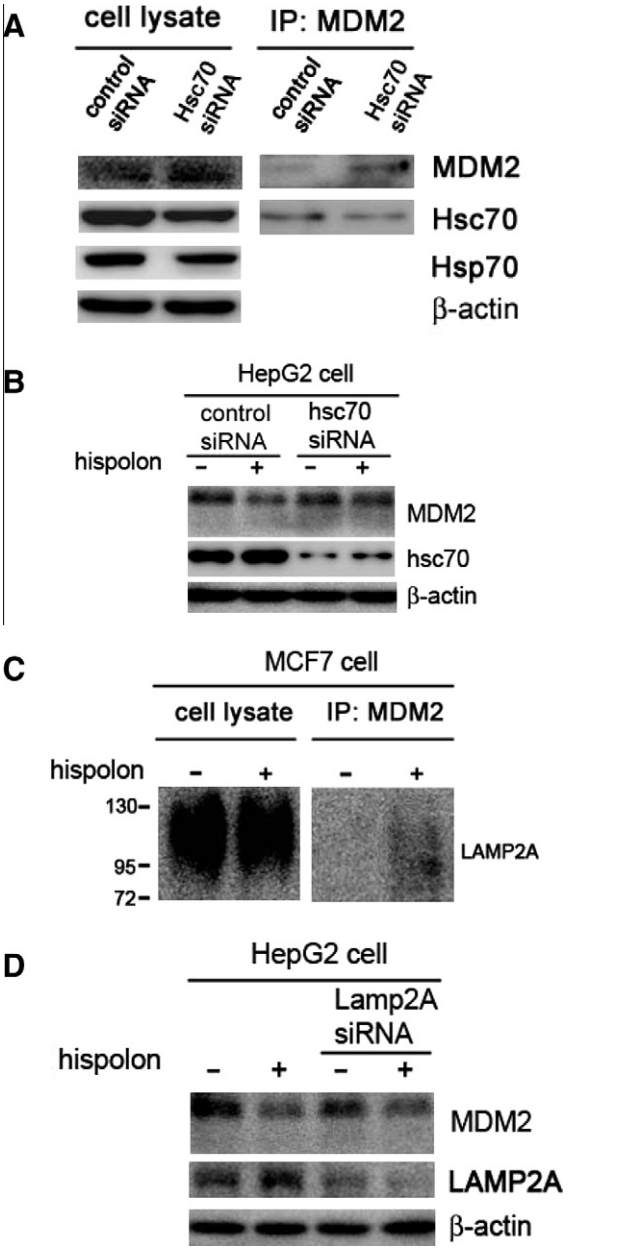


**Fig. 4.** Hsc70 and LAMP2A are involved in hispolon-induced MDM2 downregulation. HepG2 cells were transiently transfected with control or Hsc70 siRNA for 24 h and then (A) MDM2 was immunoprecipitated (IP:MDM2) with MDM2 antibody (clone SMP14). (B) Cells were treated with or without 10 μg/mL hispolon for 1 h. (C) MCF7 cells were treated with or without 10 μg/mL hispolon for 1 h. MDM2 was immunoprecipitated (IP:MDM2) with MDM2 antibody (clone IF2). (D) HepG2 cells were transiently transfected with control or LAMP2A siRNA for 24 h and then were treated with or without 10 μg/mL hispolon for 1 h. Equivalent amounts of protein from whole cell lysates (cell lysate) and the immunoprecipitates were immunoblotted with anti-MDM2 (clone SMP14), Hsc70 and Hsp70 and LAMP2A antibodies. β-Actin was used as a loading control. Similar results were obtained in three independent experiments.

tated by SMP14 antibody in Hsc70 knockdown cells (Fig. 4A). These results imply that residues 135–141 of MDM2 may be recognized by Hsc70. In addition, knockdown of Hsc70 with siRNA inhibits hispolon-induced MDM2 downregulation (Fig. 4B). These results show that hispolon downregulates MDM2 through an interaction with Hsc70 and Hsp70, which are involved in CMA.

The CMA substrate delivered by Hsc70 binds to monomers of LAMP2A. This binding promotes the formation of an approximately 700 kDa LAMP2A complex, which is required for substrate translocation [31]. Our results show that more LAMP2A interacts with MDM2 after hispolon treatment (Fig. 4C). Whether Hsc70 is involved in the interaction with LAMP2A needs to be further investigated. However, our results show that knockdown of LAMP2A by siRNA partially attenuates hispolon-induced MDM2 downregulation (Fig. 4D). These results indicate that in response to hispolon, MDM2 is downregulated by association with LAMP2A.

A recent study shows that cyclodepsipeptide toxin prevents the interaction of EGFR with Hsp90, resulting in stabilization of the

interaction of Hsc70 and Hsp70. The interaction of Hsp70 and Hsc70 with EGFR promotes EGFR degradation through CMA [29]. Our study shows that GA reduces the association of Hsp90 with MDM2 in MDM2 immunoprecipitates (Fig. 3C and D, lane 7) and induces MDM2 downregulation (Fig. 3B, lanes 3 and 7). These results are consistent with MDM2 being an Hsp90 substrate protein [19]. GA also increases the association of Hsp70 and Hsc70 with MDM2 (Fig. 3C and D, lane 7); however, there is no further increase in the association of Hsp70 and Hsc70 with MDM2 following hispolon treatment (Fig. 3C and D, lane 8). These results indicate that in response to hispolon treatment, MDM2 is recognized by Hsp90, Hsc70 and Hsp70, which directs MDM2 to LAMP2A for lysosomal degradation.

CMA plays an important role in selective degradation of cytosolic proteins in lysosomes. In many human tumors, MDM2 is overexpressed and is associated with a poor prognosis. Although the proteasome is known to mediate MDM2 degradation, our study provides the first example for the ability of hispolon to downregulate MDM2 through CMA, a lysosomal degradation system distinct from the proteasome-dependent system.

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