JBC Papers in Press. Published on May 3, 2012 as Manuscript M111.336875
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M111.336875

Hypoxia activates the tumor suppressor p53 by inducing ATR-Chk1 kinase cascade-mediated phosphorylation and consequent 14-3-3γ inactivation of MDMX

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Running Title: Hypoxia activation of p53 via inactivation of MDMX

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Key Words: Hypoxia, p53, 14-3-3γ, MDMX, ATR, Chk1, cell cycle

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Background: It remains unclear how the hypoixa-ATR-ChK1 pathway induces p53.
Results: Hypoxia suppresses MDMX activity via ATR/ChK1 phosphorylation of S367 and subsequent MDMX-14-3-3 interaction, leading to p53 activation.
Conclusion: Induction of MDMX-14-3-3 binding accounts for p53 activation by hypoxia.
Significance: Our study elucidates a molecular mechanism underlying hypoxia-induced p53 activation.

SUMMARY

It has been known that p53 can be induced and activated by hypoxia, an abnormal condition that often occurs in rapidly growing solid tumors or when normal tissues undergo ischemia. Although the ATR-Chk1 kinase cascade was associated with hypoxia-induced p53 activation, molecules that directly link this hypoxia-ATR-Chk1 pathway to p53 activation have been elusive. Here, we showed that hypoxia could induce phosphorylation of MDMX at Ser367 and enhance the binding of this phosphorylated MDMX to 14-3-3γ, consequently leading to p53 activation. A Chk1 inhibitor or knockdown of ATR and Chk1 inhibited the phosphorylation of MDMX at S367 and impaired the binding of MDMX to 14-3-3γ in addition to p53 activation in response to hypoxia. In primary mouse embryonic fibroblast cells that harbor a mutant MDMX including S367A mutation, hypoxia also failed to induce the binding of this mutant MDMX to 14-3-3γ and to activate p53 and its direct targets. These results demonstrate that hypoxia can activate p53 through inactivation of MDMX by the ATR-Chk1-MDMX-14-3-3γ pathway.

INTRODUCTION

Low oxygen tension or hypoxia is an inevitable pathophysiological status during solid tumor progression. It occurs due to the lack of blood supply in rapidly growing solid tumors, which possess anoxic (0%) or hypoxic (<8% of oxygen) regions with poor prognosis (1,2). In response to these anoxic or hypoxic conditions, cancerous cells produce several important proteins that either favor or prevent their survival and advanced progression. Among those, while HIF1α (Hypoxia-Inducible Factor 1α) is induced to promote angiogenesis and anaerobic metabolism as a tumor-promoting factor (2,3), the tumor suppressor p53 is often activated as well to stop tumor growth and progression in wild type p53 containing cancer cells (4) by inducing apoptosis and/or cell cycle arrest and repressing angiogenesis (5,6).

Over the past decade, a number of studies have been reported to elucidate how hypoxia might induce p53. The first candidate molecule that was proposed to mediate p53 induction by hypoxia is HIF1α (7,8). HIF1α was shown to directly associate with p53 and to stabilize it in response to hypoxia. However, this notion was challenged by a kinetic study showing that HIF1α and p53 were induced at distinct hypoxia stages (9). p53 was stabilized in more stringent hypoxic conditions than HIF1α and inversely proportional to HIF1α levels with repression of HIF1α activity in response to hypoxia (9-12). It seems that HIF1α is insufficient to up-regulate p53 (6) and less likely responsible for extremely low oxygen-triggered p53 induction through direct interaction. Instead, ATR, an ATM and Rad3 like serine/threonine kinase and/or Chk1, a DNA damage check-point kinase as a downstream target of ATR, have been

proposed as the responsible molecules in response to hypoxia (9,13-16). ATR was found to phosphorylate p53 at serine 15 in response to hypoxia while ATM could not (9,14). It was previously shown that serine 15 phosphorylation by UV-activated ATR or γ irradiation-activated ATM and/or DNAPK can partially stimulate p53 transcriptional activity (17-20). However, serine 15 phosphorylation could not account for the full scale of p53 induction by hypoxia, as mutation of this residue could only partially affect p53 activity in response to ionizing irradiation in animals and does not seem to affect p53 stability (21,22). Chk1 was also thought to have a role in hypoxia-responsive p53 induction (9,15,16). It is unclear whether Chk1 directly phosphorylates p53 or other components such as MDM2 or MDMX that in turn affects p53 stability. Hence, the exact mechanism of how ATR and Chk1 induce p53 activation in response to hypoxia still remains elusive.

The tight regulation of p53 has been mainly attributed to the MDM2-MDMX-p53 loop, whose direct or indirect interferences lead to p53 activation by various cellular stresses including DNA damage stress (23). Although MDM2 has been described to be a prime negative feedback regulator of p53 (24-27), another crucial regulator of p53, MDMX, also monitors the intracellular level and activity of p53 together with MDM2 (28,29). Of note, MDMX can also function independently of MDM2 (30). Like MDM2, MDMX is indispensable for p53 inactivation, as knocking out the *TP53* gene also rescues the lethality of *mdmx* null mice (31,32). Recently, in vivo studies by either deleting the C-terminal RING domain of MDMX (MDM4) or generating C462A mutation, both of which disrupt MDM2-MDMX binding, in mice demonstrate that the MDM2-MDMX binding is essential for negating p53 function in the animals (33,34) These results are consistent with the previous reports showing that MDM2 and MDMX can form a heterocomplex in vitro and in cells (35-37). Nonetheless, surprisingly, no direct connection has been unambiguously built between this loop and hypoxia-caused stress.

Several members of the 14-3-3 family, including 14-3-3γ, have been reported to bind primarily to phosphorylated MDMX at serine 367 residue in response to stress signals, such as DNA damage stimuli (38-40). Previously, we also demonstrated that UV-mediated Chk1 activation induces phosphorylation of MDMX at S367 and enhances the interaction between MDMX and 14-3-3γ, leading to p53 activation (41). This phenomenon was also demonstrated in a mouse study by knocking-in triple mutations (S341A, S367A, and S402A) at MDMX phosphorylation sites (42). Thus, we speculated that hypoxia might activate p53 by blocking the activity of MDMX via the ATR-ChK1 kinase cascade, which leads to MDMX phosphorylation at S367 and promotes MDMX interaction with 14-3-3, suppressing MDMX activity. Here, in this study, we tested this hypothesis and verified this hypoxia-ATR-ChK1-MDMX-14-3-3-p53 pathway.

EXPERIMENTAL PROCEDURES

Cell culture and hypoxia treatment. Human osteosarcoma U2OS cells and human embryonic kidney (HEK) epithelial 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 5% CO_2 humidified atmosphere. Mouse embryonic fibroblast (MEF) wild type (wt) or MDMX-triple mutant (3SA) MEF cells (37) were cultured in DMEM supplemented with 15 % FBS, 10 U/ml penicillin, 0.1 mg/ml streptomycin, 1x nonessential amino acids

(NEAA) (Cellgro) and 1x β-mercaptoethanol (Millipore). Prior to exposure to hypoxic condition, cells were cultured and incubated at 5% CO_2/37°C for 24-48 h with ~50% confluence. Hypoxia was established by incubating cells in an anaerobic system (Thermo Forma), which reduces O_2 levels less than ~0.1 % close to anoxic condition. The O_2 levels were also monitored by a methylene blue indicator strip during the incubation of cells inside the hypoxia chamber with the time periods indicated in figure legends. Corresponding normoxia control cells were incubated under a normal culture condition.

Plasmids, reagents and antibodies. Plasmids utilized here including Myc-MDMX and Flag-14-3-3γ were previously described (41,43). Myc-MDMX single mutant plasmids were generated from wild type Myc-MDMX by site-directed mutagenesis. UCN-01 was used as previously described (41). Monoclonal anti-Flag, anti-α-tubulin and anti-β-actin antibodies were purchased from Sigma. Monoclonal anti-p53 antibody (DO-1, Santa Cruz and 1C12, Cell Signaling) or polyclonal anti-p53 antibody (FL393, Santa Cruz), monoclonal anti-p21 (F-5, Santa Cruz) or polyclonal anti-p21 (M19, Santa Cruz), monoclonal anti-c-Myc (9E10, Santa Cruz), polyclonal anti-MDMX (Bethyl laboratories and H130, Santa Cruz) or monoclonal anti-MDMX (MDMX-82, Sigma and 8C6), polyclonal anti-14-3-3γ (C16, Santa Cruz), goat polyclonal anti-ATR (N19, Santa Cruz) and monoclonal anti-Chk1 (G-4, Santa Cruz) antibodies were also purchased. Monoclonal anti-pS367-MDMX was generated and purified from our laboratory and monoclonal anti-MDM2 (2A10 and 4B11) antibodies were previously described (44,45).

Transfection, Western blot and co-immunoprecipitation analyses. Cells were transfected with plasmids as indicated in figure legends using TransFectin reagents (Bio-Rad), following the manufacturer's protocol. Cells were harvested at 36-48 h post-transfection and lysed in cell lysis buffer consisting of 50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40 (NP-40), 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) in addition to protease inhibitor cocktail (Sigma). Western blot (WB) and co-immunoprecipitation (co-IP) analyses were performed as previously described (43).

RNA interference. siRNA duplex specific to human ATR or Chk1 were synthesized by Dharmacon. The target sequences, which were also utilized in the previous studies (46,47) are as follows; ATR: 5'-AAGCCAAGACAAATTCTGTGT-3', Chk1: 5'-AAGCGTGCCGTAGACTGTCCA-3'. U2OS cells were transfected with 20 nM (final conc.) ATR or Chk1 siRNA duplex or scrambled RNA duplex using siLentFect lipid reagent (Bio-Rad) and the following day, the cells were transfected once again. Approximately 24h after the second transfection, cells were subjected to hypoxic conditions and incubated for indicated time periods in figure legends followed by WB and/or co-IP analyses. In some cases for ATR knock-down, U2OS cells were transfected with shRNA or infected by lentivirus encoding shRNA and incubated for ~48 h prior to hypoxia treatment.

Cell cycle analysis. U2OS cells were double-transfected with scramble, ATR or

Chk1 siRNAs and ~24h after the second transfection, cells were subjected to normoxic or hypoxic conditions. Wild type or 3SA MEF cells were split the day before hypoxia treatment. Then cells were harvested at indicated time points and fixed with 70% EtOH (final conc.) at least for 30 min on ice. After washing with 1x PBS, cells were stained with PI staining buffer in PBS (50 μg/ml PI, 0.1 mg/ml RNase A, 0.05% Triton X-100) and incubated for 40 min at 37°C followed by fluorescence activated cell sorter (FACS) analysis.

Immunofluorescence staining and fluorescence microscopic analysis. U2OS cells were transfected with 1 mM random siRNA or Chk1 specific siRNA for 72 h or treated by 1 mM hydroxyurine for 12 h. Cells were fixed for immunofluorescence staining with rabbit anti- γ -H2AX antibody and secondary goat anti-rabbit antibody conjugated Alexa Fluor 546 (red), respectively. DNA was stained with DAPI (blue). Stained cells were analyzed under the Zeiss Axiovert 200M fluorescent microscope (Zeiss, Germany).

RESULTS AND DISCUSSION

To verify and work out the condition for the effect of hypoxia on p53 activation, U2OS cells were incubated under very low-oxygen condition (< 0.1 % O_2) up to 16h. The result showed that, indeed, p53 and its targets p21 and Puma were induced in a time dependent manner under this condition (Fig. 1A and 1B). Hypoxia-mediated p53 activation could be also extended by 18h-24h in various cancer cells, including U2OS cells [Supplemental Fig. S1A and (9,14)]. In addition, the treatment of $CoCl_2$, a hypoxia mimetic compound, which can induce biochemical and molecular responses similar to those elicited by hypoxia (8), induced p53 in MEF cells at 16h and U2OS cells in a time dependent manner up to 24h under hypoxia as well (Supplemental Fig. S1B). These results confirm that hypoxia indeed induces p53 independently of cell types. Interestingly, hypoxia led to the phosphorylation of serine 367 residue of MDMX (S367-MDMX), which is known to be the major phosphorylation site of MDMX for the binding of 14-3-3 protein(s) in response to stress signals, such as DNA damage stimuli (38-41). As expected, this phosphorylation enhanced the binding of 14-3-3γ to MDMX, leading to p53 activation (Fig. 1C). These results suggest that hypoxia can induce phosphorylation of MDMX at S367 and subsequent induction of MDMX binding to 14-3-3γ, leading to suppression of MDMX activity toward p53 and consequent p53 activation.

Since DNA damage stimuli, such as UV-irradiation, can induce p53 activation through phosphorylation of MDMX at S367 by Chk1 (41) and ATR-Chk1 kinase cascade plays an important role in hypoxic stress (9,13,14), we deduced that hypoxia-mediated MDMX phosphorylation might employ the same ATR-Chk1 signaling pathway to activate p53. To test our speculation, we utilized a Chk1 inhibitor, UCN-01 (48) to determine if it affects the phosphorylation of endogenous MDMX at S367 in response to hypoxia. When U2OS cells were subjected to hypoxia, as expected, MDMX phosphorylation was induced at S367; However, when treated with UCN-01 under the same hypoxic condition, MDMX phosphorylation at this site was dramatically reduced though the total amount of MDMX was comparable (Fig. 2A). Subsequently, the hypoxia-enhanced interaction between exogenous Myc-MDMX and Flag-14-3-3γ was considerably reduced by UCN-01 treatment (Fig. 2B). Consistently, UCN-01 treatment also reduced hypoxia-induced endogenous MDMX phosphorylation at

S367 and weakened the interaction between endogenous MDMX and 14-3-3γ, correlated well with a significant decrease in p53 activation (Fig. 2C). Thus, Chk1 plays an important role in hypoxic response, as previously reported (9,13,14), but targeting MDMX in this experiment.

To confirm this observation and to demonstrate the involvement of the ATR-Chk1 kinase cascade in MDMX phosphorylation and MDMX-14-3-3 binding, we introduced si- or sh-RNAs for Chk1 and ATR into U2OS cells. First, knockdown of Chk1 under hypoxic condition abolished MDMX phosphorylation at S367 as well as the interaction between endogenous MDMX and 14-3-3γ (Fig. 3A). To exclude the possibility that hypoxia might activate Chk1 by inducing DNA damage, we checked if knockdown of Chk1 would impair DNA damage by assessing γ-H2AX focus formation. Surprisingly, knockdown of Chk1, similar to hydroxyurea treatment, also caused DNA damage (Fig. S2). This result indicates that hypoxia-induced MDMX S367 phosphorylation is catalyzed by hypoxia-, rather than DNA damage-, activated Chk1. If not, knockdown of Chk1 would still induce S367 phosphorylation, as the depletion of this kinase also caused DNA damage (Fig. S2). However, this was not the case (Fig. 3). Then, we also performed a similar set of experiments by knocking down ATR, the upstream kinase of Chk1. The result demonstrated that the depletion of ATR also weakens the endogenous MDMX phosphorylation at S367 as well as MDMX interaction with 14-3-3γ (Fig. 3B).

If MDMX phosphorylation necessary for 14-3-3γ binding is essential for hypoxia-induced p53 activation, mutations in MDMX phosphorylation sites where 14-3-3 can bind should be expected to abolish p53 activation in response to hypoxia. Although S367 is a key phosphorylation site of MDMX for the binding of 14-3-3 proteins including 14-3-3γ, phosphorylation at S342 of MDMX was also reported to involve the interaction between 14-3-3 and MDMX (40,49). To test this idea, we first employed MDMX 3SA MEF cells, which harbor a knock-in MDMX mutant with S341A, S367A, and S402A mutations, for our experiments under hypoxic stress. When wild type MEF cells were subjected to hypoxia for 18h, as expected, p53 was activated with the induction of its direct downstream target protein p21 (Fig. 4A). Increased endogenous 14-3-3γ binding to MDMX was also detected in these cells in response to hypoxia (Fig. 4A). However, 3SA MEF cells failed to show significant induction of p53 and its target protein in response to hypoxia (Fig. 4A), similar to the previous study in which p53 activity was also considerably hampered in 3SA MEF cells compared to wild type MEF cells in response to DNA damage (42). In addition, 3SA MEF cells were unable to show enhanced interaction between MDMX and 14-3-3γ after hypoxia treatment (Fig. 4A). This result could rule out the possibilities such that ATR-Chk1-mediated direct p53 activation by N-terminal phosphorylation of p53 or hypoxia-mediated MDM2 phosphorylation, which promotes MDM2 auto-degradation since only triple mutations of MDMX are involved in this occasion. Next, wild type and 3SA MEF cells were cultured under a normoxic or hypoxic condition followed by FACS analysis. While wild type MEF cells showed more than 15% increase in G1 phase cells after hypoxia treatment, 3SA MEF cells were unable to demonstrate any significant increase in G1 arrest (Fig. 4B). This difference between wt and mutant MDMX-containing MEF cells should be due to the phosphorylation of MDMX at these three sites followed by p53 activation. Because 3SA MDMX in MEF

cells possesses three serine mutations, to determine which serine is more critical for hypoxia-induced interaction between MDMX and 14-3-3γ, we dissected and evaluated the outcome derived from individual single mutations of these MDMX phosphorylation sites in the perspective of interaction between two proteins. Co-immunoprecipitation after ectopic expression of Flag-14-3-3γ and Myc-tagged MDMX with respective single alanine mutations at S342, S367 or S403 residues showed that the S367A mutation of MDMX completely abolished the interaction between 14-3-3γ and MDMX regardless of oxygen status (Fig. 4C). Also, albeit the S342A mutation hampered this interaction, hypoxia treatment was still able to enhance the interaction between S342A-MDMX and 14-3-3γ, relatively to a less degree compared to that for wild type MDMX (Fig. 4C). In contrast, S403A-MDMX mutation did not hinder the binding of 14-3-3γ to MDMX, compared to the corresponding wild type MDMX control before and after hypoxia (Fig. 4C). These results, similar to the previous observations in response to DNA damage (38-42), again, firmly support the phosphorylation of MDMX at S367 is the most essential and responsible event for the interaction between MDMX and 14-3-3γ in response to hypoxia. Also, nearby S342 appears to possess potential to facilitate and preserve their intact interaction.

Our study as presented here unambiguously demonstrates that MDMX phosphorylation at S367 is vital to hypoxia-induced p53 activation in addition to DNA damage responses. Moreover, S342 phosphorylation of MDMX seems to function as a favorable factor conducive to S367 phosphorylation and/or 14-3-3 binding to MDMX in the course of hypoxia-mediated p53 activation. Though here we have utilized and scrutinized the function of 14-3-3γ as a binding partner of MDMX in hypoxia-mediated p53 activation in this study, it would not be surprising if other 14-3-3 family members, which have also been shown to interact with MDMX, such as 14-3-3τ or 14-3-3ε, but not 14-3-3σ (33-35), may play a role in hypoxia-caused p53 activation as well. It is likely that MDMX phosphorylation-enhanced interaction with 14-3-3 protein(s) might be a common mechanism that accounts for p53 activation via inactivation of MDMX by a number of stress signals.

In summary, our results strongly support the notion that hypoxia-mediated p53 activation is through the ATR-Chk1-MDMX-14-3-3γ signaling pathway. Specifically, hypoxia activates ATR and then ChK1, which in turn phosphorylates MDMX at S367 and induces MDMX-14-3-3 binding, consequently suppressing MDMX activity and activating p53 (Fig. 4D). Thus, this study illuminates a new role of 14-3-3γ as a functional link between the ATR-Chk1 kinase cascade and the MDMX-p53 pathway in response to hypoxia.

Acknowledgements

We thank Dr. Zaocheng Xu for allowing us to use his hypoxia chamber and the members of the Lu lab for active discussion. This work was supported in part by NIH-NCI grants CA095441, CA 079721 and CA129828 to H.L. G.W. was supported by NIH-NCI grants as well.

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FIGURE LEGENDS

FIGURE 1. Hypoxia induces endogenous p53 accompanied by increased MDMX phosphorylation at S367. ***A,*** Hypoxia induces p53 activation and MDMX phosphorylation at S367. U2OS cells were cultured in a hypoxia chamber and harvested at different time points as indicated. Cell lysates were prepared for WB analysis with antibodies as indicated. ***B,*** Hypoxia induces p53 and its targets. U2OS cells were cultured in a hypoxia chamber and harvested at different time points as indicated. 50 μg of each cell lysate was subjected to WB analysis and detected with p53, p21, Puma and β-actin antibodies. ***C,*** Hypoxia enhances MDMX and 14-3-3γ interaction. U2OS cells were cultured under normal or hypoxic conditions for 6h and 300 μg of cell lysates were subjected to co-IP with polyclonal anti-14-3-3γ antibodies. 40 μg of each lysate were loaded for WB analysis. Proteins of interest were detected by corresponding antibodies as listed on the left.

FIGURE 2. The Chk1 kinase inhibitor, UCN-01, attenuates hypoxia-induced MDMX phosphorylation at S367 and negates the interaction between MDMX and 14-3-3γ. ***A,*** UCN-01 inhibits hypoxia-induced MDMX S367 phosphorylation. U2OS cells were cultured under conditions as mentioned above. Cell pellets were lysed for SDS-PAGE and WB analyses using antibodies as indicated on the left. ***B,*** Hypoxia-enhanced interaction of exogenous MDMX and 14-3-3γ is inhibited by UCN-01 treatment. HEK293 cells were transfected with Myc-MDMX

and Flag-14-3-3γ plasmids and treated with DMSO or UCN-01 and incubated under hypoxic condition for 6h followed by preparation of cell lysates. 300 μg of each lysate were immunoprecipitated with monoclonal anti-Flag antibody. 40 μg of each lysate were loaded for WB analysis. ***C,*** UCN-01 treatment attenuates hypoxia-induced endogenous MDMX/14-3-3γ interaction. U2OS cells were treated with DMSO or UCN-01 and cultured in a hypoxia chamber for 6h. Samples were harvested and lysed for WB (40 μg) or co-IP (300 μg) with polyclonal anti-14-3-3γ antibodies. Proteins of interest were detected by corresponding antibodies as listed on the left.

FIGURE 3. Depletion of Chk1 or ATR suppresses hypoxia-induced MDMX phosphorylation at S367 and the interaction between MDMX and 14-3-3γ. ***A,*** Knock-down of Chk1 eliminates hypoxia-induced MDMX phosphorylation and MDMX interaction with 14-3-3γ. U2OS cells were double-transfected with scramble siRNA or siRNA specific to Chk1 and ~24h after the second transfection, cells were subjected to hypoxia. After 6 h or 18 h hypoxia, cell lysates were prepared for WB (40 μg) or co-IP analyses. 500 μg of cell lysates were incubated with anti-14-3-3 antibodies for co-IP and subjected to SDS-PAGE followed by WB analysis. Proteins of interest were detected by antibodies as listed on the left. ***B,*** Depletion of ATR inhibits hypoxia-induced MDMX phosphorylation and its interaction with 14-3-3γ. Knock-down of ATR was achieved by the introduction of si- or shRNA in U2OS cells ~24h after double-transfection. Then, cells were subjected to hypoxia for additional 6h or 18h followed by WB analysis (80 μg) and co-IP (500 μg) as described in the above. Corresponding antibodies were listed on the left.

FIGURE 4. Hypoxia-induced p53 activation and G1 cell cycle arrest are impeded in 3SA MEF cells and MDMX S367A single mutation abolishes the interaction between MDMX and 14-3-3γ. ***A*** *and* ***B,*** Wild type MEF and 3SA MEF cells were cultured under normoxic or hypoxic condition for 18h or 24h and subjected to WB analysis (100μg) or FACS analysis, respectively. For FACS analysis, the *p*-value was obtained by single factor ANOVA. ***C,*** 293 cells were transfected with wild type or each single Myc-MDMX mutation plasmids as indicated (2 μg/sample) along with Flag-14-3-3γ (1 μg/sample) and the next day, cells were subjected to nomorxic or hypoxic condition for 18h followed by WB analysis or co-IP (200 μg) with anti-Flag antibody (1μg). ***D,*** A model for p53 activation via the hypoxia-ATR-Chk1-MDMX-14-3-3γ pathway.

Fig. 1

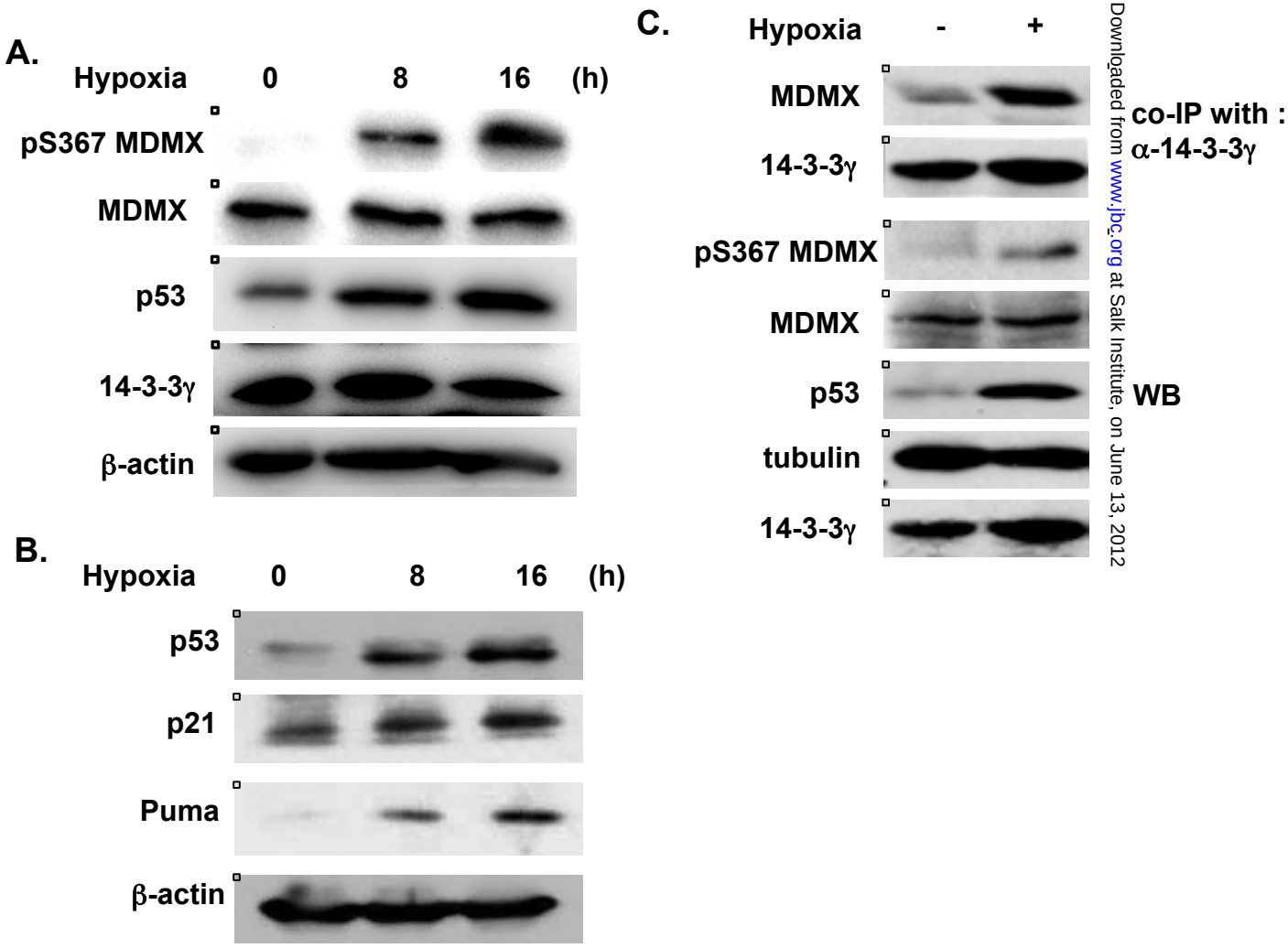

Fig. 2

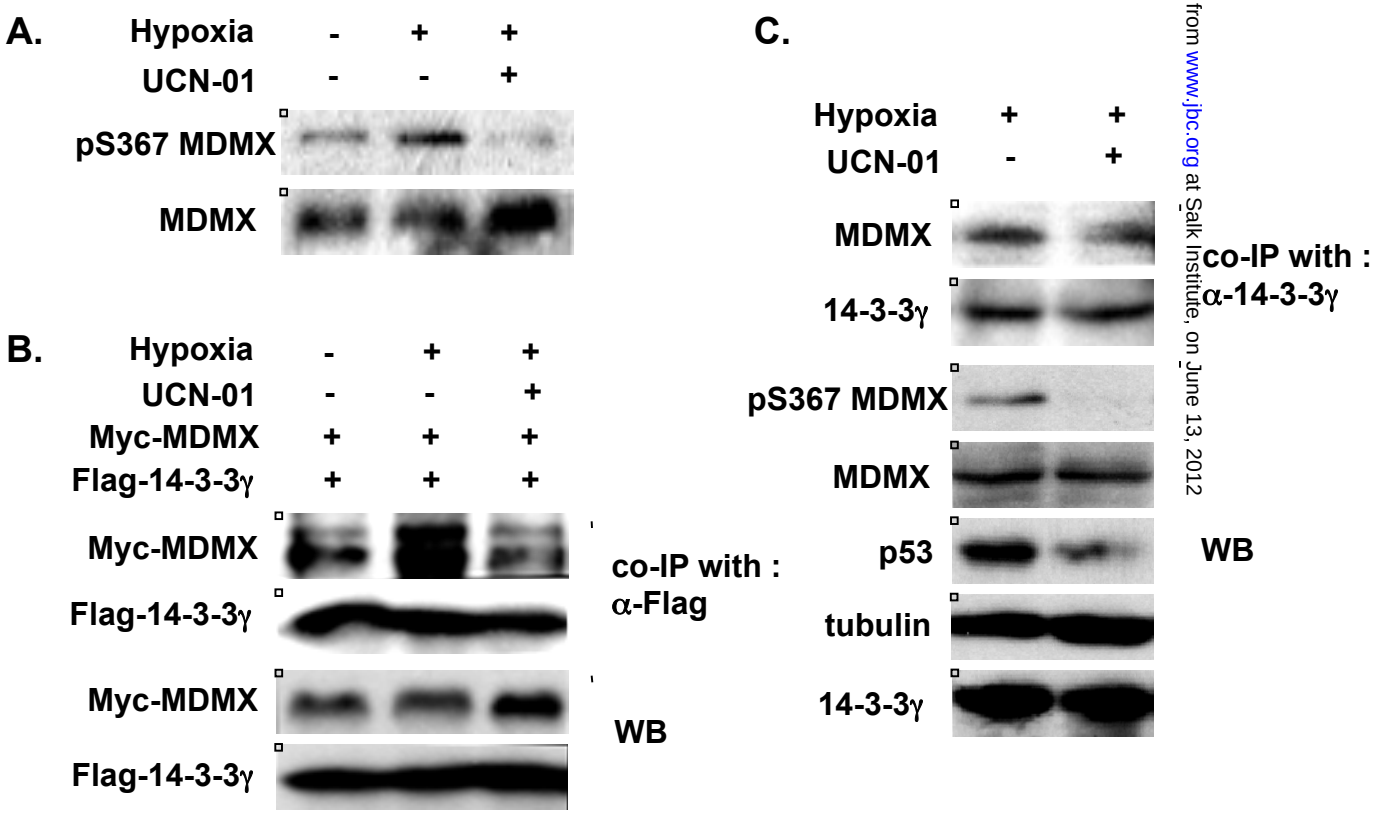

Fig. 3

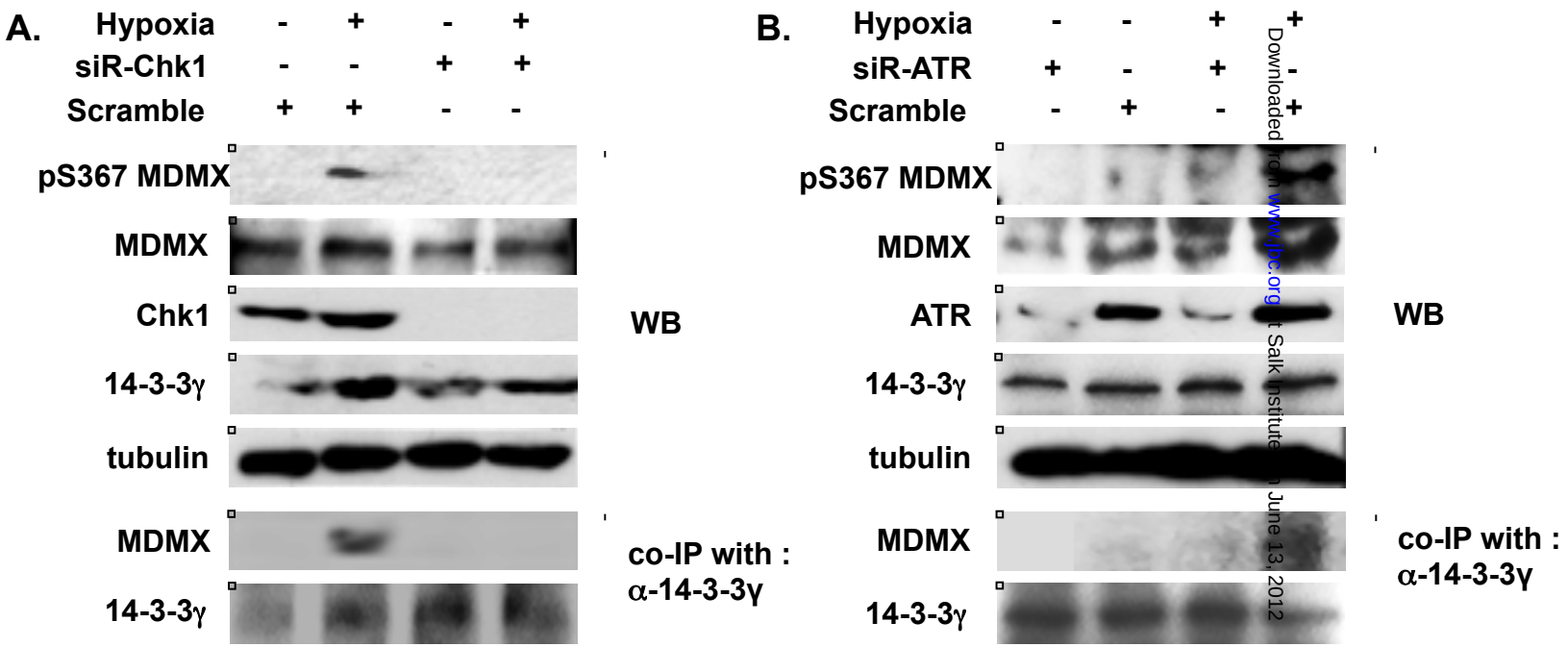

Fig. 4