Lats2 phosphorylates p21/CDKN1A after UV irradiation and regulates apoptosis

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Summary

LATS2 (Large tumor suppressor 2), a member of the conserved AGC Ser/Thr (S/T) kinase family, is a human tumor suppressor gene. Here, we show that in response to ultraviolet radiation, Lats2 is phosphorylated by Chk1 at Ser835 (S835), which is located in the kinase domain of Lats2. This phosphorylation enhances Lats2 kinase activity. Subsequently, Lats2 phosphorylates p21 at S146. p21 (CDKN1A) is a cyclin-dependent kinase (CDK) inhibitor, which not only regulates the cell cycle by inhibition of CDK, but also inhibits apoptosis by binding to procaspase-3 in the cytoplasm. Phosphorylation by Lats2 induces degradation of p21 and promotes apoptosis. Accordingly, Lats2 overexpression induces p21 degradation, activation of caspase-3 and caspase-9, and apoptosis. These findings describe a novel Lats2-dependent mechanism for induction of cell death in response to severe DNA damage.

Key words: Lats2, p21, CDKN1A, Apoptosis, UV, Phosphorylation

Introduction

A proper DNA damage response (DDR) is essential for elimination of cells harboring genotoxic lesions such as double-strand breaks (DSBs), single-strand breaks (SSBs) or stalled replication forks. The DDR is stringently regulated by DNA damage checkpoint pathways to induce cell cycle arrest for DNA repair or apoptosis for removal of damaged cells because errors in the DDR can potentially lead to genomic instability and cancer (Bartek et al., 2007; Ciccia and Elledge, 2010). The checkpoint pathways that are activated by ionizing radiation (IR), genotoxic agents, ultraviolet (UV) or DNA replication stress are mainly orchestrated by two parallel signaling cascades; the ATM–Chk2 pathway in response to DSBs and the ATR–Chk1 pathway in response to SSBs and replication errors, although crosstalk exists between these pathways. Activated ATM and ATR kinases phosphorylate Chk2 and Chk1 kinases, respectively, which in turn phosphorylate downstream effectors such as Cdc25 and p53. In addition to DDR-induced cell cycle checkpoints, Chk1 plays important roles in apoptosis, the mitotic spindle checkpoint and indirectly regulates DNA repair. Therefore, Chk1 has been noted to be an important candidate for cancer therapeutic targeting (Dai and Grant, 2010; Ma et al., 2011).

Lats2 (Large tumor suppressor 2) is one of the central kinases in the Hippo pathway, which regulates organ size and cell growth in vertebrates (Pan, 2010; Visser and Yang, 2010). In this signaling pathway, activated Lats2 phosphorylates and inhibits the transcriptional co-activators Yap and Taz, thereby limiting the expression of genes relevant to cell proliferation and anti-apoptosis to control the size of the organs. Lats2 also regulates the cell cycle by changing its subcellular localization; Lats2 localizes to the centrosome during interphase, but moves to the nucleus, chromatin and mitotic apparatus during M-phase (Toji et al., 2004; Yabuta et al., 2011). In response to mitotic stress by exposure to microtubule poisons, Lats2 accumulates in the nucleus and promotes p53 activation by direct inhibition of E3 ubiquitin ligase Mdm2, thereby preventing tetraploidization (Aylon et al., 2006). Accordingly, loss of Lats2 results in chromosome instability and mitotic defects, including centrosome fragmentation and cytokinesis failure (McPherson et al., 2004; Yabuta et al., 2007). Overexpression of Lats2 induces G1–S and G2–M arrests owing to downregulation of cyclin and CDK kinase activity (Kamikubo et al., 2003; Li et al., 2003) or apoptosis by downregulation of anti-apoptotic proteins such as Bcl-2 and Bcl-$_X$L (Ke et al., 2004). Lats2 has been demonstrated to interact with the ATR–Chk1 pathway in the DDR (by UV irradiation) and oncogenic stress (by H-RasV12) (Aylon et al., 2009; Okada et al., 2011). In the oncogenic context, Lats2 induces apoptosis by diverting p53 from cell cycle gene promoters to pro-apoptotic gene promoters through phosphorylation of ASPP1 (apoptosis-stimulating protein of p53-1) (Aylon et al., 2010). However, the detailed physiological functions of the Lats2 in DDR remain elusive.

p21$^{Cip1/WAF1/CDKN1A}$ is a key CDK inhibitor that negatively regulates cell cycle progression following DDR signaling (Abbas and Dutta, 2009; Cazzalini et al., 2010). Additionally, p21 inhibits apoptosis and DNA repair by CDK-independent mechanisms by associating with PCNA (proliferating cell nuclear antigen) (Moldovan et al., 2007; Soria and Gottifredi, 2010). Upon DNA damage, p21 protein is cleaved by activated caspase-3 (Gervais et al., 1998; Levkau et al., 1998; Jin et al., 2000). However, p21 also binds and inhibits caspase-2 and caspase-3 (Suzuki et al., 1998; Suzuki et al., 1999; Baptiste-Okoh

et al., 2008). Moreover, p21 also interacts with and inhibits the pro-apoptotic protein Ask1 (apoptosis signal regulating kinase 1) (Asada et al., 1999). These results suggest that p21 functions as a linch pin to govern cell fate that is poised between cell cycle arrest and apoptosis.

p21 expression is regulated transcriptionally by p53, which is activated during DDR. At the post-translational level, the p21 protein is phosphorylated to modulate its subcellular localization and degradation rate (Jung et al., 2010). Akt, PKCζ, PKCδ and Pim-1 are kinases that phosphorylate p21 on S146 within the PCNA binding domain, in close proximity to its nuclear localizing signal (Scott et al., 2002). PKC-mediated phosphorylation on S146 of p21 reduces its binding to PCNA (Scott et al., 2002). Because p21 is degraded by the proteasome through interaction with its PCNA binding domain (Sheaff et al., 2000; Touitou et al., 2001), PKCζ phosphorylation of p21 leads to its destabilization (Scott et al., 2002), However, phosphorylation on the same S146 site by Akt or PKCδ has been shown to increase p21 stability (Li et al., 2002; Oh et al., 2007). Additionally, S114 phosphorylation of p21 by GSK3β destabilizes p21 to facilitate repair following UV irradiation (Bendjennat et al., 2003; Lee et al., 2007; Abbas et al., 2008; Nishitani et al., 2008). Notably, a recent report showed that NDR1 (nuclear-DBF2-related 1), belonging to the Dbf2/Lats kinase family, phosphorylates S146 to destabilize p21, which together with stabilization of c-Myc, could promote G1–S cell cycle transition under conditions of activated Mst3 kinase (Cornils et al., 2011). In sum, the roles of p21 phosphorylation in relation to its protein stability, cell cycle regulation and apoptosis in DDR, especially upon UV irradiation, remain poorly understood.

Here, we show that Chk1-mediated phosphorylation of Lats2 upon UV irradiation enhances Lats2 autophosphorylation and Lats2 kinase activity towards p21 S146, thereby leading to apoptosis through activation of caspase-3/9. We propose that the Chk1–Lats2–p21 axis defines a novel DDR pathway that regulates apoptosis upon severe UV-induced damage.

Results

Downregulation of Lats2 causes stabilization of p21

Recently, we reported that Lats2 has an important role in the formation of processing bodies (P-bodies) after UV irradiation (Okada et al., 2011), indicating that Lats2 has a role in the DDR after UV irradiation. Other studies have shown that, in contrast to gamma-radiation, p21 is destabilized after UV irradiation (Lee et al., 2007; Abbas et al., 2008; Nishitani et al., 2008). To investigate whether Lats2 affects p21 degradation following UV irradiation, we used short interfering RNA (siRNA) to deplete Lats2 in UV-irradiated U2OS cells. After UV irradiation, p21 protein levels decreased rapidly; however, p21 protein levels remained markedly higher in cells transfected with Lats2 siRNA (siLats2) compared with control siRNA (siCTL) (Fig. 1A,B). To

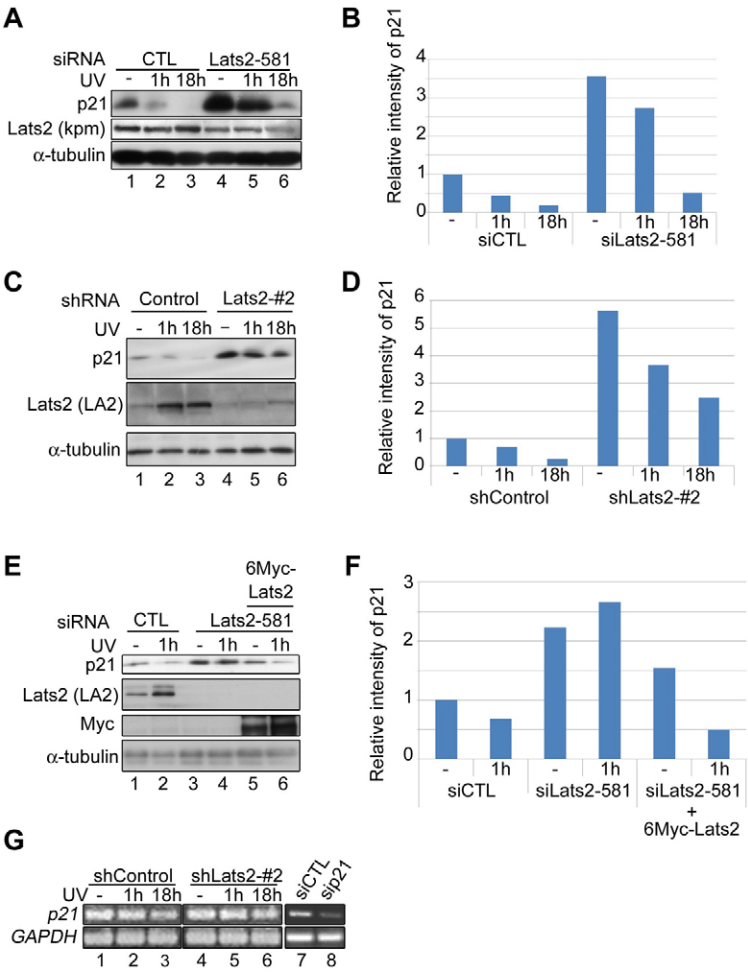

Fig. 1. p21 protein is stabilized by depletion of Lats2. (A) U2OS cells were transfected with siRNA (siCTL or siLats2-581), irradiated with UV (50 J/m^2) 48 hours after transfection and then incubated for 1 hour or 18 hours. Cell lysates were analyzed by western blotting with the indicated antibodies. siCTL, negative control. (B) Relative intensity of p21 in A. The intensity of p21 was quantified using the Image J software and was normalized to that of α-tubulin. (C) U2OS cell lines stably expressing shRNAs (U2OS/shControl or U2OS/shLats2-#2) were irradiated with UV and analyzed by western blotting in a manner similar to A. (D) The bar graph represents the relative intensity of p21 in C. (E) U2OS cell lines expressing 6Myc–Lats2 (lanes 5 and 6) and parent U2OS cells (lanes 1–4) were transfected with siRNAs (siCTL or siLats2-581), irradiated with UV (50 J/m^2) 48 hours after transfection and then incubated for 1 hour. Cell lysates were analyzed by western blot analysis with the indicated antibodies. (F) The bar graph shows the relative intensity of p21 in E. (G) RT-PCR analysis to assess p21 mRNA levels in U2OS/shControl cells, U2OS/shLats2-#2 cells, and U2OS cells transfected with siCTL or sip21. Total RNA (3 ng) from each cell line was reverse-transcribed and amplified by PCR using specific primer pairs for p21 and *GAPDH*.

minimize potential side effects of siRNA transfection, we obtained similar results using derivatives of U2OS cells in which endogenous Lats2 was stably knocked down by an integrated plasmid expressing Lats2-specific short hairpin RNA (shRNA) (Aylon et al., 2006) (Fig. 1C,D). Notably, Lats2 depletion elevated p21 protein levels even in non-treated cells (Fig. 1A,C; lane 4). A similar result was also obtained using HeLa S3 cells (supplementary material Fig. S1). To confirm that the increased p21 level was due to Lats2 depletion, we overexpressed 6Myc–Lats2 in Lats2-knockdown cells, which showed that overexpression of 6Myc–Lats2 suppressed the increase in the p21 level in Lats2-knockdown cells (Fig. 1E,F). Moreover, RT-PCR analysis demonstrated that p21 mRNA levels were only minimally affected either by Lats2 knockdown or by UV irradiation (Fig. 1G), supporting the conclusion that the observed effects on p21 protein levels were not due to changes in the corresponding mRNA. Taking into account that the p21 protein is known to be destabilized by UV irradiation, these results indicate that Lats2 potentially contributes to the decrease in p21 protein stability under these conditions.

Lats2 phosphorylates p21 on S146

Because Lats2 is a serine/threonine kinase that belongs to the AGC kinase family, we hypothesized that Lats2 negatively regulates p21 through phosphorylation. Moreover, p21 contains two putative RxxS/T (Mah et al., 2005; Oka et al., 2008) consensus sequences for Lats2: KRRQ^{145}T^{146}SMTDF. *In vitro* activation of Lats2 kinase activity requires both the addition of Mob1A and phosphatase inhibition by okadaic acid (OA) (Yabuta et al., 2007). To examine whether Lats2 phosphorylates p21, we first performed *in vitro* kinase assays using wild-type, Thr145Ala (T145A) or Ser146Ala (S146A) mutant forms of p21 as substrates (Fig. 2A). Although Lats2-WT phosphorylated WT and T145A efficiently, it failed to phosphorylate the S146A mutant and the T145A-S146 double mutant. To confirm Lats2 phosphorylation of p21 on S146, an *in vitro* Lats2 kinase assay was performed in the absence of [γ-^{32}P]ATP, followed by western blot analysis using the S146 phospho-specific antibody against p21 (anti-pS146). As expected, we obtained similar results, namely, phosphorylation of p21-S146 was dependent on Lats2 kinase activity and was specific to S146 (Fig. 2B). These results demonstrate that Lats2 phosphorylates p21 on S146 *in vitro*.

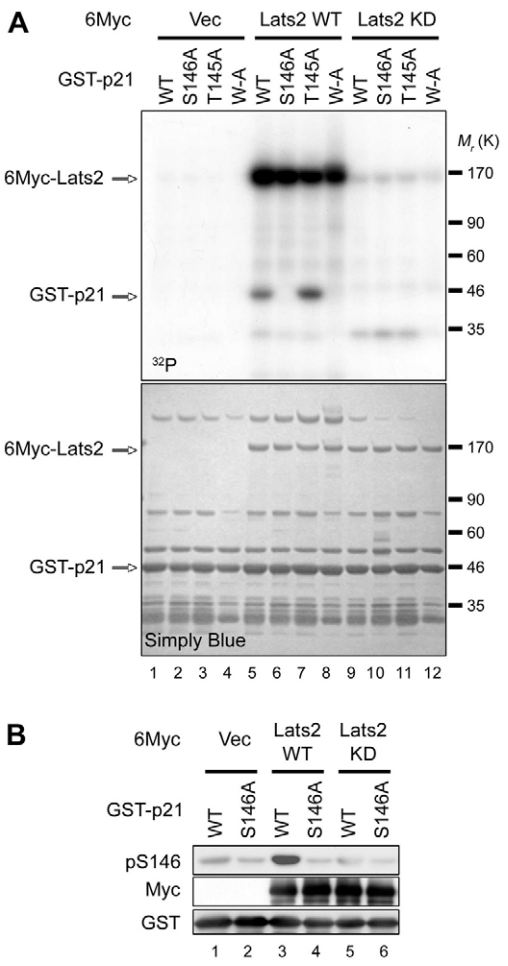

Fig. 2. Lats2 phosphorylates p21 at Ser146 *in vitro*. (A) An *in vitro* Lats2 kinase assay was performed with immunoprecipitated 6Myc vector or 6Myc-tagged-Lats2 (WT or KD) together with 3×FLAG-tagged Mob1A. GST–p21 WT, T145A, S146A or T145A/S146A (W–A) mutants were used as the substrates. These proteins were labeled with ^{32}P and resolved by SDS-PAGE. ^{32}P-labeled proteins were detected by autoradiography. Simply Blue staining was used as a control gel for gel loading. (B) *In vitro* Lats2 kinase assays without [γ-^{32}P]ATP. Western blot analysis was performed using anti-pS146-p21, anti-Myc and anti-GST antibodies.

Lats2 phosphorylates p21 *in vivo* and regulates p21 stability

To confirm Lats2 phosphorylation of p21 *in vivo*, U2OS cells were co-transfected with 3×FLAG–p21 and 6Myc–Lats2, then treated with OA before lysis to activate Lats2 kinase. The OA-treated lysates were immunoprecipitated with anti-FLAG antibody and detected by western blotting using anti-pS146 (Fig. 3A). Notably, overexpression of Lats2-WT markedly increased the phosphorylation of p21 upon OA treatment (Fig. 3A, lane 2), whereas Lats2-KD correlated with only slight p21 phosphorylation (Fig. 3A, lane 4).

Next, we examined the biological significance of p21 phosphorylation by Lats2. Since PKCζ and NDR1 phosphorylation of S146 destabilizes p21 (Scott et al., 2002; Cornils et al., 2011), we hypothesized that Lats2 also decreases p21 stability in response to DDR. To test this, U2OS cells were transfected with 6Myc–Lats2 and *de novo* translation of p21 was inhibited by cycloheximide (CHX) (Fig. 3B). Lats2-WT significantly reduced p21 protein stability, whereas Lats2-KD only moderately affected p21 stability. Moreover, biochemical fractionation demonstrated that the UV-induced p21 degradation occurred in the cytoplasm rather than in the nucleus (Fig. 3C, top panel), which is consistent with previous reports (Jung et al., 2010). NDR1 phosphorylates p21 at S146 and regulates p21 stability during cell cycle progression (Cornils et al., 2011), and both Lats1/2 and NDR1/2, which belong to the same kinase family, play similar signaling roles downstream of Hippo/MST kinases. Thus, we examined the contribution of NDR1 to p21 degradation after UV irradiation. As expected, p21 was increased by knockdown of NDR1 in the absence of UV irradiation (supplementary material Fig. S2, top panel, lane 4), which is consistent with the results of a previous report (Cornils et al., 2011). However, at 1 hour after UV irradiation, the p21 protein level was the same as before irradiation, despite the knockdown

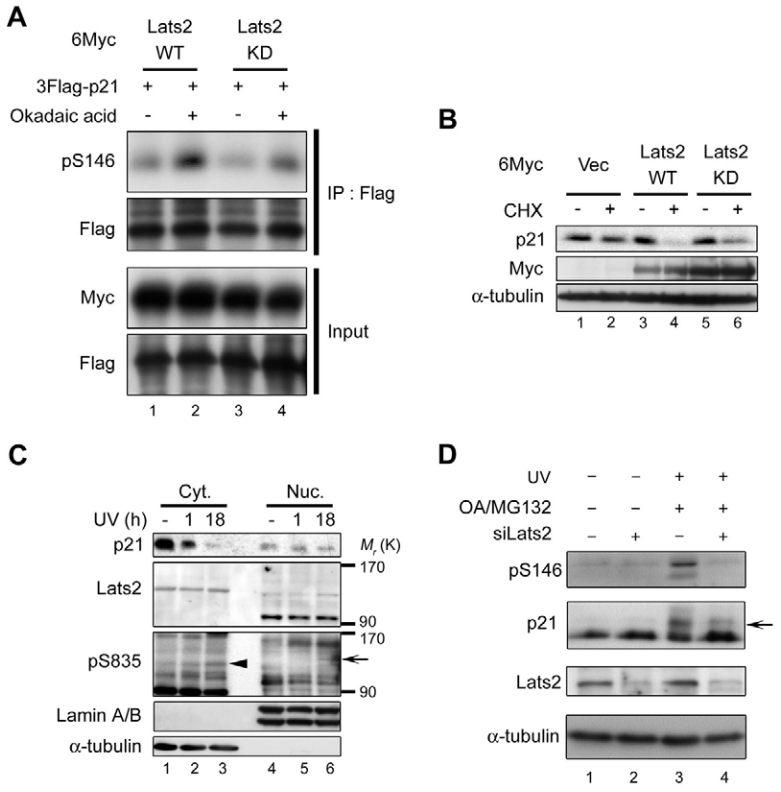

Fig. 3. Lats2 phosphorylates p21 *in vivo* and regulates p21 stability. (A) 293T cells were transfected with 6Myc-tagged Lats2 (WT and KD) and 3×FLAG-tagged p21. Cells were then treated with both okadaic acid (OA) and UV irradiation (50 J/m^2) for 3 hours before lysis. 3×FLAG-tagged p21 was immunoprecipitated with an anti-FLAG antibody (IP), followed by western blot analysis with anti-pS146-p21 and anti-FLAG antibodies. (B) U2OS cells were transfected with the 6Myc-tagged vector or 6Myc-tagged Lats2 (WT and KD). Cells were treated with 50 μg/ml cycloheximide (CHX) for 1 hour before lysis. Western blot analysis was then performed using the indicated antibodies. α-tubulin is shown as a loading control. (C) U2OS cells were irradiated with UV for the indicated times. Cell lysates were then fractionated into cytosolic (Cyt.) and nuclear (Nuc.) components, followed by western blotting with the indicated antibodies. Arrowhead and arrow indicate cytoplasmic and nuclear forms of phosphorylated Lats2 (pS835-Lats2), respectively (see also Fig. 4). α-tubulin and Lamin A/B are cytoplasmic and nuclear markers, respectively. (D) U2OS cells were transfected with siRNAs (−, siCTL; +, siLats2-581), irradiated with UV (50 J/m^2) and treated with OA and MG132 48 hours after transfection, and then incubated for 1 hour or 18 hours. Cell lysates were analyzed by western blot analysis with the indicated antibodies.

of NDR1 (supplementary material Fig. S2, lanes 4 and 5), suggesting that NDR1 contributes, at least partially, to p21 degradation after UV irradiation. To confirm that p21 is a direct substrate of Lats2 during the UV-induced DDR *in vivo*, we examined the phosphorylation status of endogenous p21-S146 in the absence or presence of Lats2 knockdown after UV exposure (Fig. 3D). Because phosphorylation of S146 could lead to p21 degradation, the cells were treated with a proteasome inhibitor, MG132, and OA after UV irradiation. The phosphorylated band of endogenous p21-S146 (pS146) was successfully detected in control cells after these treatments (Fig. 3D, lane 3), whereas this band was almost completely absent in Lats2-knockdown cells (Fig. 3D, lane 4). These results suggest that Lats2 plays a major role in the phosphorylation of p21 after UV irradiation. Moreover, western blotting using the antibody against total p21 protein revealed that endogenous p21 migrated to a higher molecular weight, probably reflecting post-translational modifications such as phosphorylation, which are inhibited by Lats2 knockdown (Fig. 3D, lanes 3 and 4, black arrow). Taken together, these results suggest that Lats2 phosphorylates p21 on S146 *in vivo*, and this phosphorylation destabilizes cytoplasmic p21.

Lats2 is actively phosphorylated on S835 following UV irradiation

We previously reported that Chk1, activated by DNA damage, phosphorylates Lats2 after UV irradiation (Okada et al., 2011). To investigate whether Chk1 could mediate the Lats2-dependent effect on p21 stability, cells were transfected with control siRNA (siCTL) or Chk1 siRNA (siChk1), UV irradiated, and cell extracts were analyzed by western blotting (Fig. 4A). Chk1 knockdown stabilized p21 protein (Fig. 4A, upper panel, lane 4) in a similar manner to Lats2 depletion. Consistent with this, Lats2 protein levels were slightly reduced in Chk1-knockdown cells (Fig. 4A, third panel, lanes 3 and 4), implying that Chk1 is also involved in regulation of Lats2 expression or protein stability. These results suggest that a Chk1–Lats2 pathway plays a role as a negative regulator of p21 after UV irradiation. Thus, it is likely that Chk1, similarly to Lats2, diminishes p21 stability after UV irradiation. To address the role of Chk1 in more depth, we further investigated the phosphorylation of Lats2 by Chk1. Although Lats2 harbors numerous putative Chk1 phosphorylation sites (RxxS) (Okada et al., 2011; O'Neill et al., 2002), we focused on S835 as a potential candidate site because it is the sole Chk1 consensus sequence within the Lats2 kinase domain. To perform *in vitro* kinase assays, we raised a phospho-S835 specific antibody against (anti-pS835). We confirmed the specificity of this antibody against phosphorylated S835-Lats2 peptides by dot-blot analysis (supplementary material Fig. S3A). Then, we constructed Lats2-WT and Lats2-S835A mutant peptides (amino acids 644–855) as substrates, and used wild-type (WT) Chk1 or a kinase-dead Chk1 mutant (KD) as a kinase (Fig. 4B). *In vitro* kinase assays in the absence of [γ-^{32}P]ATP were examined by western blot analysis using the anti-pS835 antibody. We found that the active form of Chk1 (Chk1 WT) directly phosphorylated Lats2 at S835, whereas the Lats2-S835A mutant was only marginally phosphorylated by either Chk1-WT or KD (Fig. 4B, top panel, empty arrowhead), suggesting that Lats2-S835 is a novel site for phosphorylation by Chk1. Next, we examined whether endogenous Lats2-S835 is phosphorylated *in vivo* after UV irradiation (Fig. 4C). Lats2-S835 phosphorylation could be detected following UV irradiation, but not in untreated cells. To validate the specificity of the anti-pS835 antibody, we examined the intensity of the pS835 signals in Lats2 shRNA-mediated

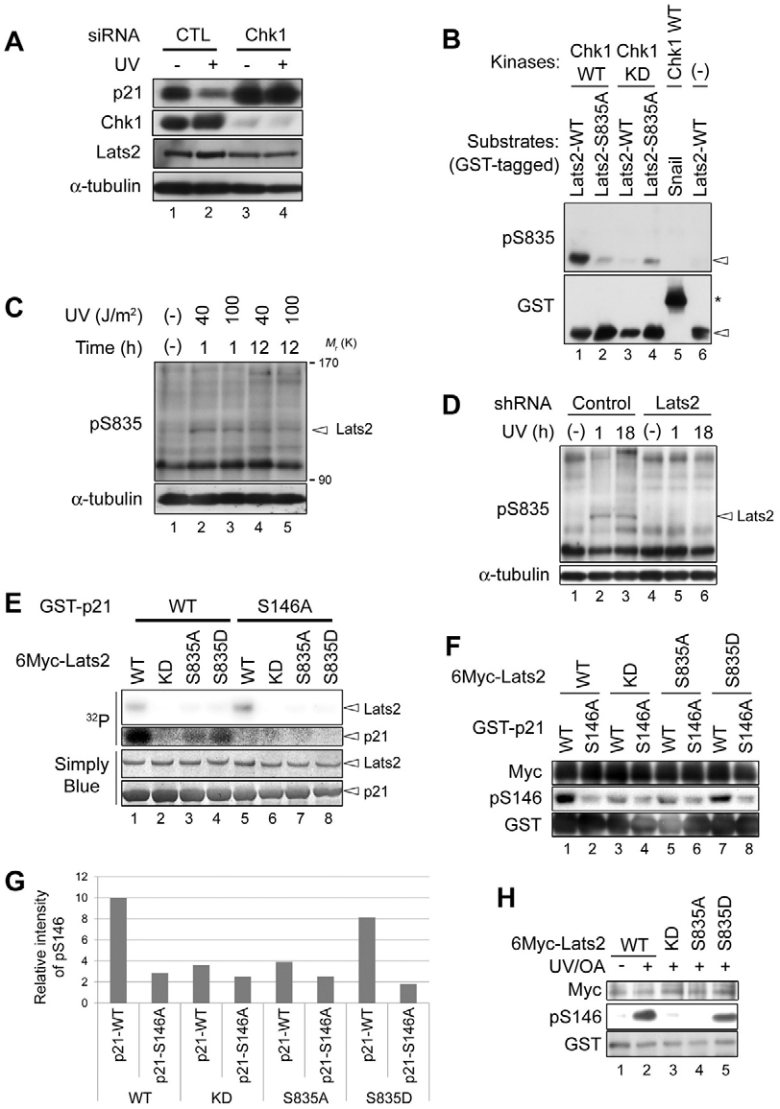

Fig. 4. Chk1 phosphorylates Lats2 at S835 after UV irradiation and induces Lats2 activity. (A) U2OS cells were transfected with siRNA (siCTL or siChk1), irradiated with UV (50 J/m^2) at 48 hours after transfection and then incubated for 1 hour, followed by western blot analysis using the indicated antibodies. α-tubulin is shown as a loading control. (B) *In vitro* kinase assays were performed using active Chk1-WT or Chk1-KD as the kinases, and GST–Lats2$^{644–855}$-WT and GST–Lats2$^{644–855}$-S835A as the substrates. GST-Snail was used as a negative control (asterisk). Western blot analysis was performed using the anti-pS835-Lats2 and anti-GST antibodies. Empty arrowheads indicate GST-Lats2$^{644–855}$ proteins. (C) U2OS cells were exposed to UV irradiation with the indicated doses and incubated for the indicated times. Western blot analysis was performed using the anti-pS835-Lats2 and α-tubulin antibodies. Empty arrowhead indicates pS835-Lats2. (D) U2OS/shControl and U2OS/shLats2-#2 cells were irradiated with UV (50 J/m^2) and then incubated for 1 hour or 18 hours, followed by western blot analysis using the indicated antibodies. Empty arrowhead indicates pS835-Lats2. (E) The *in vitro* Lats2 kinase assay was performed with immunoprecipitated 6Myc-tagged Lats2 (WT, KD, S835A and S835D) together with 3×FLAG-tagged Mob1A. GST–p21 (WT and S146A) were used as substrates. These proteins were labeled with ^{32}P and resolved by SDS-PAGE. ^{32}P-labeled proteins were detected by autoradiography. Simply Blue staining was used as a control for gel loading. (F) The *in vitro* Lats2 kinase assay was performed using 6Myc-tagged Lats2 (WT, KD, S835A, and S835D)-3×FLAG-tagged Mob1A immunoprecipitates as the kinases and GST-p21 (WT and S146A mutant) as the substrates. Western blot analysis was performed using the anti-pS146-p21, anti-Myc and anti-GST antibodies. (G) The bar graphs represent the relative intensities of the bands revealed by anti-pS146-p21 in F. (H) An *in vitro* Lats2 kinase assay was performed using 6Myc-tagged Lats2 (WT, KD, S835A, and S835D)-3×FLAG-tagged Mob1A immunoprecipitates as the source of kinase activity and GST-p21 WT as substrates. Western blot analysis was performed using anti-pS146-p21, anti-Myc and anti-GST antibodies. Except for lane 1, the cells transfected with 6Myc-Lats2 and Mob1 were treated with OA and exposed to UV irradiation before lysis and immunoprecipitation.

knockdown cells (U2OS/shLats2-#2). Western blotting with anti-pS835 antibody revealed that control cells (shControl/U2OS) exhibit enhanced signals of pS835-specific bands after UV irradiation; whereas the anti-pS835 signals disappeared in Lats2-knockdown cells, even after UV damage (Fig. 4D, empty arrowhead).

Moreover, the UV-induced pS835 signals were accumulated in the cytoplasm (Fig. 3C, third panel, black arrowhead), in correlation with the degradation of cytoplasmic p21 (Fig. 3C, top panel). Although a small fraction of pS835 signals also exists in the nucleus at 18 hours after UV damage, this nuclear pS835 band migrated more slowly than cytoplasmic pS835 (third panel, black arrow), suggesting that the nuclear pS835-Lats2 is a different form from cytoplasmic pS835-Lats2.

Interestingly, Lats2-S835 is not only a Chk1 phosphorylation site but also a Lats2 trans-autophosphorylation site. In fact, the band of Lats2 kinase-dependent trans-autophosphorylation could be detected in the *in vitro* kinase assay depicted in Fig. 2A. To examine the direct phosphorylation of S835-Lats2 by itself, we performed *in vitro* Lats2-kinase assay by using GST-fused Lats2 (644–855 aa)-WT and Lats2 (644–855 aa)-S835A as the substrates. Lats2 immunoprecipitates phosphorylated GST-fused Lats2 (644–855 aa)-WT but not Lats2 (644–855 aa)-S835A, whereas Lats2-KD did not phosphorylate either substrate (supplementary material Fig. S3B). Thus, Lats2 directly phosphorylates S835 on Lats2 itself *in vitro*. This observation suggests that after UV irradiation Chk1 might initially phosphorylate Lats2-S835 which could activate Lats2 kinase activity towards itself and its other kinase targets. In other words, it is likely that the kinase activity of phospho-S835 Lats2 is stronger than that of non-phosphorylated Lats2. To verify this hypothesis, we constructed mutants in which the phosphorylated serine residue was replaced by a nonphosphorylatable alanine (Lats2-S835A) or a phosphorylation-mimic aspartic acid (Lats2-S835D). First, we verified the direct binding of p21 to Lats2 and its mutants (supplementary material Fig. S4). Immunoprecipitation and GST pull-down assays revealed that Lats2 interacted with p21 both *in vivo* (supplementary material Fig. S4A) and *in vitro* (supplementary material Fig. S4B,C), regardless of the phosphorylation state of S835. Next, we performed *in vitro* kinase assays with Lats2 immnoprecipitates from UV-irradiated cells expressing 6Myc–Lats2-WT, 6Myc–Lats2-KD, 6Myc–Lats2-S835A and 6Myc–Lats2-S835D with

GST–p21-WT or GST–p21-S146A as substrates in the presence of [γ-^{32}P]ATP. The band of Lats2 trans-autophosphorylation was detected with Lats2-WT only (Fig. 4E, lanes 1 and 5). This result demonstrates that Lats2 trans-autophosphorylation predominantly occurs on S835 and that this site is important for Lats2 kinase activity towards p21 (Fig. 4E, lanes 1 and 4). Surprisingly, the Lats2-S835D mimic mutant seems to have diminished the kinase activity toward p21 compared with Lats2-WT (Fig. 4E, second panel, lane 4), probably because the Asp substitution does not fully mimic the phosphorylated form of Lats2 in this context. Therefore, to validate more convincingly the phosphorylation site specificity on p21, we performed a similar *in vitro* kinase assay using the anti-pS146 antibody (Fig. 4F,G). Similar to the result in Fig. 4E, S146 of p21 was phosphorylated by Lats2-S835D as well as by Lats2-WT (Fig. 4F, lanes 1 and 7), whereas it was barely phosphorylated by Lats2-S835A (Fig. 4E, lane 3 and 4F, lane 5). In agreement with these results, the phosphorylation of p21 by Lats2-WT was activated by both UV irradiation and OA treatment (Fig. 4H, middle panel, lane 2). These results suggest that the Lats2-S835A/D mutants are not trans-autophosphorylated, but Lats2-S835D is capable of phosphorylating p21 on S146.

Taken together, our findings imply that S835 phosphorylation is essential for Lats2 activation, and this site is crucial for the Lats2-mediated destabilizing phosphorylation of p21 in response to UV.

S835-phophorylated Lats2 induces apoptosis

Next, to examine the cellular consequences of Lats2-S835 phosphorylation status, we generated U2OS cell lines in which the expression of 6Myc–Lats2-WT, 6Myc–Lats2-S835A, 6Myc–Lats2-S835D and vector alone (Vec) are induced by doxycyclin treatment (Dox). Interestingly, after these cells were treated with Dox for 48 hours, the cells expressing Lats2-S835D spontaneously underwent cell death and displayed reduced levels of p21 protein, even without UV irradiation (Fig. 5A, red arrow and Fig. 5B, second panel, lanes 20–24). Furthermore, after UV irradiation, the cells expressing Lats2-WT and Lats2-S835D underwent more conspicuous cell death than the cells expressing Lats2-KD or Lats2-S835A (supplementary material Fig. S5A). Notably, although cell death could be induced by UV irradiation in all transfected cells in a time-dependent manner, it occurred more quickly in the constitutive phospho-mimetic mutant S835D than in the WT after UV damage (supplementary material Fig. S5A). It is possible that p21-independent pathway(s) other than the Lats2–p21 pathway, acting via the phosphorylation of Lats2-S835, regulate UV-induced cell death *in vivo* because the phosphorylation of p21 by Lats2-S835D was less strong than that by Lats2-WT *in vitro* (Fig. 4E). These results suggest that the S835 phosphorylated form of Lats2 facilitates p21 degradation and cell death, which is limited by Chk1. To confirm that this cell death is due to apoptosis, the cells were assessed by TUNEL staining (Fig. 5C). Only cells expressing Lats2-S835D were positive for TUNEL staining (Fig. 5C,D). In fact, ~25% of cells expressing Lats2-S835D were TUNEL positive (Fig. 5E). We also confirmed cell death by Annexin-V labeling using FACS analysis (Fig. 5F). Approximately 46% of the cells expressing Lats2-S835D underwent early apoptosis. The other half of the cell population stained positive for both PI and Annexin V indicating that they had already undergone late stage apoptosis and death. These results indicate that apoptosis was induced by Lats2-S835D but not by Lats2-WT and Lats2-S835A, suggesting that phosphorylation of S835 on Lats2 promotes apoptosis.

The Lats2-p21 pathway induces caspase-mediated apoptosis

Apoptosis is triggered by the activation of the CED-3/caspase protease family members (Steller, 1995; Alnemri et al., 1996). Apoptosis is induced upon activation of these caspases by cleavage of their inactive forms (pro-caspases). In mammals, caspase-3 and caspase-9 play important roles in inducing apoptosis after DNA damage (Xue et al., 1996; Alnemri, 1997). Thus, we examined the relationship between caspase activation and p21 degradation in U2OS cells expressing Lats2-WT or the S835A/D mutants. Only cells expressing Lats2-S835D showed markedly reduced levels of p21 expression (Fig. 6A, second panel, lane 8). Moreover, caspase-3 and caspase-9 were activated only in cells expressing Lats2-S835D. These results suggest that Lats2-S835D induces caspase-3- and caspase-9-dependent apoptosis (Fig. 6A, fourth and fifth panels, lane 8). Indeed, the Lats2-S835D-induced apoptosis was inhibited by pre-treatment with the pan-caspase inhibitor Z-VAD-FMK (Fig. 6B).

Previous reports showed that p21 inhibits apoptosis through direct binding to caspase or CDK (Levkau et al., 1998; Hakem et al., 1999). When we examined the UV sensitivity of cells expressing empty FLAG vector or FLAG-tagged p21 (supplementary material Fig. S5B, right panel, lanes 3 and 4), survival of UV-treated cells expressing p21 is augmented compared with vector-only cells (denoted by large and small red arrows, respectively, in the left panel); from this, we conclude that p21 has a function to inhibit apoptosis. We also examined the UV sensitivity of cells in which p21 mRNA was knocked down by siRNA (sip21). Compared with the negative control (siCTL), no significant difference was observed between the cells treated with sip21 and siCTL (denoted by blue arrows in supplementary material Fig. S5C, left panel); this might be due to degradation of p21 by following UV irradiation (supplementary material Fig. S5C). Taken together, we propose the following model; when DNA damage level is low, the presence of p21 inhibits apoptosis (supplementary material Fig. S5Di). When the level of DNA damage is severe, Lats2-mediated p21 degradation leads to the induction of apoptosis (supplementary material Fig. S5Dii).

On the basis of these findings, we hypothesized that Lats2-S835D-induced apoptosis is accelerated by p21 destabilization. In fact, overexpression of p21 in cells expressing Lats2-S835D decreased apoptosis to a similar level to that caused by the pan-caspase inhibitor (Fig. 6C). Furthermore, overexpression of p21-S146A, a mutant refractory to phosphorylation by Lats2, inhibited S835D-induced apoptosis more strongly than p21-WT (Fig. 6C). These results suggest that phosphorylation of S835 on Lats2 causes apoptosis through degradation of p21 protein. Consistent with this, cell viability was slightly reduced by p21 knockdown in each cell line expressing Lats2-WT, S835A or vector alone (Fig. 6D). Moreover, S835D-expressing cells exhibited reduced viability irrespective of levels of p21 or Chk1, strengthening the notion that Lats2-S835D functions epistatically with both genes and suggesting that S835D mimics the effect of UV irradiation.

Lats2 also plays a role in phosphorylation of Yap through the Hippo pathway (Pan, 2010). We examined whether Dox-induced Lats2 expression influences S127-phosphorylation of endogenous

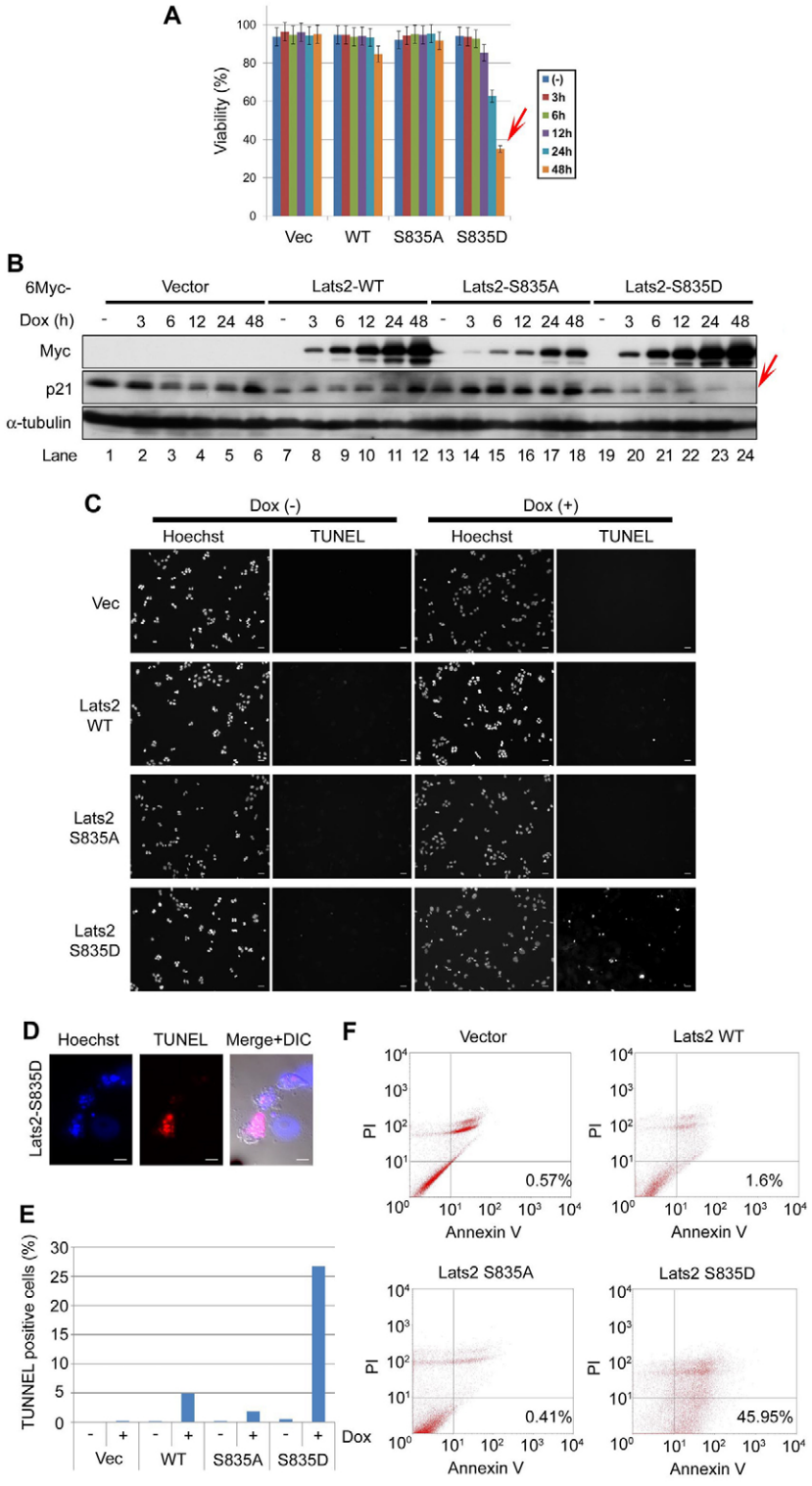

Fig. 5. Phosphorylated Lats2 degrades p21, activates caspase-3 and induces apoptosis. (A) U2OS/pTRET-6Myc-tagged vector or Lats2 (WT, S835A or S835D) cells were treated with Dox for the indicated times. Cell viability was measured in a Trypan Blue exclusion assay. The histograms show the average viability (%) calculated from three independent experiments. The error bars represent s.d. Red arrow indicates that overexpression of Lats2-S835D markedly decreased cell viability at 48 hours after addition of Dox. (B) Cell lysates were analyzed by western blotting with the indicated antibodies. α-tubulin is shown as a loading control. Red arrow indicates that overexpression of Lats2-S835D markedly decreased p21 protein levels at 48 hours after addition of Dox. (C) TUNEL assay. U2OS/pTRET-6Myc-tagged vector or Lats2 (WT, S835A or S835D) cells were treated with Dox for 24 hours. Apoptosis was determined using TUNEL assay. DNA was counterstained with Hoechst 33258. Scale bars: 10 μm. (D) Enlarged images of TUNEL-positive cells in D expressing Lats2-S835D. DIC, differential interference contrast. Scale bars: 10 μm. (E) The graph shows the percentage of TUNEL-positive cells expressing the WT or Lats2 mutants (average of three independent experiments). More than 200 cells were counted for each experiment. (F) Annexin V assay. U2OS/pTRET-6Myc-tagged vector or Lats2 (WT, S835A or S835D) cells were treated with Annexin V. The flow cytometry profiles show Annexin-V–FITC staining on the *x* axis and PI staining on the *y* axis. The number in the lower right quadrant represents the percentage of early apoptotic cells under each treatment condition.

Yap in U2OS cells with or without UV irradiation (Fig. 6E). Interestingly, the phosphorylation levels of Yap (pYap) in vector-transfected control cells were decreased after UV irradiation (Fig. 6E, top panel, lane 2). The pYap levels in wt Lats2-expressing cells were further markedly decreased after UV irradiation (Fig. 6E, top panel, lane 4). Notably, the pYap levels were dramatically increased by overexpression of Lats2-S835A (Fig. 6E, lane 5), and were not affected by UV irradiation (Fig. 6E, lane 6). These results suggest that the S835 phosphorylation of Lats2 is not required for the Lats2 kinase activity toward Yap, and that the phosphorylation targets of Lats2 might be converted from general targets, such as Yap, to DDR-specific targets, such as p21, when the Chk1–Lats2 axis is activated by UV irradiation.

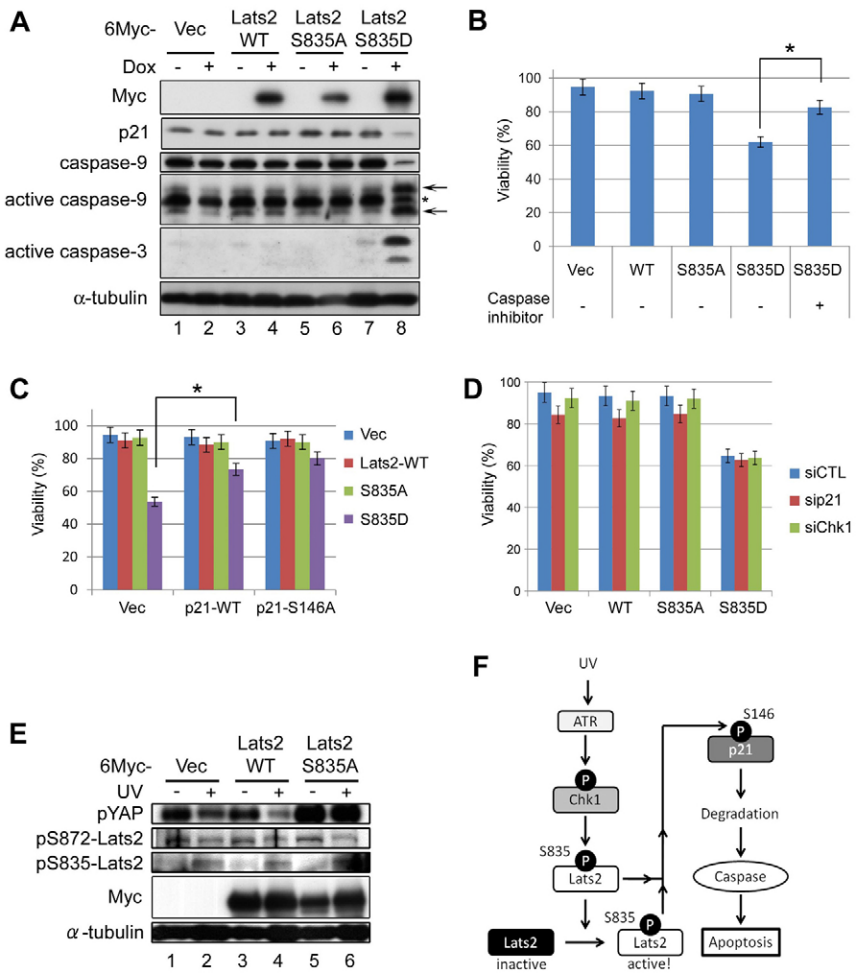

Fig. 6. p21 inhibits apoptosis to the same extent as a caspase inhibitor. (A) U2OS/pTRET-6Myc-tagged vector or Lats2 (WT, S835A or S835D) cells were treated with Dox for 48 hours. Cell lysates were then analyzed by western blotting with the indicated antibodies. Arrows indicate cleaved caspase-9 (active form). Asterisk indicates non-specific bands. (B) U2OS/pTRET-6Myc-tagged vector or Lats2 (WT, S835A or S835D) cells were treated with Dox for 24 hours and 25 μM pan-caspase inhibitor Z-VAD-FMK. Cell viability was measured in a Trypan Blue exclusion assay. The histograms show the average viability (%) calculated from three independent experiments. (C) U2OS/pTRET-6Myc-tagged vector or Lats2 (WT, S835A or S835D) cells were transfected with a 3×FLAG-tagged vector, p21-WT or p21-S146A, and treated with Dox for 24 hours. Cell viability was measured in a Trypan Blue exclusion assay. The histograms show the average viability (%) calculated from three independent experiments. (D) U2OS/pTRET-6Myc-tagged vector or Lats2 (WT, S835A or S835D) cells were transfected with siRNA (siCTL, sip21 or siChk1) and treated with Dox for 24 hours. Cell viability was measured by a Trypan Blue exclusion assay. The histograms show the average viability (%) calculated from three independent experiments. (E) U2OS/pTRET-6Myc-tagged vector or Lats2 (WT or S835A) cells were treated with Dox for 24 hours, irradiated with UV (50 J/m^2) and then incubated for 8 hours. Western blot analysis was then performed using the indicated antibodies. (F) Model summarizing the regulation of apoptosis by Lats2 and p21 following UV damage. Chk1 is activated by UV damage and induces Lats2 activation through phosphorylation at Lats2 S835. Activated Lats2 trans-autophosphorylates and activates other Lats2 molecules. Activated Lats2 then phosphorylates p21 at S146, which degrades p21. Degradation of p21 activates caspase-3 and caspase-9, which induce apoptosis (intact p21 inhibits caspase activation). The error bars represent s.d.; *P<0.01.

Because Lats2 also possesses an additional autophosphorylation site (S872) that is phosphorylated in response to Mst1/2-mediated phosphorylation of Lats2 on T1041 during Hippo pathway activation (Pan, 2010), we examined the impact of UV damage on the S872 phosphorylation of Lats2 (pS872). We generated antibodies that specifically recognize pS872 Lats2 (supplementary material Fig. S3A, second panel). Levels of pS872 decreased following UV irradiation, and were not affected by ectopic overexpression of wt Lats2 or Lats2-S835A (Fig. 6E, second panel, lanes 2, 4 and 6). These results suggest that UV irradiation promotes the S835 phosphorylation levels of endogenous Lats2, whereas it reduces the phosphorylation levels of another autophosphorylation site, S872 (Fig. 6E, second panel compare with third panel).

Taken together, our data are consistent with a scenario in which, following UV irradiation, Chk1 phosphorylates Lats2 at S835 in the kinase domain and activates Lats2 via autophosphorylation. Activated Lats2 then phosphorylates and destabilizes p21, thereby inducing apoptosis through caspase activation (Fig. 6F).

Discussion

In the present study, we suggest that the Chk1–Lats2–p21 axis plays an important role in a phosphorylation cascade in response

to UV irradiation. We demonstrate that Lats2 is phosphorylated and activated by Chk1 after UV irradiation. This ignition of signaling causes trans-autophosphorylation of Lats2, which augments Lats2 activation. Activated Lats2 phosphorylates p21, which promotes degradation of p21 protein, thereby releasing caspase-3 and caspase-9 from a p21-dependent blockade to induce apoptosis.

Our present report reveals that Lats2 phosphorylates p21 on S146, which decreases p21 stability (Fig. 3) in accordance with previous reports that PKCζ and NDR1-mediated phosphorylation of S146 induces p21 degradation by the proteasome in a ubiquitin-independent manner (Scott et al., 2002; Cornils et al., 2011). By contrast, other reports show that Akt and PKCδ-mediated phosphorylation at S146 increases p21 stability (Li et al., 2002; Oh et al., 2007). Although Akt can potentially phosphorylate both S146 and the adjacent T145 of p21, it preferentially phosphorylates T145 (Li et al., 2002). Moreover, the phosphorylation of T145 prevents p21–PCNA interaction and plays a crucial role in relocalization of p21 from the nucleus to the cytoplasm. In fact, the T145A mutant of p21 (unphosphorylated form) retains its nuclear localization despite Akt activation, whereas a T145D mutant (phosphomimetic form) is readily translocated to the cytoplasm in the cells expressing dominant-negative Akt (Zhou et al., 2001). These data suggest that the subcellular localization of p21 is regulated by T145 phosphorylation, whereas its protein stability is negatively regulated through S146 phosphorylation. It is noteworthy that Lats2 phosphorylates S146, but not T145, of p21 (Fig. 2). This suggests that Lats2 primarily regulates p21 through S146 phosphorylation-dependent protein degradation.

Previous studies demonstrate that ubiquitin-independent degradation of cytoplasmic p21 leads to apoptosis by cancelling its inhibitory function against apoptotic proteins such as caspases, whereas ubiquitin and PCNA binding-dependent degradation of nuclear p21 promotes DNA repair and S-phase progression (Abbas and Dutta, 2009; Jung et al., 2010). Moreover, these distinct mechanisms of p21 degradation are stringently regulated by distinct phosphorylation events; namely, S146 (probably also T145) phosphorylation(s) of p21 contributes to its degradation in the cytoplasm by preventing p21-PCNA interaction (Scott et al., 2000), whereas S114 phosphorylation of p21 contributes to its degradation in the nucleus by augmenting the binding with PCNA and the subsequent polyubiquitylation (Abbas et al., 2008). Consistently, Lats2-mediated p21 degradation is regulated by the cytoplasmic S146 phosphorylation, thereby inducing caspase-dependent apoptosis (Fig. 3). Thus, it is probable that Lats2-mediated p21 degradation is required for apoptosis but not DNA repair and DNA replication.

In the context of the DDR, p21 was shown to be immediately degraded after UV irradiation, which contributes to DNA repair and cell cycle arrest (Bendjennat et al., 2003). In this case, p21 degradation is regulated by S114 phosphorylation by GSK3β, PCNA binding and subsequent polyubiquitylation, which is dependent on ATR activation (Lee et al., 2007). However, we found that UV-induced p21 degradation also leads to apoptosis through the Chk1–Lats2 signaling cascade, probably downstream to ATR. Taken together with previous reports, we suggest that p21 degradation induced by GSK3β upon low doses (~5 J/m^2) of UV irradiation contributes to the restoration of normal cell growth after DNA repair and cell cycle arrest, whereas p21 degradation induced by Chk1–Lats2 upon high doses (50 J/m^2) of UV irradiation facilitates the removal of cells with irreparably damaged DNA by inducing apoptosis. Therefore, the Chk1–Lats2–p21 axis may be a novel pathway to remove damaged cells with high levels of DNA lesions after high doses of UV irradiation.

How is Lats2 activity restricted prior to UV irradiation? How does Lats2 efficiently induce apoptosis subsequent to UV damage? These mechanisms might be attributed to the fact that Lats2 is activated by trans-autophosphorylation. We surmise that Chk1 initially phosphorylates S835 to activate Lats2, then a subset of phosphorylated (and activated) Lats2 phosphorylates other Lats2 molecules in turn. Finally, a threshold of intracellular Lats2 is activated to phosphorylate p21 (Fig. 6F). In fact, a Lats2-KD mutant was unable to undergo auto-phosphorylation (Fig. 2) and only S835 phosphorylated Lats2 was able to phosphorylate p21 (Fig. 4). Thus, it is likely that Chk1 is a priming kinase to amplify gross Lats2 kinase activity through trans-autophosphorylation of Lats2 in response to UV damage. Interestingly, overexpression of Lats2-S835D but not Lats2-WT induced apoptosis through caspase activation even without UV irradiation. (Fig. 5). Thus, even in the absence of any intentional DNA damage, Lats2-S835D acts as a phosphorylated and activated Lats2, induces trans-autophosphorylation for activation of intracellular Lats2, and consequently p21 is phosphorylated and destabilized. Because phosphorylation on S835 induces apoptosis via p21 phosphorylation, we speculate that this phosphorylation also influences other substrates and pathways.

Our previous report revealed that Lats2 induces the formation of P-bodies after UV irradiation (Okada et al., 2011). However, our report here shows that Lats2 also induces apoptosis under the same stimulation (UV irradiation). From these findings, we propose that Lats2 plays an important role in many events under DDR. It is conceivable that Lats2 also functions as a crucial mediator of other types of DDR signaling, such as γ-ray radiation, chemical compounds, oncogenic stress and so on. Further studies are required to identify additional functions of Lats2 in DDR.

Taken together, the Chk1–Lats2–p21 axis we propose here is a novel DDR pathway that facilitates apoptosis upon high levels of UV damage, thereby eliminating damaged cells and potentially preventing tumor malignancy, making this pathway a promising candidate for targeted cancer therapies in the future.

Materials and Methods

Plasmid DNA constructs

6Myc–Lats2-WT and 6Myc–Lats2-KD have been described previously (Yabuta et al., 2011). For construction of Lats2 (amino acids 644–855)-S835A/D, a *Bsp*EI-digested Lats2-S835A/D (2273–2950; 683bp) were synthesized encompassing S835 and then inserted into the *Bsp*EI sites in 6Myc–Lats2-WT. The cDNAs of human p21 WT, T145A, S146A and T145A-S146A containing both *Asc*I and *Not*I sites were synthesized (GenScript, NJ) and then the *Asc*I- and *Not*I-digested fragments were subcloned into the *Asc*I and *Not*I sites of pGST6P+AscI, a modified version of pGEX6P-2 (GE Healthcare, Piscataway, NJ) and p3FLAG+AscI, a modified version of p3×FLAG-CMV-7.1 (Sigma, St Louis, MO) containing the linker *Hin*dIII–*Asc*I–*Bmg*BI–*Not*I. Lats2-WT, KD, S835A and S835D were ligated into the *Asc*I and *Xho*I sites in pTRET3–6Myc, a modified version of pTRE-Tight (Clontech, Palo Alto, CA) containing a *Hin*dIII–6Myc–*Not*I linker.

Cell culture and transfection

Human osteosarcoma U2OS cells and embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, UT), penicillin (100 U/ml) and streptomycin (100 μg/ml). Human cervical cancer HeLa S3 cells were maintained in DMEM supplemented with 5% FBS, penicillin (100 U/ml) and streptomycin

(100 μg/ml). U2OS/shLats2-#2 and U2OS/shControl cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and Blasticidin S (10 μg/ml; InvivoGen, San Diego, CA) to select for stably transfected clones (Aylon et al., 2006). The 293T and U2OS cells were transiently transfected with the indicated plasmids using the Lipofectamine and PLUS reagent (Invitrogen, San Diego, CA), according to the manufacturer's instructions.

Cell irradiation

For UV irradiation of cells using a germinal lamp (Stratalinker 2400, Stratagene, La Jolla, CA) at a rate of 0.22 J/m^2/second, the culture medium and cell plate cover were first removed and washed with PBS without calcium and magnesium [PBS(−)]. After UV irradiation, the culture medium was returned to the cell plate and incubated at 37℃ for the indicated times.

Generation of U2OS pDox-On cell lines

U2OS pDox-On cells were generated by the stable transfection of U2OS cells with pTet-On Advanced (Clontech) according to the manufacturer's protocol and grown in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and G418 (800 μg/ml). After selection for ~14 days, colonies were isolated and expanded. To generate stable clones, U2OS pDox-On cells were co-transfected with each of the pTRET3–6Myc plasmids and the Linear Hygromycin Marker (Clontech) using the Lipofectamine and PLUS reagent according to the manufacturer's instructions. Transfected cells were diluted and selected with Hygromycin B (InvivoGen; 200 μg/ml). Single colonies were isolated and checked for the correct expression patterns in the presence or absence of doxycycline (Dox; 5 μg/ml). U2OS/pTRET3–6Myc-Vec, Lats2-WT, Lats2-S835A and Lats2-S835D cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), G418 (800 μg/ml) and Hygromycin B (200 μg/ml) to select for stably transfected clones.

siRNA

siRNA duplexes specific for human Lats2, Chk1, p21, NDR1 or control (CTL) siRNA (GL2) were used to knock down each gene in U2OS cells and HeLa S3 cells. siNDR1a, siNDR1b and siNDR1c were purchased from Origene (Rockville, MD). The sequences are shown in supplementary material Fig. S6. siRNAs were transfected into U2OS cells using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Cells were incubated at 37℃ for 48 hours.

Generation of anti-phospho-specific Lats2 antibodies

To generate the novel polyclonal antibody against the phosphorylated S835 and S872 sites in Lats2, rabbits were injected with the KLH-conjugated phospho-peptide CHVQD{pSer}MEPSDLWD (pS835) or CQHQRCLAH{pSer}LVGTP (pS872). The antiserum was then affinity-purified using and the Linear Hygromycin Marker (Clontech) a phospho-antigen peptide column. To eliminate nonspecific antibodies that could react with the unphosphorylated peptide antigen, the antibody preparation was passed through a non-phospho-peptide column [CHVQDSMEPSDLWD(S835) or CQHQRCLAHSLVGTP(S872)]. These experiments were supported by GenScript Corp. The specificities of these antibodies were estimated by dot-blot analysis (supplementary material Fig. S3A) and ELISA (data not shown). The dot-blot analysis was performed as described previously (Yabuta et al., 2011).

Antibodies and chemicals

Anti-Chk1 monoclonal antibody (anti-Chk1), anti-α-tubulin monoclonal, anti-FLAG monoclonal and anti-FLAG polyclonal antibodies were purchased from Sigma; anti-Myc monoclonal (PL14), anti-Myc-tag HRP-DirecT, anti-caspase-9 monoclonal and anti-GST monoclonal antibodies were purchased from MBL (Nagoya, Japan); anti-p21 monoclonal antibody was purchased from BD Bioscience (Franklin Lakes, NJ); anti-GAPDH polyclonal was purchased from Fitzgerald Industries International (Concord, MA); anti-p21-pS146 polyclonal antibody was purchased from Abgent (San Diego, CA); anti-cleaved caspase-3 polyclonal and anti-Lamin A/B monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA); and anti-NDR1/STK38 goat polyclonal antibody was purchased from OriGene. Lats2 was detected by LA-2 (Okada et al., 2011), anti-kpm polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Lats2 polyclonal (Bethyl Laboratories, Montgomery, TX) antibodies. A general caspase inhibitor Z-VAD-FMK and a protein synthesis inhibitor CHX were purchased from R & D Systems (Minneapolis, MN) and Sigma, respectively.

Western blot analysis

Protein lysates were prepared by incubating cells in TNE250 lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.2% NP-40, 1 mM PMSF, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatine A, 1 mM NaF, 1 mM Na_3VO_4 and 10 mM β-glycerophosphate) at 4℃ for 30 minutes. Subcellular fractionation was performed as described previously (Yabuta et al., 2000). The cellular extracts and immunoprecipitates were resolved by SDS-PAGE and transferred to Immobilon PVDF (polyvinylidene difluoride) membranes (Millipore, Bedford, MA). Western blotting was performed in TBST (100 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) containing 5% non-fat milk or 5% BSA. Immunoreactive protein bands were visualized using western Lightning Chemiluminescence Reagent Plus-ECL (Perkin-Elmer, San Jose, CA).

RT-PCR

Total RNA was prepared from U2OS cells using the *mir*Vana™ miRNA Isolation kit (Ambion, Austin, TX), according to the manufacturer's instructions. cDNA was synthesized from 3 ng RNA using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). PCR was performed with the primer pairs shown in supplementary material Fig. S6, using the following cycle profile: 95℃ for 3 minutes, followed by 30 cycles of 95℃ for 30 seconds, 55℃ for 30 seconds and 72℃ for 2 minutes, followed by extension at 72℃ for 5 minutes. PCR products were then resolved by agarose gel electrophoresis, followed by ethidium bromide staining.

In vitro Chk1 and Lats2 kinase assays

In vitro Chk1 kinase assays were performed as described previously (Okada et al., 2011). Briefly, 150 ng of active Chk1 (Upstate-Millipore, Bedford, MA) was incubated with 2 μg of GST-purified substrate for 30 minutes at 30℃ in Chk1 kinase buffer containing 25 μM ATP. For the *in vitro* Lats2 kinase assays (Yabuta et al., 2007), 293T cells were co-transfected with 6Myc–Lats2 or GFP–Lats2 kinases together with 3FLAG–Mob1A, and treated with the serine/threonine phosphatase inhibitor okadaic acid (0.1 μM) for 3 hours and irradiated UV (50 J/m^2) irradiation before harvesting the lysates. Immunoprecipitates obtained from the cell extracts using anti-Myc antibody were mixed with GST-purified substrates and incubated in Lats2 kinase buffer containing 20 μM ATP and 10 μCi [γ-^{32}P]ATP for 30 minutes at 30℃. The proteins were then separated by SDS-PAGE. The gels were used for western blot analysis or stained with Simply Blue safe stain (Invitrogen).

Cell viability assay

U2OS cells were seeded in 6 cm dishes. After overnight incubation, cells were treated with Dox for the indicated times. The cells were then trypsinized and resuspended in the appropriate medium. The cell suspension was mixed 1:1 (v/v) with 0.4% (w/v) Trypan Blue stain. Viable cells, which excluded the trypan blue dye, were counted using Countess™ cell counting chamber slides and a Countess™ Automated Cell Counter (Invitrogen).

TUNEL assay

Apoptotic cells were detected using the DeadEnd colorimetric TUNEL assay (Promega, Madison, WI), which end-labels fragmented DNA. U2OS cells were plated on cover glasses and fixed by sequential incubation with 4% formaldehyde in PBS(−), 0.1% Triton X-100 in PBS(−) and 0.05% Tween-20 in PBS(−), each for 10 minutes at room temperature. After washing, cells were incubated with recombinant terminal deoxynucleotidyl transferase (TdT) and biotinylated nucleotides for 1 hour at 37℃. Cells were then washed with PBS(−), stained with Hoechst 33258 (Sigma), and observed using a BX51 microscope (Olympus).

Annexin V staining

Apoptotic cells were also detected using the MEBCYTO apoptosis kit (MBL) using FITC-conjugated Annexin V and propidium iodide (PI). U2OS cells were cultured for 24 hours in the presence of FBS (10%) and then harvested. After Dox-on, specific binding of Annexin V-FITC was performed by incubating the cells for 15 minutes at room temperature in a binding buffer according to the manufacturer's instructions containing a saturating concentration of Annexin-V–FITC and PI according to the manufacturer's instructions. Analysis was performed using a FACScalibur with CellQuest software (BD bioscience).

Pull-down assays

GST-tagged human p21 proteins were produced in *E. coli* BL21 RIL and purified with Glutathione Sepharose 4B (GE Healthcare). 6Myc-tagged human Lats2 wild-type, kinase-dead, S835A and S835D mutant proteins were extracted with TNE250 lysis buffer from 293T cells transfected with each plasmid. The cleared lysates (0.5 mg proteins in 1 ml lysis buffer) were incubated at 4℃ for 4 hours with 10 μg of each GST-tagged p21 protein immobilized on Glutathione Sepharose 4B. After six washes with 1 ml TNE250 lysis buffer, bound proteins were eluted by boiling in SDS-PAGE sample buffer. The eluates were resolved by SDS-PAGE and subjected to western blot analysis.

Statistics analysis

For all cell viability assays, $>8\times10^5$ cells were counted and each experiment was repeated independently at least three times. The error bars represent the s.d. P values were calculated using the Student's two-tailed t-test and are indicated as follows: $*P<0.01$.

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Author contributions

H.S., N.Y. and H.N. designed the research; H.S., N.Y., N.O. and K.T. performed research and analyzed data; Y.A. and M.O. discussed the research data and provided materials; and H.S., N.Y., Y.A., M.O. and H.N. wrote the paper.

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