JNK phosphorylates Yes-associated protein (YAP) to regulate apoptosis

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Yes-associated protein (YAP) regulates DNA damage and chemosensitivity, as well as functioning as a pro-growth, cell size regulator. For both of its roles, regulation by phosphorylation is crucial. We undertook an in vitro screen to identify novel YAP kinases to discover new signaling pathways to better understand YAP’s function. We identified JNK1 and JNK2 as robust YAP kinases, as well as mapped multiple sites of phosphorylation. Using inhibitors and siRNA, we showed that JNK specifically phosphorylates endogenous YAP in a number of cell types. We show that YAP protects keratinocytes from UV irradiation but promotes UV-induced apoptosis in a squamous cell carcinoma. We defined the mechanism for this dual role to be YAP’s ability to bind and stabilize the pro-proliferative ΔNp63α isoform in a JNK-dependent manner. Our report indicates that an evaluation of the expression of the different isoforms of p63 and p73 is crucial in determining YAP’s function.

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Conversely, YAP has also been identified recently as a pro-growth, cell size regulator in both Drosophila melanogaster and mammalian cells.15 In contrast to regulating apoptosis by activation of p73, the growth control role of YAP or its fly homolog, Yorkie (Yki), is due to inactivation by the MST2 (HIPPO in fly) pathway.16,17 Here, the tumor-suppressor LATS1 kinase (WTS in fly) directly phosphorylates YAP (Yki), inhibiting its co-activation of the TEAD (Scalloped in fly) transcription factor to upregulate pro-growth genes.18,19 However, phosphorylation of YAP by MST2/LATS1 has also been shown to enhance p73 binding and subsequent apoptosis downstream from Fas in human breast cancer cells and chemotherapy in leukemia cells, as well as overexpression of pathway members in HEK293 cells.20–22

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Clearly, phosphorylation is a key regulatory mechanism for YAP. To further understand the role of YAP, we sought to discover new signaling pathways that regulate YAP’s function. We wished to identify kinases that directly phosphorylate YAP and then functionally characterize the phosphorylation in cells in the context of apoptosis. To this end, we performed an in vitro screen using recombinant YAP and a panel of recombinant, active kinases. We selected the kinases on the basis of their putative phosphorylation site motifs expressed in YAP. Here we report the identification of JNK1 and JNK2 as kinases that robustly phosphorylate YAP and regulate its function in apoptosis.

Results

Identification of JNK as a YAP kinase. To find novel YAP kinases, a panel of 29 recombinant, candidate kinases was

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Abbreviations: ΔN, N-terminal truncated; PML, pro-myelocytic leukemia; siRNA, short interfering RNA; TA, transactivation domain; YAP, Yes-associated protein; Yki, Yorkie

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screened for in vitro phosphorylation of recombinant YAP1. YAP phosphorylation was visualized by autoradiography of the SDS-PAGE fractionation of $^{32}$P-labeled ATP incorporation and quantified (Figure 1 and Supplementary Table 1). Specific activities of candidate kinases were validated by using phosphorylation of control peptides (Supplementary Table S1). We identified JNK1 (variant JNK1a1) and JNK2 (variant JNK2a2) as strong YAP kinases (Figure 1 and Supplementary Table 1). In addition, ERK2 and PKC$\alpha$ were also identified as moderate, and CaMKII, PKC$\gamma$ and PKC$\varepsilon$ as weak, YAP kinases (Figure 1 and Supplementary Table 1). On the basis of these initial findings and the well-characterized role of JNKs in regulating apoptosis and diseases such as cancer, we focused our efforts to pursue JNKs as putative YAP kinases. We performed time courses of phosphorylation to determine whether both JNK1 and JNK2 phosphorylated YAP stoichiometrically in vitro (Figure 2a).

**JNK phosphorylates YAP on multiple sites.** To identify these sites, mass spectrometry (LC-MS/MS) was used to analyze recombinant YAP that had been incubated with either JNK1 or JNK2 for 60 min for full phosphorylation, as evidenced by pronounced MW shift (Figure 2b). In total, five novel phosphorylation sites on YAP were identified: T119, S138, T154, S317 and T362 (Figure 2b). Each of the serines and threonines are followed by a proline and therefore represent canonical JNK phosphorylation sites.

To ascertain if JNK phosphorylates YAP on the same sites in vivo, we expressed a Flag-tagged YAP1 construct in 293 cells, followed by treatment with the potent JNK signaling activator, anisomycin. Immunoprecipitation (IP) of YAP from these cells showed a clear anisomycin-induced MW band shift in YAP, similar to that observed in vitro, by both Western blot analysis and Coomassie staining (Figure 2c). LC-MS/MS analysis of the in vivo JNK-stimulated YAP identified three phosphorylation sites, S138, S317 and T362, all of which were also found from in vitro analysis (Figure 2c). We mutated these residues individually to alanine to generate phospho-deficient constructs, along with the other two sites identified in vitro, T119 and T154. The wild-type and five mutated Flag–YAP constructs were expressed in U2OS cells, followed by treatment with anisomycin to determine what effect these mutations would have on the JNK-signature YAP band shift. The mutated constructs showed varying degrees of band shift upon anisomycin treatment, with the S317A and T362A mutants showing minimal shift, indicating that these two sites are most responsible for in vivo phosphorylation induced MW shift (Figure 2d).

**JNK specifically phosphorylates YAP in vivo.** To determine whether JNK phosphorylates endogenous YAP1, we activated JNK by treating cells with anisomycin and detected the YAP band shift in a number of cells, including the HaCaT keratinocyte, U2OS osteosarcoma, MCF-7 breast...
cancer and 293 human embryonic kidney cell lines (Figure 3a). We confirmed anisomycin activation of JNK by phosphorylation of its target, c-Jun.\(^2^7\) (Figure 3a). This showed that JNK phosphorylation of YAP is not cell line-specific. To further confirm that the anisomycin-induced YAP band shift was due to JNK activation, U2OS cells were treated with a JNK-specific chemical inhibitor, SP600125,\(^2^8\) before anisomycin treatment. The inhibitor prevented JNK activation, as observed by a reduction in the phosphorylation of c-Jun, and prevented the YAP band shift induced by anisomycin (compare lanes 2 and 3 in Figure 3b, upper panel). Although our \emph{in vitro} screen indicated that only JNK1 and JNK2, and not other stress-activated MAPKs, that is, the different p38 isoforms, phosphorylate YAP (Figure 1 and Supplementary Table S1), we wished to rule out the possibility that the YAP band shift is due to anisomycin-induced p38 MAPK activation. HaCaT cells were pretreated with the specific p38 inhibitor, SB203580,\(^2^9\) before anisomycin treatment. Western blot analysis showed an anisomycin-induced shift in YAP in both untreated and treated cells, confirming that p38MAPK does not phosphorylate YAP (Figure 3b, lower panel). The same lysates were immunoblotted for the phosphorylated form of the direct
p38 target, MAPKAPK230 to show that SB203580 was effective in inhibiting p38’s activity (Figure 3b, lower panel). Furthermore, although we had identified ERK2 as a moderate YAP kinase (Figure 1 and Supplementary Table S1), as expected, anisomycin did not activate ERK1 or ERK2 in these cells, as shown by immunoblotting with a phospho-ERK antibody (Supplementary Figure 1). Our in vivo results confirm that JNK but not p38 MAPKs phosphorylates endogenous YAP in cells.

We attempted to raise phospho-specific antibodies against the S317 and T362, the two sites that were most responsible for in vivo phosphorylation-dependent band shift of YAP (Figure 2d), and were successful in generating a T362-specific antibody (pT362). The pT362 antibody was effective when used on immunoprecipitated YAP from anisomycin-treated cells (Figure 3c), confirming our mass spectrometry results (Figure 2c). In these experiments, we monitored the anisomycin activation of JNK using a phospho-JNK antibody.

Figure 3  JNK specifically phosphorylates YAP in vivo. (a) HaCaT, U2OS, MCF7 and 293T cell lines were treated with anisomycin or DMSO (control) for 60 min before harvesting. YAP band shift was analyzed as described before and lysates were analyzed by Western blotting with the indicated antibodies. (b) Upper panel: U2OS cells were treated with SP600125 or an equivalent volume of DMSO for 3 h, followed by treatment for 15 min with anisomycin or DMSO. Lower panel: HaCaT cells were treated with SB203580 or DMSO for 3 h prior followed by a 15 min treatment with anisomycin or DMSO, harvested and lysates were analyzed using the indicated antibodies. (c) U2OS cells were transfected with Flag–YAP and either siControl (siCTL) or siJNK1 and siJNK2 (siJNK). The cells were then treated with anisomycin for 1 h and subsequently Flag–YAP was immunoprecipitated from lysates. IP inputs and eluates were analyzed by Western blotting using the indicated antibodies. (d) BWT cells were transfected with Flag–YAP, Flag-mut2AYAP and Flag-mut2DYAP. The cells were irradiated with 30 J/m² UV-C and harvested 1 h later for Western blot analysis with the indicated antibodies. (e) HaCaT cells were irradiated with 50 J/m² UV-C and harvested at 10, 20, 30 min and 2 h. Endogenous YAP was immunoprecipitated and IP inputs and eluates were analyzed by Western blotting using the indicated antibodies.
radiation and treatment with Fas ligand, but the role of YAP chemotherapeutic agents such as cisplatin, as well as ionizing c-Jun phosphorylation. Together, these results show that YAP phosphorylation. To verify that UV exposure results in detectable band shift in either mutant YAP construct, showing resolved at an MW similar to untreated mut2DYAP (Figure 3d). Moreover, this shift (Figure 3d). Similar to anisomycin, UV-C treatment caused a JNK phosphorylation-dependent band shift from previous experiments (Figure 3d). To study the effects of YAP expression in these cells, we generated control and YAP shRNA stable cell lines and subjected upon UV radiation in HaCaT cells (Figure 3e). To study the effects of YAP expression in these cells, we generated control and YAP shRNA stable cell lines and subjected them to 50 J/m² UV irradiation. At 24 h after treatment, 40% apoptosis was observed in the control cells (as measured by FACS analysis of Annexin-V and propidium iodide (PI) staining, both early and late apoptosis), YAP silencing resulted in considerably more death, with 70% apoptosis evident in YAP shRNA cells (Figure 4a and Supplementary Figure 2a). Noticeably, stable YAP loss alone did not cause death in these cells (Figure 4a and Supplementary Figure 2a). YAP silencing and JNK activation as shown by phosphorylated c-Jun levels were maintained at 24 h of UV treatment (Supplementary Figure 2b). Western blot analysis of lysates from similarly treated cells showed that YAP silencing enhanced UV-induced PARP cleavage, a hallmark of apoptosis, at time points as early as 2 h (Figure 4b). Interestingly, anisomycin treatment, which resulted in comparable levels of JNK activity, as shown by phosphorylated c-Jun levels, also similarly enhanced PARP cleavage in YAP shRNA stable cells (Figure 4b). These results indicate that YAP expression protects from UV-induced, JNK-mediated apoptosis in keratinocytes. As described earlier, this finding is contrary to what has been reported previously for YAP's role in other DNA-damaging stimuli in tumor-derived cells. We next examined how YAP's expression regulated UV-induced apoptosis in a skin cancer cell line. BWT squamous cell carcinoma cells treated with control siRNA subject to 30 J/m² UV for 24 h resulted in over 40% total apoptosis as measured by FACS analysis. YAP siRNA-treated cells irradiated similarly were markedly protected from UV-induced cell death, showing only 18% apoptosis (Figure 4c). As shown in Figure 4d, efficient silencing of YAP in these cells also inhibited UV-induced PARP cleavage at 6 h. To investigate whether JNK phosphorylation of YAP regulates its proapoptotic role in this skin cancer cell line, we expressed either an empty vector or the mut2DYAP plasmid and irradiated with UV as before. Tellingly, expression of the JNK-site phosphomimetic YAP construct alone resulted in a level of apoptosis similar to that observed with UV irradiation of the empty vector control cells (Figure 4e). This corroborated with our results in Figure 3d where we show that expression of mut2DYAP alone mimics the UV-induced band shift of wild-type YAP in BWT. In contrast to our results in HaCaT keratinocytes (Figure 4a and b), these findings show that endogenous YAP expression promotes UV-induced apoptosis in BWT, in a JNK phosphorylation-dependent manner.

JNK phosphorylation of YAP enhances its stabilization of ΔNp63α by direct binding. We have recently reported that YAP promotes cisplatin-induced, c-Jun-mediated apoptosis, in part by stabilizing the proapoptotic TAp73α isoform, a mechanism also shown by others.12,13 HaCaT cells more abundantly express the ΔNp63α isoform, which has been shown to be crucial for proliferation, differentiation and actually protective from p73-dependent cell death.33–36 However, the PPXY motif required for YAP binding present on the longer isoforms of p73 is also conserved on ΔNp63α.4 We examined whether YAP can also stabilize this protective p53-family member in a JNK-dependent manner. In HaCaT cells, silencing of endogenous YAP resulted in a striking decrease in the levels of endogenous ΔNp63α (Figure 5a). Within the time frame indicated, UV treatment did not decrease the expression of ΔNp63α in control cells but markedly did so in YAP-knockdown cells (Figure 5a). Silencing of YAP had no effect on UV-induced JNK activation itself, as evidenced by phospho-c-Jun immunoblotting (Figure 5a). We wished to verify whether YAP-dependent stabilization of ΔNp63α is due to their direct binding, as was shown for YAP and TAp73α.12,13 Unstimulated HaCaT cells showed basal
binding of endogenous YAP and ΔNp63x, as detected by co-IP with an anti-YAP antibody (Figure 5b). Strikingly, UV treatment induced rapid and sustained increase in the binding of endogenous YAP and ΔNp63x (Figure 5b), following the kinetics of YAP phosphorylation by UV radiation in these cells (Figure 3e).

It was shown that YAP stabilized p73 in part by displacing the E3 ligase ITCH by competitive binding at the PPXY site on p73. ItCH was recently shown to bind and lead to degradation of ΔNp63x, as well as TAp73x. To further understand the mechanism behind the YAP-mediated stabilization of ΔNp63x, we assessed whether YAP stabilizes ΔNp63x by interfering with ITCH binding; HaCaT cells were transfected with either control or siRNA targeting ITCH. Consistent with our observations in Figure 5b, co-IP with anti-p63 antibody reveals endogenous YAP-p63 binding in control HaCaT cells. Significantly, silencing endogenous ITCH in HaCaTs resulted in greater YAP-p63 binding as shown by increased YAP co-immunoprecipitated with p63 (Figure 5c).

To ensure that these finding were not specific to HaCaT cells, we assessed endogenous YAP stabilization of ΔNp63x...
...siRNA (siYAP) and 48 h later irradiated with 50 J/m² UV. Cells were harvested at the indicated time points after UV irradiation and analyzed by Western blotting using the indicated antibodies. (c) H357 cells were transfected with control siRNA (siCTL) or ITCH siRNA (siITCH) and 72 h later the cells were harvested for IP of endogenous p63. p63 IP inputs and eluates were analyzed by Western blotting using the indicated antibodies...
UV-induced cell death (Figure 6b), while ectopically expressing ΔNp63α in BWT (Figure 6c) resulted in decrease in apoptosis upon UV treatment.

Discussion

Our results show that YAP is a direct substrate for JNK phosphorylation in a number of cell lines, both tumor-derived (U2OS, MCF-7, BWT) and immortalized (293, HaCaT) (Figure 3a). We further show that JNK is stimulated to phosphorylate YAP upon UV irradiation (Figure 3e), one of the most potent stress activators of the JNK pathway.23,24,31

The studies that show YAP to promote apoptosis most often used chemotherapeutics, such as cisplatin, to induce cell death in tumor-derived cell lines.7,9,11–13 Consistent with this role, YAP has recently been shown to function as a tumor suppressor in certain breast cancers.39 However, YAP has been shown to be a pro-growth signal in development, most likely that to fully appreciate YAP's function in a particular context, an integrative approach to phosphorylation, as well as the other post-translational modifications reported for YAP,10,43 will be required.

Materials and Methods

YAP Kinase screen. See Supplementary Methods for details on YAP kinase screens.

Cell culture, transfections and treatments. U2OS and HaCaT cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, l-glutamine and penicillin/streptomycin. The BWT human skin squamous cell carcinoma line (a gift from Dr Karin Purdie (ICMS, Barts and The London School of Medicine, Queen Mary University of London, London, UK)) was grown in Dulbecco’s modified Eagle’s medium and F12 3:1 supplemented with 10% fetal bovine serum, adenosine, hydrocortisone, insulin, epidermal growth factor, cholera toxin, transferrin, lipothryione and penicillin/streptomycin. H357 head-and-neck cancer cells were grown in Dulbecco’s modified Eagle’s medium supplemented as BWT medium but excluding transferrin or lipothryione. Transfections of cDNA and shRNA constructs were performed using Effectene (Qiagen, Crawley, West Sussex, UK), according to the manufacturer’s instructions, either in six-well plates or 10-cm dishes using 0.4 or 2 μg of DNA, respectively. Cells were harvested 24 or 48 h after transfection. Interferin (Polyplus Transfection, New York, NY, USA) was used for transfecting siRNA at a final concentration of 10–25 nM, according to the manufacturer’s instructions, and cells were harvested 48 h later. Anisomycin (Sigma, Gillingham, Dorset, UK) was used at a final concentration of 10 μM for 30–60 min before harvesting. The SP600125 and SB203580 inhibitors (Calbiochem, Merck Chemicals Ltd., Nottingham, UK) were used for 3 h before 15-min anisomycin treatment at a concentration of 20 and 10 μM, respectively. For UV-C treatments, cells were washed in PBS and irradiated with 50 J/m² (HaCaT) and 30 J/m² (BWT) of UV-C and then incubated with normal media for the indicated time periods.

Plasmids and siRNAs. Flag–YAP was cloned from GFP–YAP as described elsewhere.7 into pCMV-Tag2B purchased from Stratagene (Agilent Technologies UK Ltd., Stockport, UK). pcDNAHA–ΔNp63α was a gift from Dr Eleonora Candi (Università di Tor Vergata, Roma, Italy). Mutagenesis was performed by using the Quikchange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The primers used in the mutagenesis of YAP at JNK phosphorylation sites to alanine and aspartic acid can be found in Supplementary Methods. pRemoSuper was used to stably express shRNA against YAP (YAP shRNA); also used for YAP siRNA7 and a non-targeting control (control shRNA)44. The JNK siRNA, which targets both JNK1 and JNK2, and a non-targeting sequence #2 were both purchased from Dharmacon (Thermo Scientific, Northumberland, UK).

IP and Western blotting. Cells were harvested and lysed in either NP40 lysis buffer or, for Flag IPs, in Flag lysis buffer (Sigma) containing protease and phosphatase inhibitors. For Flag IPs, Flag beads (Sigma) were washed twice in 0.5% TBS and the lysates were incubated with the beads in 0.5–1 ml of Flag lysis buffer for 2 h at 4 °C. After washing, proteins were eluted off the beads using 3 × Flag peptide in a total volume of 50 μl for 30 min at 4 °C. For endogenous YAP IPs, a monoclonal YAP1 antibody (MO1; Abnova Corporation, Heidelberg, Germany) was used at 1.4 μg/ml protein. For endogenous p63 IP, p63α (H129; Santa Cruz Biotechnology, Heidelberg, Germany) was used at 1 μg/ml protein. Protein-G–Sepharose beads (Sigma) were used according to the manufacturer’s protocol. Proteins were analyzed by 8% SDS-PAGE and probed overnight with primary antibodies, followed by incubation with HRP-coupled secondary antibodies and chemiluminescence detection (ECL). The following antibodies were used: YAP, p63α, (Santa Cruz Biotechnology); Flag–; β-tubulin (Sigma); c-Jun, p-c-Jun, JNK, PARP and cleaved PARP (Cell Signaling Technology, NEB UK, Hitchin, UK); YAP1 (Abnova Corporation); and ITCH (BD Transduction Laboratories, Oxford, UK). The YAP pT362 antibody was made by Kinasonic (Dundee, UK) and was used in conjunction with a dephospho-peptide.

In vitro kinase assay. Active JNK1x1 or JNK2x2 kinases (Upstate, Millipore (U.K.) Limited, Watford, UK) were incubated with recombinant YAP protein along with 100 μM ATP (magnesium/ATP cocktail; Upstate) in a kinase buffer (10 × 500 mM Tris (pH 7.5), 100 mM 2-ME) for 0 or 60 min.

Mass spectrometry. Samples from in vitro kinase assays of Flag IPs were run on a pre-cast 4–12% NuPage Bis–Tris gel (Invitrogen, Paisley, UK), which was then stained with Coo massie (GelCode Blue Stain Reagent; Pierce, Thermo Scientific, Northumberland, UK). The bands containing YAP was excised, digested with trypsin and chymotrypsin, and the resultant peptides were subjected to LC-MS/MS analysis at the Protein Analysis Unit (WHRI, Barts and The London School of Medicine and
Dentistry, Queen Mary University of London, London, UK) and the Taplin Mass Spectrometry Facility (Harvard University). Spectra were analyzed for the phosphorylation signature.

Annexin-V staining and flow cytometry. HaCaT control shRNA (control shRNA) or a YAP-targeted shRNA (YAP shRNA) stables were treated with 50 μM UV-C. BWT cells were transiently transfected with the constructs and siRNAs indicated, and irradiated 24 h later with UV at 30 μJ/cm². Twenty-four hours after UV irradiation, media and PBS wash as well as trypsinized cells were pelleted and washed in PBS before a 15-min incubation with Annexin-V-Alexa-488 and PI according to the manufacturer’s instructions (Molecular Probes, Invitrogen). Flow cytometry was performed using a FACSCalibur instrument (Becton Dickinson, BD Biosciences, Oxford, UK) following the manufacturer’s instructions.

Conflict of interest

The authors declare no conflict of interest.

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