Yes-Associated Protein (YAP) is a critical mediator of c-Jun-dependent apoptosis

Cell Death and Differentiation (2008) **15,** 217–219; doi:10.1038/sj.cdd.4402226; published online 7 September 2007

Dear Editor,

c-Jun plays an important role in the cellular response to DNA damage. Following UV irradiation, c-Jun is rapidly induced and blocks p53-mediated upregulation of p21. c-Jun is required for re-entering the cell cycle following p53-mediated G1 arrest,[1] as c-Jun-null MEFs undergo a prolonged growth arrest following UV irradiation. Consequently, these cells show increased resistance to p53-dependent apoptosis.[1] Moreover, c-Jun is also important for the DNA repair response following cisplatin treatment. Following DNA damage, c-Jun/ATF complexes are rapidly recruited to the promoters of known DNA repair genes such as MSH2 and 6, Rad50 and ATM.[2] Although c-Jun promotes efficient DNA repair upon DNA damage, c-Jun also contributes to DNA damage-mediated apoptosis. This is evident because c-Jun-deficient MEFs demonstrate enhanced resistance to cisplatin-induced apoptosis,[3,4] indicating a role of c-Jun in chemosensitivity.

The cellular response to chemotherapy is critically dependent on functional p73. Reduction of p73 levels in tumour cells by siRNA, or the expression of dominant-negative mutants, leads to a strong reduction of apoptosis induced by DNA damaging agents such as cisplatin, camptothecin or doxorubicin.[5] Importantly, this is irrespective of p53 status.[5] p73 function relies on the presence of Yes-Associated Protein (YAP). Like the loss of p73, downregulation of YAP similarly suppresses cisplatin-mediated cell death.[6,7]

Recent studies suggest that c-Jun promotes cell death by stabilising p73 following cisplatin treatment.[3] Little is known regarding the precise molecular mechanism of c-Jun-mediated stabilisation of p73, although the fact that both the transactivation and DNA binding domains of c-Jun are required, suggests the existence of c-Jun targets with specific roles in cisplatin-induced apoptosis.

Since YAP plays an important role in the p73-dependent response to cisplatin, we investigated whether YAP could be a possible mediator of c-Jun-dependent apoptosis. Sequence analysis of the YAP promoter revealed the presence of several putative AP-1 binding sites within 5000 bp relative to the transcriptional start site of YAP. Interestingly, using quantitative PCR (q-PCR) we found that YAP mRNA levels were induced in U2OS cells following transient transfection of c-Jun (Figure 1a). To validate these observations, we assessed YAP mRNA levels in c-Jun $-/-$ MEFs and found that these cells expressed reduced YAP mRNA levels as compared to wild-type MEFs, and that retroviral-mediated with re-introduction of even small amounts of c-Jun restored YAP mRNA expression levels (Figure 1b). The levels of YAP mRNA correlated well with its protein expression. Thus, downregulation of c-Jun in U2OS cells using siRNA resulted in reduced levels of endogenous YAP (Figure 1c). Furthermore, c-Jun $-/-$ MEFs showed much reduced YAP protein expression as compared with wild-type controls (Figure 1d). Taken together, our data indicate that YAP expression is c-Jun-dependent.

Next, we tested whether c-Jun-mediated stabilisation of p73 is dependent on YAP. To this end, we depleted YAP by siRNA and assessed the ability of c-Jun to promote p73 stabilisation. Interestingly, c-Jun-dependent protein stabilisation of p73 was impaired in cells transfected with two different siRNAs to YAP (Figure 1e). We then examined whether YAP alone was sufficient to influence p73 steady-state levels. In agreement with the recent finding that YAP promotes p73 stabilisation,[8] we found that ectopic expression of YAP efficiently stabilised p73 in U2OS cells (Supplementary Figure 1a), consistent with previous observations.[7] Conversely, loss of YAP through YAP siRNA resulted in markedly reduced levels of p73 (Supplementary Figure 1b). Thus, these results strongly suggest that YAP is a key mediator of c-Jun-dependent p73 protein stability.

We further assessed the protein half-life of TAp73α and γ in the presence or absence of YAP. While TAp73α readily interacts with YAP, TAp73γ lacks the PPXY motif, which is indispensable for YAP binding. Consequently, TAp73γ is unable to bind to YAP.[7] In the presence of cycloheximide, which blocks *de novo* protein synthesis, and the absence of YAP, TAp73α and γ protein levels were rapidly depleted. However, in the presence of YAP, TAp73α levels persisted, indicating that the protein stability of TAp73α was increased in the presence of YAP. In contrast to TAp73α, the protein stability of TAp73γ, which fails to interact with YAP, was unaltered by the presence of YAP (Supplementary Figure 1c). Our data therefore indicate that the YAP-mediated stabilisation of p73 requires physical interaction between YAP and p73. Taken together, these data indicate that YAP regulates the levels of TAp73α via a post-translational mechanism.

We have recently demonstrated that p73 is targeted for degradation by binding to the E3 ubiquitin-protein ligase Itch, which ubiquitinates p73 promoting its proteasomal degradation.[9] Interestingly, Itch and YAP both interact with the same PPXY motif in p73. We therefore reasoned that YAP stabilises p73 by competing with Itch for p73 binding. According to this scenario, YAP would stabilise p73 by liberating/protecting p73 from Itch, thus abrogating Itch-mediated proteasomal degradation of p73. We co-expressed Itch, YAP and p73 constructs in cells to assess the effect of YAP on Itch-mediated p73 stability. Similar to the recent findings of Shaul and co-workers,[8] ectopic expression of YAP stabilised p73 compared

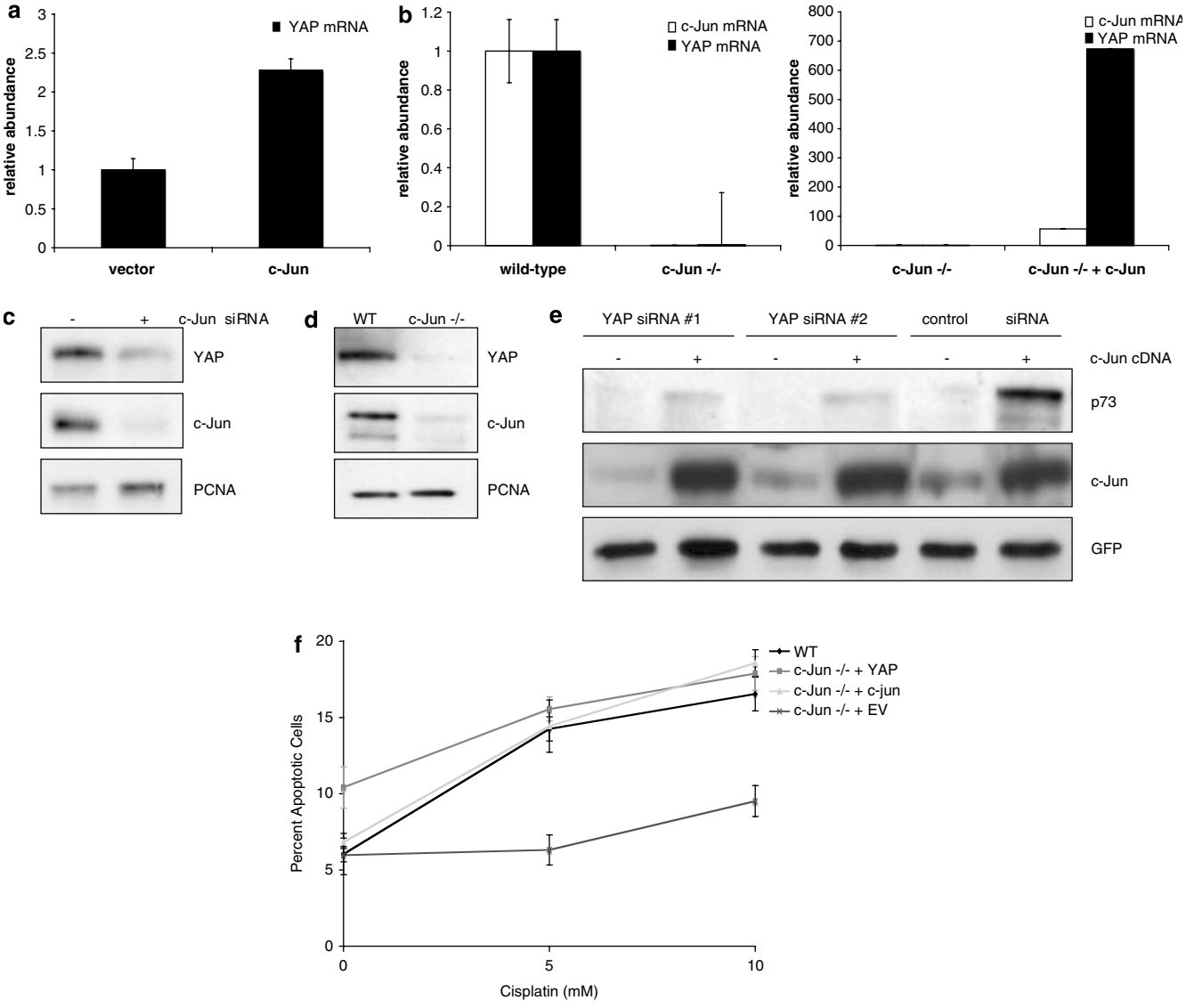

Figure 1 c-Jun upregulation of YAP promotes p73 protein stability and cisplatin-induced apoptosis. (**a**) U2OS cells were transfected with 2 μg of p-EGFP c-Jun or pEFGP-C2 control vector. Twenty-four hours post transfection, cells were harvested and analysed by q-PCR. (**b**) YAP and c-JUN mRNA levels from wild-type and c-Jun −/−MEFs, or c-Jun −/− MEFs retrovirally infected with pBabe or pBabe c-Jun, as indicated, were assessed by q-PCR. (**c**) U2OS cells were transfected with siRNA to c-Jun and harvested 24 h later. Cell extracts were analysed with c-Jun, YAP and PCNA antibodies. (**d**) Cell extracts from wild-type and c-Jun −/− MEFs were blotted as in (**c**). (**e**) U2OS cells were transiently transfected with either control, YAP siRNA no. 1 (as described[6] or YAP siRNA no. 2 (Dharmacon Yap no. 7) as indicated. Six hours later, cells were further transfected with 100 ng TAp73-HA, 300 ng of either pcDNA3.1 or c-Jun, as indicated, and with 200 ng pEGFP-C2 as a control for transfection. Twenty-four hours after transfection, total cell extracts were prepared and subjected to Western blot analysis with anti-p73, anti-c-Jun antibodies to assess levels of expression of p73 and c-Jun, and with anti-GFP antibody to ensure changes of expression were not due to differential transfection and/or protein loading. (**f**) Wild-type and c-Jun −/− MEFs retrovirally infected with either pBabe, pBabe-c-Jun or pBabe YAP. Infectants were treated with cisplatin as indicated. Twenty-four hours following treatment, cells were analysed by flow cytometry for apoptosis using Annexin V and PI. All error bars as given as S.E.M.

to empty vector control in a pulse–chase experiment. Whereas expression of Itch enhanced degradation of p73, coexpression of YAP attenuated Itch's effect on p73, especially at early time points where Itch expression had already degraded p73 by greater than 50% (Supplementary Figure 1d). Finally, using recombinant proteins, we see that YAP attenuates the binding of p73 by Itch, indicating that YAP and Itch compete for binding of p73 (Supplementary Figure 1e). Degradation of p73 may be mediated by other E3 ligases besides Itch,[10] as well as by ubiquitin-independent pathways.[11] However, these data indicate that YAP stabilises p73 at least in part by physically interfering with Itch binding and degrading p73.

To assess the role of YAP in c-Jun-mediated cell death, we exposed c-Jun −/− MEFs, which express low levels of YAP (Figure 1d) and are very resistant to cisplatin treatment,[3,4] retrovirally infected with pBabe, pBabe-c-Jun or pBabe-YAP, to a dose response of cisplatin. Expression of YAP in c-Jun −/− MEFs to levels similar to that of wild-type MEFs resulted in cisplatin-induced cell death comparable to those of c-Jun −/− MEFs re-expressing c-Jun and wild-type MEFs (Figure 1f). This shows that c-Jun −/− MEFs can be re-sensitised to cisplatin by

restoration of YAP expression. Interestingly, c-Jun−/−MEFs re-expressing YAP showed a slightly higher background level of apoptosis than the other cell lines, in keeping with our earlier data that overexpression of YAP, even in the absence of DNA damage, can cause apoptosis.[6] This indicates that expression of YAP in c-Jun −/− MEFs primes them for pro-apoptotic signalling. Our data strongly suggest that YAP is a critical component of c-Jun-mediated induction of apoptosis.

In this study, we identify YAP as a novel and critical downstream effector of c-Jun-mediated apoptosis following cisplatin treatment. Our data indicate that YAP contributes to cell death by stabilising p73 protein by competing with the E3 ubiquitin-protein ligase Itch for p73 binding, as seen recently by others.[8] Thus, YAP blocks p73 degradation by Itch. These results further corroborate the importance of YAP in p73 regulation. YAP appears to control p73 function at two levels. Through competing with Itch, YAP regulates the overall levels of p73 protein. Moreover, YAP enhances p73 function by promoting its transcriptional potential,[7] and recruits p73 to promoters of apoptotic genes following apoptotic stimuli, and is also able to promote p300-mediated acetylation of p73.[7] These observations further strengthen the role of YAP in p73-dependent apoptosis and also implicate YAP as an important factor in c-Jun-mediated chemosensitivity. YAP may be a key indicator in predicting response to chemotherapy and thus treatment outcome.

Recently, Whitmarsh and co-workers[12] have indicated that c-Jun N-terminal kinase (JNK), the upstream activator of c-Jun, can regulate p73 stability and activity. Although their study suggests that this is via direct phosphorylation of p73 by JNK, JNK activity may also regulate p73 stability by activating c-Jun to upregulate YAP as outlined above. Further study will be required to assess the relative contribution of both these paths to p73 stability and their possible synergy downstream of JNK activation. Another exciting, recent study has shown that p73 can augment c-Jun transcriptional activity to support cellular growth, demonstrating not only a possible feedback loop back to c-Jun, but also a novel role in proliferation for p73.[13] It remains to be seen if YAP is also required for p73-dependent cellular growth via c-Jun, as well as c-Jun-dependent apoptosis via p73, but it may at least partially explain the proto-oncogenic role of YAP seen in a mouse model of liver cancer.[14]

Our data also raise two interesting questions. First, can YAP mediate and regulate other functions of c-Jun? Our findings demonstrate the importance of YAP in the c-Jun-dependent cisplatin response, but its role in other c-Jun-regulated processes such as proliferation and differentiation will require further elucidation. Interestingly, c-Jun is also a substrate for Itch and is degraded in an Itch-dependent manner in response to T-cell stimulation.[15] It remains to be seen if this also occurs in response to chemotherapy and if YAP is similarly involved in attenuating Itch degradation of c-Jun.

Second, as our observations show that YAP effectively stabilises p73 through competitive binding with Itch, can YAP stabilise other PY motif containing proteins by similar means? Given the diverse plethora of transcription factors whose activities YAP is known to regulate, the possibility of an additional role in maintaining the integrity of these protein by acting as a global competitor of Itch and other WW domain-bearing E3 ligases remains an exciting prospect for further studies.

SA Danovi[1], M Rossi[2], K Gudmundsdottir[1], M Yuan[1], G Melino[2] and S Basu[,1]*

[1] Cell Survival Signalling Laboratory, Centre for Molecular Oncology, Institute of Cancer, Barts and The London School of Medicine and Dentistry, John Vane Science Centre, Charterhouse Square, London EC1M 6BQ, UK and
[2] Medical Research Council, Toxicology Unit, Leicester University, Hodgkin Building, PO Box 138, Lancaster Road, Leicester LE1 9HN, UK
* Corresponding author: S Basu, Cell Survival Signalling Laboratory, Centre for Molecular Oncology, Institute of Cancer, Barts and The London School of Medicine and Dentistry, John Vane Science Centre, Charterhouse Square, London EC1M 6BQ, UK.
Tel: +44 207 014 0422; Fax: +44 207 014 0431;
E-mail: Subham.Basu@cancer.org.uk

1. Shaulian E, Schreiber M, Piu M *et al. Cell* 2000; **103**: 897–907.
2. Hayakawa J, Mittal S, Wang Y *et al. Mol Cell* 2004; **16**: 521–535.
3. Toh WH, Siddique MM, Boominathan L *et al. J Biol Chem* 2004; **279**: 44713–44722.
4. Sanchez-Perez I, Perona R. *FEBS Lett* 1999; **453**: 151–158.
5. Irwin MS, Kondo K, Marin MC *et al. Cancer Cell* 2003; **3**: 403–410.
6. Basu S, Totty NF, Irwin MS, Sudol M, Downward J. *Mol Cell* 2003; **11**: 11–23.
7. Strano S, Monti O, Pediconi N *et al. Mol Cell* 2005; **18**: 447–459.
8. Levy D, Adamovich Y, Reuven N *et al. Cell Death Differ* 2007; **14**: 743–751.
9. Rossi M, De Laurenzi V, Munarriz E *et al. EMBO J* 2005; **24**: 836–848.
10. Oberst A, Rossi M, Salomoni P *et al. Biochem Biophys Res Commun* 2005; **331**: 707–712.
11. Asher G, Tsvetkov P, Kahana C *et al. Genes Dev* 2005; **19**: 316–321.
12. Jones EV, Dickman MJ, Whitmarsh AJ. *Biochem J* 2007; **405**: 617–623.
13. Vikhanskaya F, Toh WH *et al. Nat Cell Biol* 2007; **9**: 698–705.
14. Zender L, Spector MS, Xue W *et al. Cell* 2006; **125**: 1253–1267.
15. Gao M, Labuda T, Xia Y *et al. Science* 2004; **306**: 271–275.

Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)