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YAP Dysregulation by Phosphorylation or ΔNp63-mediated Gene Repression Promotes Proliferation, Survival and Migration in Head and Neck Cancer Subsets*

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Abstract

Over-expression of Yes-associated protein (YAP), and TP53 family members ΔNp63 and p73 with which YAP may serve as a nuclear co-factor, have been independently detected in subsets of head and neck squamous cell carcinomas (HNSCC). Their potential relationship and functional role of YAP in HNSCC are unknown. Here we reveal that in a subset of HNSCC lines and tumors, YAP expression is increased but localized in the cytoplasm in association with increased AKT and YAP phosphorylation, and decreased expression of ΔNp63 and p73. Conversely, YAP expression is decreased but detectable in the nucleus in association with lower AKT and YAP phosphorylation, and increased ΔNp63 and p73 expression, in another subset. Inhibiting AKT decreased Serine-127 phosphorylation and enhanced nuclear translocation of YAP. ΔNp63 repressed *YAP* expression and bound its promoter. Transfection of a YAP-Serine-127-Alanine phosphoacceptor-site mutant or ΔNp63 knockdown significantly increased nuclear YAP and cell death. Conversely, YAP knockdown enhanced cell proliferation, survival, migration, and cisplatin

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Conflicts of Interest

The authors declare no conflicts of interest.

chemoresistance. Thus, YAP function as a tumor suppressor may alternatively be dysregulated by AKT phosphorylation at Serine-127 and cytoplasmic sequestration, or by transcriptional repression by ΔNp63, in different subsets of HNSCC. AKT and/orΔNp63 are potential targets for enhancing YAP-mediated apoptosis and chemosensitivity in HNSCC.

Keywords

YAP; p53; ΔNp63; p73; Apoptosis; Cancer

Introduction

We previously detected increased expression of mRNA encoding Yes-associated protein (YAP) by molecular profiling among genes differentially expressed in both murine skin and a subset of human head and neck squamous cell carcinoma lines (HNSCC) (Dong *et al.*, 2001b; Dong *et al.*, 1997; Lee *et al.*, 2007). Amplification of the chromosomal region that encodes YAP, 11q21-22, is frequently detected in human HNSCC lines and tumors (Carey *et al.*, 1993; Snijders *et al.*, 2005). We also noted differences in intensity associated with cytoplasmic or nuclear distribution of YAP protein among different HNSCC tumor specimens by immunohistochemistry (IHC) (Lee *et al.*, 2007). These observations suggested that altered expression and cellular distribution of YAP could be important in the molecular pathogenesis of HNSCC.

YAP protein consists of two isoforms, containing one or two conserved WW domains, which mediate binding to PPxY motif proteins, including the Src family kinase, Yes, for which it is named (Chen and Sudol, 1995; Sudol, 1994; Sudol *et al.*, 1995). Other important cancer-related binding partners of YAP identified include isoforms of the tumor suppressor TP53 family transcription factors, p63α, p73α and β, but not TP53 itself (Strano *et al.*, 2001). YAP was shown to serve as a co-factor for p73α-p300 mediated target gene transcription of the proapoptotic gene Bax, and p73-dependent apoptosis in response to DNA damage (Strano *et al.*, 2005; Strano *et al.*, 2001). YAP has been reported to function as a transcriptional co-regulator of p73-mediated apoptosis in certain non-malignant and cancer cells (Basu *et al.*, 2003; Danovi *et al.*, 2008; Howell *et al.*, 2004; Levy *et al.*, 2007; Levy *et al.*, 2008; Matallanas *et al.*, 2007; Oka *et al.*, 2008; Strano *et al.*, 2005; Strano *et al.*, 2001; Yuan *et al.*, 2008). Paradoxically, however, YAP has been implicated as an oncogene in other primary or immortalized cells (Baldwin *et al.*, 2005; Overholtzer *et al.*, 2006; Snijders *et al.*, 2005; Zender *et al.*, 2006; Zhang *et al.*, 2008; Zhao *et al.*, 2007).

The basis for differences in function of YAP in these different contexts appears to be complex; potentially involving post-translational modification by phosphorylation by different signal proteins, and interaction with different transcription factors (Bertini *et al.*, 2009; Downward and Basu, 2008). Of potential relevance to HNSCC, phosphorylation of YAP serine-127 by AKT was reported to induce cytoplasmic sequestration by 14-3-3 and attenuation of p73 mediated apoptosis (Basu *et al.*, 2003), and PI3K-AKT activation is prevalent and implicated in pathogenesis of HNSCC (Amornphimoltham *et al.*, 2008; Amornphimoltham *et al.*, 2004; Bancroft *et al.*, 2002; Bian *et al.*, 2009; Massarelli *et al.*,

2005; Molinolo *et al.*, 2007; Moral *et al.*, 2009). Further, p63 and p73 with which YAP has been reported to interact (Strano *et al.*, 2001), includes both pro-apoptotic and anti-apoptotic isoforms (Barbieri and Pietenpol, 2006; Rosenbluth and Pietenpol, 2008), and overexpression of isoform ΔNp63α has been implicated in dysregulation of p73 function, apoptosis, and cell survival in a subset of HNSCC (Rocco and Ellisen, 2006; Rocco *et al.*, 2006). Thus, the role of AKT, ΔNp63, p73 and YAP in dysregulation of apoptosis may be of particular significance in HNSCC, where the tumor suppressor function of TP53 is frequently dysregulated by mutation or inactivation (Forastiere *et al.*, 2001; Friedman *et al.*, 2007).

We hypothesized that differences in YAP expression and cellular distribution we observed among HNSCC cell lines and tumor specimens could be related to alterations in AKT, ΔNp63 and p73, and affect function of YAP in HNSCC.

Results

Increased expression and cytoplasmic distribution of YAP is associated with increased YAP and AKT phosphorylation in HNSCC tissue array and cell lines

Based on previous observations indicating that YAP expression and/or cellular localization varies in murine and human HNSCC (Dong *et al.*, 2001b; Lee *et al.*, 2007), we analyzed YAP expression and distribution patterns in a human tissue microarray with 20 HNSCC and 6 normal mucosa specimens. This revealed two major subsets of tumors: one subset showing high and predominantly cytoplasmic YAP staining (e.g., Fig. 1A, higher resolution Suppl Fig. 1A, left panels), and another showing relatively lower YAP staining with predominantly nuclear localization (e.g., Fig. 1A, Suppl, Fig. 1A, right panels). By a semiquantitative histoscore, higher overall YAP staining intensity was observed in those tumors with a predominantly cytoplasmic pattern (Fig. 1B, upper panel bar graphs). AKT is reported to be a potential modulator of Ser127 phosphorylation and cellular distribution of YAP (Basu *et al.*, 2003), and exhibits increased phosphorylation in a subset of UMSCC lines (Bancroft *et al.*, 2002) and HNSCC tumors (Amornphimoltham *et al.*, 2004). To investigate if increased YAP expression and cytoplasmic distribution is potentially related to YAP and AKT phosphorylation, we compared phospho-AKT (serine-473) and phospho-YAP (serine-127) in the same 20 HNSCC tissue microarray specimens. Increased cytoplasmic YAP in tumor tissue was associated with higher phospho-AKT and phospho-YAP staining (Fig. 1A, left panel; Fig. 1B, lower panel bar graphs). Ordinary least squares analysis revealed a significant relationship between increasing histoscores for phospho-AKTserine-473 and phospho-YAPserine-127 (Supplemental Fig. 2A; $r=0.5$; $p<0.05$).

To determine if HNSCC cell lines reflect and provide a model for study of the differences observed in human tumors, we examined YAP mRNA and protein expression among a panel of 9 UMSCC and a primary keratinocyte cell line (Fig. 1C). Compared to non-malignant human keratinocytes (H), YAP mRNA and protein was increased in UMSCC lines 1-11B, and at relatively lower levels in UMSCC 22A, 22B, 38 and 46 (Fig. 1C; YAP mRNA Expression; Whole Cell Extracts). Similar to HNSCC tumors, increased YAP expression was associated with both higher phospho-YAPserine-127 and phospho-AKTserine 473 levels. Total AKT as well as phospho-AKT was relatively lower in two lines

with lower phospho-YAP, UMSCC 22A and B. Also like HNSCC tumors, most of the UMSCC lines with high overall YAP expression, displayed higher cytoplasmic and lower nuclear YAP, while cells with low overall YAP mRNA and protein expression, displayed relatively greater nuclear YAP (Fig. 1C: Cytoplasmic and Nuclear Extracts). Furthermore, for those with increased overall and cytoplasmic YAP, localization of phospho-YAPserine-127 was enriched in the cytoplasm (Fig. 1D; Cytoplasmic and Nuclear Extracts). The YAP cytoplasmic chaperone 14-3-3 did not differ between the UMSCC cell lines (Supplemental Fig. 2B, lower panels), excluding differences in 14-3-3 expression as a basis for the distribution observed. Immunofluorescent analysis of HNSCC cell lines expressing higher (UMSCC-11A) and lower levels (UMSCC-22B) of YAP (Fig. 1C) revealed a similar pattern of YAP staining intensity and cellular distribution as observed in tumor tissue and subcellular extracts from cell lines (Supplemental Fig. 3).

Pharmacologic AKT inhibition or overexpression of a YAPserine-127 phosphoacceptor mutant enhances nuclear localization of YAP and cell death

To investigate the potential role of AKT activation in the phosphorylation and cellular distributon of YAP, the effect of inhibitor AKT-X previously shown to specifically inhibit both AKT phosphorylation and activation was examined (Thimmaiah *et al.*, 2005). After exposure to 5 μM AKT-X for 1 hour, AKTserine-473 as well as YAPserine-127 phosphorylation was inhibited (Fig. 2A; upper and middle panels). The inhibition of phospho-AKT and phospho-YAP correlated with an increase in detection of YAP in the nucleus, with minimal change in the overall cytoplasmic levels of YAP (Fig. 2A; middle and lower panels). To further investigate if the limited nuclear YAP detected in cell lines with high YAP expression was due to the serine-127 phosphorylation, we transfected UMSCC-11A cells with expression vectors encoding YAP2 or YAP2-S127A mutant proteins, since YAP2 with a second WW domain can be distinguished from endogenous YAP1 by its slower mobility (Fig. 2B). An ~50% increase in nuclear localization of YAP protein was seen with mutant compared to wild-type protein when normalized to OCT-1 (Fig. 2B). We assessed the functional effect of increased nuclear S127A mutant YAP2 on viability of UMSCC cells by DNA cell cycle analysis of sub-G0/G1 fragmented DNA (a measure of % Cell Death) three days post-transfection with control vector (CV), Y2 or Y2M (Figure. 2C). The increase in nuclear YAP2 mutant was accompanied by a relatively greater increase in cell death compared with empty control or wt YAP vectors, indicating that with phospho-site inactivation and increased nuclear localization, transfected YAP2 may function as a proapoptotic factor.

YAP and p53-family members ΔNp63 and p73 are differentially expressed in an inverse pattern in subsets of HNSCC cell lines, tumors, and mucosal epithelia

The cell lines with higher YAP mRNA and protein expression in Fig. 1C were previously found to be of wild-type *TP53* genotype but weakly expressing TP53 protein (UMSCC-1, 6, 9, and 11A), or a functionally deficient mutant (mt) TP53 protein (UMSCC-11B) (Friedman *et al.*, 2007) (Fig. 3A). In contrast, those with lower YAP expression in Fig. 1C were found to overexpress mutant TP53 (Friedman *et al.*, 2007) (Fig. 3A). Examining the expression of other p53-family members in UMSCCs revealed lower or undetectable expression of p63, p73 and wt TP53 together in one subset, while p63, p73 and mt TP53 were strongly

expressed together in the other subset of UMSCC lines (Fig. 3A). The identities of major p63 and p73 isoforms detected in UMSCC lines included ΔNp63α and TAp73, as determined by qRT-PCR with specific primers; by comparison with electrophoretic mobilities of over-expressed plasmids containingΔNp63α TAp63α and TAp63γ; and by blotting with TAp73 specific antibody (H. Lu, unpublished data, not shown).

To examine if the expression patterns of YAP, ΔNp63, p73 and/or TP53 detected *in vitro* are observed in HNSCC specimens *in situ*, we performed an IHC staining of YAP, ΔNp63, p73 and TP53 in a panel of frozen human HNSCC and squamous mucosal tissue specimens (Fig. 3B). The strong and predominately cytoplasmic pattern of YAP staining was associated with relatively weak ΔNp63 and p73 staining in a subset of tumors (I–III). The relatively weak, but predominantly nuclear YAP staining pattern was observed together with strong ΔNp63 and p73staining in another subset (IV–VI). While the inverse relationship between YAP and p63/p73 family members seen in UMSCC lines was observed in these tumor specimens, no clear association with TP53 immunostaining was observed in tumor specimens. A similar pattern was observed in different hyperplastic non-malignant mucosa (Fig. 3B, right panels, compare HNSCC I–III and mucosa I; HNSCC IV–VI and mucosa II). Together, the data suggest that the expression and cytoplasmic distribution of YAP is often inversely associated with p63 and/or p73 in subsets of HNSCC and hyperplastic squamous mucosa.

ΔNp63 inhibits YAP expression, binds the *YAP* promoter, and suppresses cell death

To examine if the apparent inverse relationship between YAP and ΔNp63/p73 expression observed was potentially due to repression of YAP expression by ΔNp63 and/or p73, we explored the effects of siRNA knockdown of p63 isoforms or p73 on YAP expression in UMSCC-11A, 6, or 22B. In pilot experiments, ≥50% inhibition of targeted mRNA isoforms was observed after ΔNp63, TAp63, total p63 or p73 siRNA knockdown (Suppl Fig. 4A–C, upper panels). ΔNp63 knockdown resulted in a marked increase in YAP mRNA in UMSCC-11A, 6 and 22B at 48 h (Supplemental Fig. 4A–C, lower panels). Knockdown of p73 had a relatively weaker but detectable effect in enhancing YAP expression (Suppl. Fig 4A, C). After ΔNp63 knockdown, UMSCC-11A and UMSCC-6 lines both displayed a greater increase in YAP expression when compared to UMSCC-22B (Suppl Fig. 4D).

Further experiments were conducted in UMSCC-11A, since this line expressing ΔNp63 at an intermediate level (Fig. 3A) could be used for both knockdown and over-expression experiments with high transfection efficiencies. ΔNp63 knockdown resulted in a greater increase of YAP mRNA expression when compared to TAp63 knockdown during days two and three post-treatment (Fig 4A). This was consistent with detection of only the ΔNp63 isoform protein by western in UMSCC lines by us (Fig. 3A, H. Lu, data not shown) and in other HNSCC lines by independent investigators (Rocco *et al.*, 2006). ΔNp63 knockdown also had a greater effect on YAP protein expression at three days post-treatment, and particularly in increasing the proportion of a faster migrating band that potentially represents non-phosphorylated YAP capable of enhanced nuclear distribution (Fig. 4B, middle lane). Based on the effect of ΔNp63 knockdown in increasing YAP expression, we hypothesized that ΔNp63 may serve as a negative regulator for YAP gene expression. To further investigate this possibility, we also over-expressed either ΔNp63 or TAp63 in UMSCC-11A.

We observed a significantly greater decrease in *YAP* mRNA after expression of ΔNp63 than TAp63 (Fig 4C). Further support for direct transcriptional repression of YAP by ΔNp63 was obtained by demonstration of p63 binding to two regions of the *YAP* gene promoter containing predicted p63 binding sites by chromatin immunoprecipitaton (ChIP) assay (Fig. 4D; Supplemental Fig 5). Thus, our data are consistent with a regulatory interaction underlying the inverse relationship between YAP and ΔNp63 expression observed in UMSCC cell lines and tumors, specifically, the knockdown or overexpression results which establish ΔNp63 as a repressor of *YAP* gene expression.

Since ΔNp63 is the dominant isoform, is able to repress YAP expression, and p63 has also previously been shown to bind to YAP and p73 and hinder their pro-apoptotic function (Basu *et al.*, 2003; Rocco *et al.*, 2006), we compared the effects of knockdown of ΔNp63, via total p63 siRNA, with that of transfection of YAP2S127A mutant vector on apoptosis. Knockdown of ΔNp63 or transient expression of mutant YAP vector both increased cell death by a similar increment (Fig 4E). No significant increase in cell death was observed by combining upregulation of YAP2S127A with ΔNp63 knockdown (Fig 4E). The knockdown of ΔNp63 resulted in an increase in annexinV and propidium iodide double-positive cells, consistent with cell death by apoptosis (Fig. 4F). Knockdown of ΔNp63 over a longer term significantly decreased cell density in 5 day MTT assay (XP Yang, data not shown).

YAP knockdown promotes cell proliferation, survival, migration and cisplatin chemotherapy resistance

To directly characterize the functional role of endogenous YAP, we inhibited YAP gene expression by siRNA in UMSCC-11A cells. Efficient inhibition of YAP mRNA was observed for up to four days, and knockdown of YAP protein was also confirmed three days post-transfection (Fig. 5A). YAP inhibition was accompanied by effects on important genes implicated in the malignant phenotype of HNSCC (Fig. 5B), including a significant decrease in mRNA expression of p53/p73 targets *p53AIP1* and *p21*, which function to promote apoptosis and inhibit cell growth, as well as an increase in the expression *BCL-XL* and *VEGF*, which promote cell survival and angiogenesis, respectively (Bancroft *et al.*, 2002; Lee *et al.*, 2008; Oda *et al.*, 2000).

Upon YAP siRNA knockdown, a relative increase in proliferation was observed, indicating that YAP has residual anti-proliferative and/or pro-apoptotic functional activity even in UMSCC11A cells which express relatively low levels of p73 and nuclear YAP (Fig. 5C). The pro-apoptotic function of YAP was supported by a decrease in the percentage of sub-G0/G1 fragmented DNA (% cell death) and annexinV and propidium iodide double positive cells (% apoptosis) (Fig. 5D). An *in vitro* wound healing assay revealed a significant increase in the rate of wound closure, also implicating YAP as a potential inhibitor of cell migration (Fig. 5E). Inhibiting YAP expression also decreased the sensitivity of the cells to the DNA damaging agent cisplatin (Fig. 5F). Taken together, these data indicate that YAP expressed in UMSCC cell lines inhibits cell proliferation, survival, migration and enhances cisplatin chemosensitivity.

Discussion

Here, we show that in HNSCC where YAP is overexpressed, it is predominantly located in the cytoplasm, while in tumors expressing lower levels, it is predominantly detected in the nucleus. An independent survey of YAP expression in four cancer types suggests that similar subsets may exist within other cancers of the same histologic classification (Steinhardt *et al.*, 2008). Here, we provide evidence that in HNSCC these different patterns of YAP expression and cellular distribution are linked with increased AKT and YAP phosphorylation orΔNp63 and p73 overexpression in subsets of tumors and cell lines (Figs. 1, 3). Where YAP expression is increased, it is predominantly detected in the cytoplasm in HNSCC exhibiting increased AKT and YAP phosphorylation, but relatively low levels of ΔNp63 and p73. By contrast, YAP expression was markedly reduced but was detectable in the nucleus in another subset of tumors and lines with lower AKT and YAP phosphorylation, but increased ΔNp63 and p73. This previously unrecognized relationship suggested that alternative mechanisms may predominantly contribute to regulation and function of YAP among these major subsets of HNSCC tumors and cell lines.

Cytoplasmic sequestration of YAP by 14-3-3 and inactivation as a co-factor for p73-mediated apoptosis was previously shown to involve phosphorylation of YAPser127, and YAP was reported to be a direct substrate of AKT in cells and by kinase assay of recombinant proteins *in vitro* (Basu *et al.*, 2003). In HNSCC, we found a strong relationship between increased AKTser473 and YAPser127 phosphorylation, and cytoplasmic distribution in a subset of both tumors and cell lines (Fig. 1). Further, a selective AKT inhibitor, AKT-X, inhibited YAPser127 phosphorylation and enhanced nuclear YAP in HNSCC lines (Fig. 2). Further supporting the hypothesis that AKT-dependent phosphorylation of YAPser127 contributes to the predominant sequestration of YAP in the cytoplasm and attenuation of apoptosis, expression of a YAP S127A phospho-acceptor mutant enhanced nuclear YAP and apoptosis. Consistent with these findings, we have shown that increased activation and phosphorylation of the PI3K and AKT signal axis in HNSCC may occur as a result of overexpression and activation of the Epidermal Growth Factor Receptor (EGFR), Hepatocyte Growth Factor Receptor (c-MET) or their ligands (Bancroft *et al.*, 2002; Dong *et al.*, 2001a). Further, inhibitors of PI3K-AKT were potent inhibitors of growth (Bancroft *et al.*, 2002). Activation of the PI3K-AKT pathway has subsequently been strongly linked with development, cell survival and decreased prognosis of HNSCC (Amornphimoltham *et al.*, 2008; Amornphimoltham *et al.*, 2004; Bian *et al.*, 2009; Massarelli *et al.*, 2005; Molinolo *et al.*, 2007; Moral *et al.*, 2009). However, a role for AKT in cytoplasmic inactivation of YAP as a contributing mechanism to cell survival in a subset of HNSCC as described here, has not been previously reported.

In the same subset where YAP is most strongly expressed in the cytoplasm, decreased expression of members of the p53-family may also represent important co-determinants of dysfunction of YAP and apoptosis. The significant decrease in expression of both TP53 and p73 (Fig. 3) represents a further significant deficiency and compromise of the necessary components of the p73/p53 pathways critical for induction of apoptosis. YAP has previously been shown to be an important factor in preventing the degradation of p73 (Levy *et al.*, 2007; Levy *et al.*, 2008; Strano *et al.*, 2005), hence study of how YAP cytoplasmic

sequestration contributes to decreased p73 protein levels observed in this subset of UMSCC lines and HNSCC tumor specimens is warranted.

Protein-protein interactions between ΔNp63, p73 and/or YAP have been reported, and suggest that ΔNp63 can inhibit p73-YAP function (Strano *et al.*, 2001; Rocco *et al*., 2006). We also examined the possibility that ΔNp63 and p73 overexpression potentially mediate negative feedback inhibition of expression of their co-factor. Supporting this hypothesis, knockdown of ΔNp63, and to a lesser extent, p73, enhanced YAP mRNA and protein expression, while overexpression of ΔNp63 repressed *YAP* mRNA expression. Analysis of the YAP promoter revealed p63 binding sites, and p63 was detected to bind to the regions of the YAP promoter containing these sequences, supporting ΔNp63 as a transcriptional regulator of YAP. Expression of other key growth arrest and apoptotic genes are inhibited by ΔNp63, including *p21Cip1*, *NOXA* and *PUMA* (Rocco and Ellisen, 2006; Rocco *et al.*, 2006; Westfall *et al.*, 2003), indicating *YAP* may be one of several important tumor suppressor genes repressed by overexpression of ΔNp63 in this HNSCC subset. These findings indicate that further analysis for common mechanisms of interaction of ΔNp63, p73 and YAP on the promoter of *YAP* and these other genes are warranted.

While ΔNp63 is reported to repress expression of YAP and other proapoptotic genes, promoting survival of HNSCC (this study; Rocco *et al.*, 2006), other reports suggest p63 may repress other features of the malignant phenotype that affect prognosis of SCC. p63 is principally immunolocalized in the basilar layer as in Fig. 3B, where it plays a role in regulating both regeneration and differentiation of normal squamous epithelia (reviewed in Barbieri & Pietenpol, 2006). p63 depletion by siRNA targeting total p63 was linked with modulation of multiple genes that enhanced malignant conversion, invasiveness and epithelial-mesenchymal-transition of immortalized keratinocyte and HNSCC lines (Barbieri *et al.*, 2006). Thus, the differential expression of p63 and phenotype of SCC in HNSCC subsets could reflect origination from p63 positive or negative progenitors within the epithelia, or different stages of invasive SCC. However, we find that with specific knockdown of ΔNp63, migration is inhibited (Yang et al, manuscript in preparation), consistent with the inhibitory effects of YAP (Fig. 5E).

The role of YAP as a tumor suppressor in HNSCC was directly supported by the finding that increased YAP S127A mutant or endogenous YAP after ΔNp63 knockdown enhanced apoptosis, while YAP siRNA knockdown enhanced proliferation, survival, migration, and resistance to the chemotherapeutic agent cisplatin. Consistent with these observations, overexpression of YAP has recently been implicated to enhance induction of apoptosis and detachment (Oka *et al.*, 2008), while knockdown of YAP suppressed anoikis, increased migration and invasiveness, inhibited the response to taxol, and enhanced tumor growth in nude mice (Yuan *et al.*, 2008). With regard to chemotherapy sensitivity, YAP has been reported to be a critical component of DNA damage induced cell death (Basu *et al.*, 2003; Danovi *et al.*, 2008; Levy *et al.*, 2007; Oka *et al.*, 2008; Strano *et al.*, 2005; Strano *et al.*, 2001). In addition, YAP has been reported to positively influence the expression of pro-apoptotic proteins BAX and PIG3 (Howell *et al.*, 2004; Levy *et al.*, 2008; Strano *et al.*, 2001) and p53AIP1 (Oda *et al.*, 2000), that play important roles in mediating either TP53- or p73-dependent apoptosis.

The role of YAP seems likely to be dependent on the molecular and tissue context, since there is now considerable evidence that YAP can serve a pro-oncogenic function in other non-malignant and malignant cell types (reviewed in Bertini *et al.*, 2009). In these contexts, cytoplasmic homeostasis of YAP, and its drosophila orthologue Yorkie (Yki), was shown to be regulated by a conserved pathway that includes MST1/2/Hippo and LATS1/2/Wrts kinases, which when mutated or physiologically inactivated, result in reduced phosphorylation and nuclear translocation of YAP/Yki, and promotion of tissue overgrowth or tumorigenesis. Further, several studies provided evidence that LATS1/2, but not AKT, directly phosphorylates YAPser127. Additionally, YAP is reported to associate with and undergo tyrosine phosphorylation by the YES/SRC family kinases, promoting interaction with transcription factor RUNX2 and osteoblastogenesis, or c-ABL, promoting interaction with p73, and apoptosis. Together, these observations suggest that YAP is a target and co-factor for several signal-activated transcription factors with opposing functions that are critical in cell survival. Hopefully, better understanding of the functions of YAP in different experimental systems will emerge from characterization of the net effects of upstream signaling that modulate YAP phosphorylation and localization, and alterations in TP53/ΔNp63/p73 that affect YAP expression or apoptosis, which together may determine YAP function.

In summary, we identified at least two subsets of HNSCC with predominantly different mechanisms of dysregulation of YAP tumor suppressor function (Fig. 6). The mechanisms of dysregulation include AKT-dependent serine-127 phosphorylation and cytoplasmic sequestration, and a previously unreported mechanism, wherein overexpression of ΔNp63 represses YAP gene expression. Targeting YAP phosphorylation or ΔNp63 may enhance YAP nuclear expression, inhibit cell growth and migration, and enhance apoptosis and chemosensitization in HNSCC.

Materials and Methods

Cell lines

UMSCC cell lines (from Dr. T.E. Carey; University of Michigan, Ann Arbor, MI) were previously shown to reflect molecular and phenotypic alterations important in the pathogenesis of HNSCC (Chen *et al.*, 1999; Dong *et al.*, 2001a; Lee *et al.*, 2006; Worden *et al.*, 2005; Yan *et al.*, 2007; Yu *et al.*, 2006). Normal human epidermal keratinocytes (HEKA) were purchased from Invitrogen (Carlsbad, CA). Cells were cultured and used as previously described (Chen *et al.*, 1999; Yan *et al.*, 2007).

Plasmids, siRNA and drug treatment

Human YAP expression vectors were constructed as previously described (Komuro *et al.*, 2003). The siRNAs specifically targeting YAP and TAp63 were from ON-TARGET plus of SMART pool selection (Dharmacon, Lafayette, Colorado). The sequence of p63 siRNA targeting the unique exon in the N-terminal of ΔNp63 was as previously published (Thurfjell *et al.*, 2005), and was synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The knockdown specificities and efficiencies were confirmed (Yang et al, manuscript in preparation). Transfections were conducted with Lipofectamine 2000 (Invitrogen) per

manufacturer's instructions. Cisplatin (Bedford Laboratories) was used as indicated. AKT inhibitor X (AKT-X, Calbiochem) used is 10-(4′-(N-diethylamino)butyl)-2-chlorophenoxazine, HCl; compound 10B, (Thimmaiah *et al.*, 2005).

Immunohistochemical staining of HNSCC tissue specimens

A tissue microarray with HNSCC and normal mucosa described in Suppl Table 1 was stained for H&E, pan-cytokeratin, YAP, p-YAP and p-AKT. Antibodies and methods used for immunostaining of the TMA and frozen HNSCC specimens for YAP and p53 family members are described in Supplemental Methods. IHC histoscores based upon the products of 0–3+ stain intensity multiplied by percentage positive cells per 100 in three different fields for each tumor section are described in Supplemental Methods.

Western blot

Western blot analysis was performed as previously described (Friedman *et al.*, 2007) with primary antibodies listed in supplemental methods.

Real-time reverse transcription-PCR (QRT-PCR)

RNA isolation, cDNA synthesis and QRT-PCR were performed as previously described (Lee *et al.*, 2006), as specified in supplemental methods.

Chromatin Immunoprecipitation Assay (ChIP)

ChIP assays were performed using the EZ ChIP assay kit (Upstate Biotechnology, Waltham, MA) according to manufacturer's protocol, and supplemental methods.

MTT cell proliferation assay

Cells were trypsinized 24hrs post-siRNA transfection, transferred to a 96-well plate in quadruplicate. Treatment and/or analyzed 24 post-plating using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation kit (Roche Diagnostics, Indianapolis, IN).

Cell cycle and apoptotic analysis by flow cytometry

Cells were processed as per Cycletest Plus DNA Regent Kit (BD Biosciences, San Jose, CA) protocol or as per Annexin V- FITC and PI Apoptotic Kit (Invitrogen) manufacturer's instructions. All samples run on a FACS Canto analyzer, and data processed with Diva (BD Biosciences) or Flow-Jo (Tree Star Inc., Ashland, Oregon).

Wound/Scratch migration assay

Forty eight hours post-siRNA transfections wounds were made through confluent cell sheets. Measurements at preset distances on the wound were averaged and wound healing was quantified relative to the control siRNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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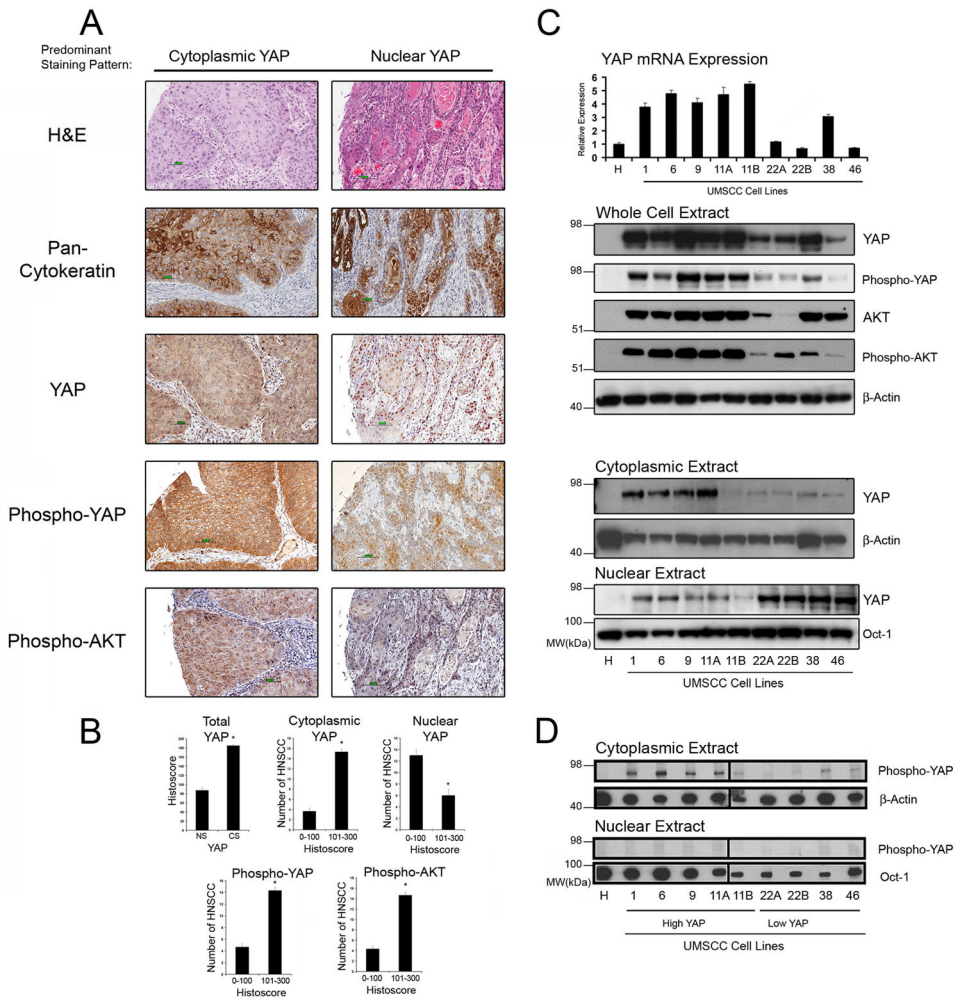

Figure 1. Predominantly cytoplasmic versus nuclear YAP localization is associated with YAP (Ser127) and AKT (Ser473) phosphorylation in HNSCC tumor and cell line subsets

(A,) Comparison of two HNSCC specimens representing predominantly cytoplasmic and nuclear YAP distribution, stained for Hematoxylin and Eosin (H&E), Pan-Cytokeratin, YAP, phospho-YAP (Ser127) and phospho-AKT (ser473). Sections from specimens #4 and #13 of a 20 HNSCC specimen tissue microarray (TMA) are shown (Suppl. Table 1). Original magnification 400X, shown at higher resolution with 100μM bars in Suppl. Fig. 1. (B.) Upper panels, semi-quantitative average histoscore staining intensity for total YAP, and number with lower and higher cytoplasmic and nuclear YAP histoscores from a 20 HNSCC specimen TMA. Lower panels: number of tumor samples with low or high phospho-AKT and phospho-YAP (serine 127) histoscores. Mean +/− SD. * indicates statistical difference (student t-test, $p<0.05$). (C) Upper panels: Increased YAP mRNA and protein is associated with increased phospho-YAP, AKT and phospho-AKT in whole cell extracts of HNSCC lines (UMSCC) compared with normal human keratinocytes (H). Lower panels: Predominantly cytoplasmic or nuclear distribution of YAP is associated with above expression of YAP, p-YAP and p-AKT. (D) Phospho-YAP is predominantly sequestered in the cytoplasmic extracts of UMSCC with high YAP. Cytoplasmic and nuclear extracts were probed for phospho-YAP (Ser127). For C, D, β-actin was used as a loading control for

whole and cytoplasmic extracts. Nuclear OCT-1 was used as loading control for nuclear extracts. The UMSCC panel included lines with deficient wild-type p53 expression (1, 6, 9, 11A), a functionally deficient mt p53 (11B) and mt p53 (UMSCC 22A, 22B, 38, 46).

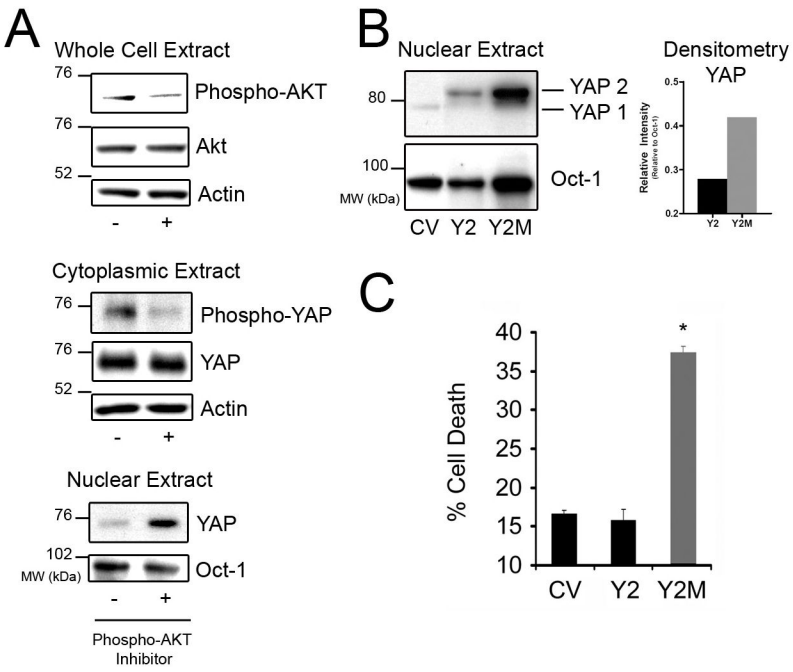

Figure 2. AKT inhibition or overexpression of a YAP serine 127 phosphoacceptor mutant enhances nuclear localization of YAP and cell death

(A) Western blot of whole cell, cytoplamsic, and nuclear extracts of UMSCC 11A cells following no treatment (−) or with AKT inhibitor AKT-X (5 μM) for 1 hour (+). Whole cell extract was probed for phospho-AKT (Ser473) and total AKT, with actin as a loading control. Cytoplasmic extract was probed for phospho-YAP (Ser127) and total YAP with actin as a loading control. Nuclear extract was probed for total YAP with Oct-1 as a loading control. (B) Western blot of nuclear extract from UMSCC 11A shown three days post-transfection with wild-type YAP 2 vector (Y2) or phosphoacceptor-site mutant YAP2 vector (Y2M). Nuclear extract was probed for total YAP with Oct-1 as a loading control. Densitometry measurements adjusted for loading revealed a quantitative increase nuclear localization of 50% with the Y2M vs. Y vector. (C) DNA cell cycle analysis of the percentage of Sub-G0/G1 cells (% Cell Death) three days post-transfection with control vector (CV), Y2 or Y2M.

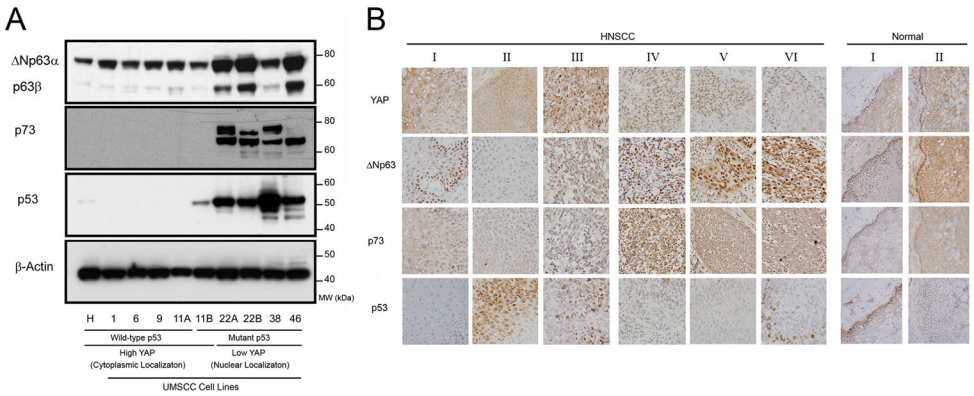

Figure 3. Low expression and nuclear localization of YAP is associated with high p53-family member expression in HNSCC tissue specimens and UMSCC cell lines

(A) Western blot probed for p63, p73, and p53 protein expression in whole cell extracts of a panel with human keratinocytes (H) and HNSCC with wild-type p53 (UMSCC 1, 6, 9, 11A), a functionally deficient mt p53 (UMSCC 11B) and mt p53 (UMSCC 22A, 22B, 38, 46) expression. UMSCC 1, 6, 9, 11A, and 11B displayed high overall YAP expression while UMSCC 22A, 22B, 38, and 46 displayed low overall YAP expression (Fig. 1C). β-actin was used as a loading control. (B) Frozen tissue sections of HNSCC from six patients (HNSCC I-VI) and normal hyperplastic tissue for two patients (Normal I–II) immunostained for YAP, ΔNp63, p73, and p53 (Original magnification, 400X).

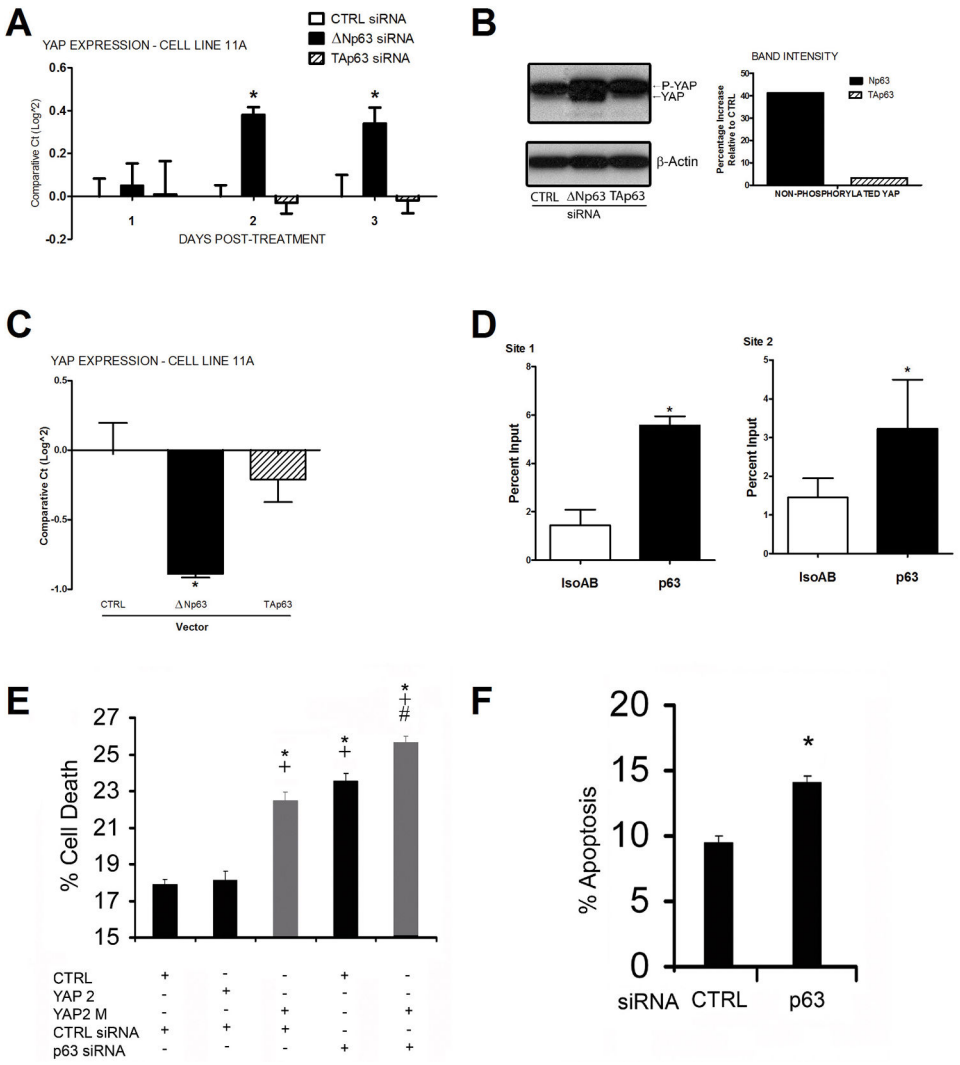

Figure 4. ΔNp63 negatively regulates YAP expression and inhibits programmed cell death

(A) Effect of ΔNp63 and TAp63 siRNA one, two, and three days post-treatment on YAP mRNA expression by QRT-PCR. (B) Left panel, Western blot of whole cell extract from UMSCC 11A three days post-transfection of indicated siRNA. Right panel, Densitometry results of the band representing unphosphorylated YAP adjusted to loading and relative to control (CTRL) siRNA (right panel). (C) QRT-PCR of YAP expression two days post-transfection of CTRL, ΔNp63 and TAp63 vectors in UMSCC 11A cells. (D) p63 binding to predicted p63 binding sites on the YAP promoter (Suppl. Fig. 5) were detected by ChIP analysis using anti-p63 versus isotype control antibody. Mean +/− SD. * indicates statistical difference (student t-test, $p<0.05$). (E) DNA cell cycle analysis of percent sub-G0/G1 DNA (% Cell Death), two days post-transfection with indicated vectors and/or siRNA. * indicates statistical difference (student t-test, $p < 0.05$) vs. control transfections; + indicates statistical difference vs. YAP2 transfection ($p<0.05$); # indicates statistical difference vs. YAP2 M or p63 siRNA transfection ($p<0.05$). (F) Flow cytometric analysis of changes in the percentage of cells undergoing apoptosis two days post-treatment with CTRL or p63 siRNA, as

revealed by an increase in annexinV and propidum iodide double positive cells. Mean +/− SD. * indicates statistical difference (student t-test, $p<0.05$).

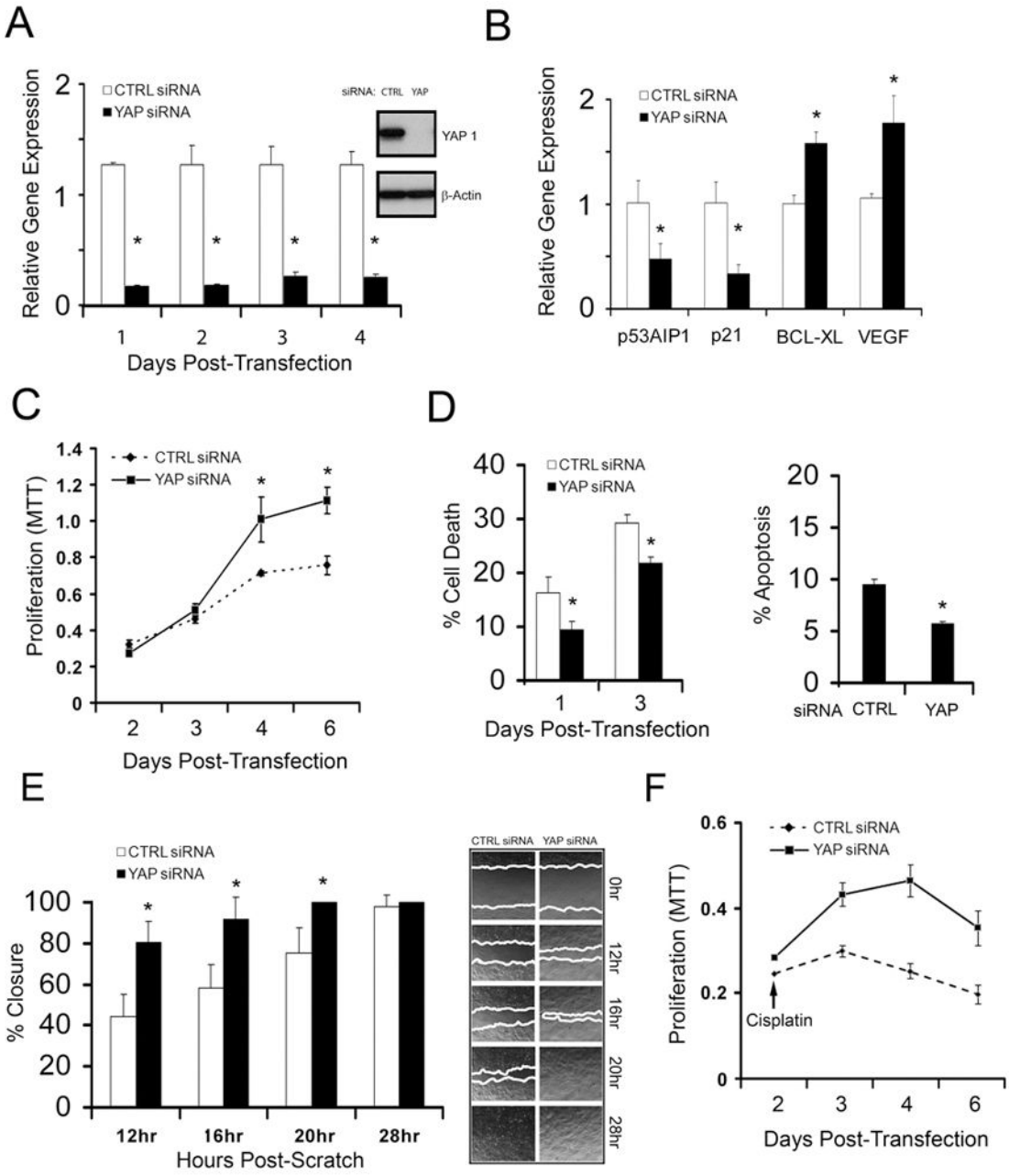

Figure 5. Knockdown of YAP alters gene expression, increases cell proliferation and migration, and decreases programmed cell death and cisplatin cytotoxicity

(A) QRT-PCR demonstrates knockdown efficiency of YAP mRNA days 1–4, and YAP protein (inset) day 3 post transfection of UMSCC 11A with YAP siRNA. (B) QRT-PCR probe for pro-apoptotic *p53AIP* and *BAX*, anti-apoptotic *BCL-XL*, and pro-angiogenic *VEGF* genes two days post-YAP knockdown. (C) MTT assay showing increased density of UMSCC-11A after knockdown of YAP. (D) Left panel: DNA cell cycle analysis of the percentage of sub-G0/G1 DNA (% Cell Death) by UMSCC-11A one and three days post-YAP siRNA treatment. Right panel: Flow cytometric analysis of the percentage apoptosis as indicated by the percentage of annexinV and propidium iodide double positive cells, two

days post-transfection with indicated siRNA. (E) Wound healing/cell migration time course assay of the effects of YAP knockdown in UMSCC 11A. Left panel: measurements of the scratch/wound, using borders highlighted by the white lines in the timecourse images (right panel). (F) MTT assay of UMSCC-11A cell density after knockdown of YAP and treatment with 5 μM of cisplatin. * indicates significant difference (student t-test, $p < 0.05$).

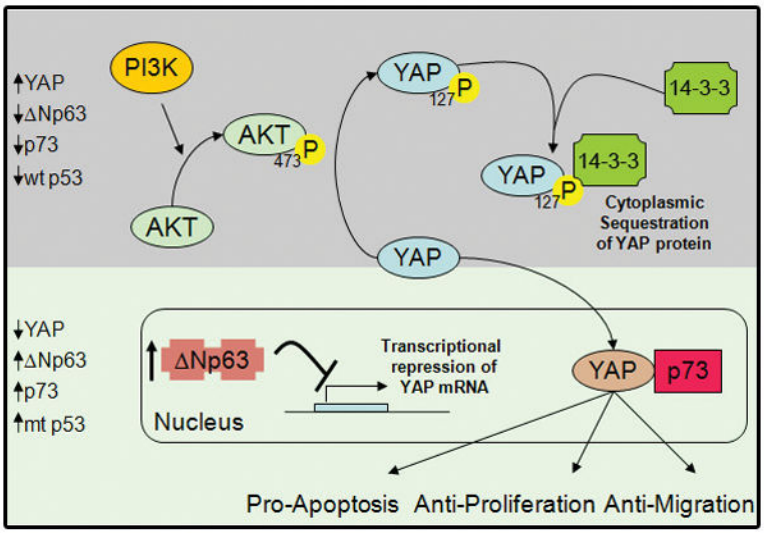

Figure 6. Model of YAP dysregulation in HNSCC

The HNSCC subset expressing high overall YAP and decreased ΔNp63, p73 −/+TP53 exhibits serine-127 phosphorylation and cytoplasmic sequestration of YAP modulated by activated AKT. HNSCC exhibiting decreased overall YAP expession overexpress ΔNp63, p73 −/+TP53, and transcriptional repression of YAP by ΔNp63. DNp63 has also been reported to interact and inhibit p73 finction (Rocco *et al.*, 2006).