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ACTL6A is co-Amplified with *p63* in Squamous Cell Carcinoma to Drive YAP Activation, Regenerative Proliferation and Poor Prognosis

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SUMMARY

Loss-of-function mutations in SWI/SNF chromatin remodeling subunit genes are observed in many cancers, but an oncogenic role for SWI/SNF is not well established. Here we reveal that *ACTL6A*, encoding a SWI/SNF subunit linked to stem and progenitor cell function, is frequently co-amplified and highly expressed together with the p53 family member *p63* in head and neck squamous cell carcinoma (HNSCC). ACTL6A and p63 physically interact, cooperatively controlling a transcriptional program that promotes proliferation and suppresses differentiation, in part through activation of the Hippo-YAP pathway via regulators including *WWC1.* Ectopic ACTL6A/p63 expression promotes tumorigenesis, while *ACTL6A* expression and YAP activation are highly correlated in primary HNSCC and predict poor patient survival. Thus, ACTL6A and p63 collaborate as oncogenic drivers in HNSCC.

Graphical abstract

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AUTHOR CONTRIBUTIONS

S.V.S., M.K., P.R.T., and H.M. conducted experiments, conceived and designed experiments, and interpreted data.
S.V.S., K.R., and S.R. performed bioinformatic analysis and data interpretation.
L.W.E., J.R. and S.R. conceived and designed experiments, interpreted data, and provided funding.
L.W.E. wrote the manuscript. All authors approved the final submitted manuscript.

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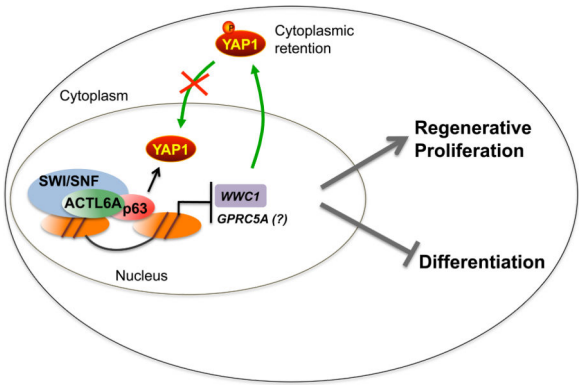

Keywords

squamous cell carcinoma; p63; SWI/SNF (BAF) complex; chromatin remodeling; Hippo pathway; HNSCC

INTRODUCTION

Squamous Cell Carcinoma is an aggressive malignancy arising within the stratified epithelium of the skin, lung, esophagus, and upper aerodigestive tract (so-called head and neck SCC, HNSCC). Histologically, while most proliferating cells within SCC resemble undifferentiated basal epithelia, a remarkable feature of these tumors is variable degrees of ongoing terminal differentiation and growth arrest, recapitulating that observed in the normal stratified epithelium (Leemans et al., 2011). The degree of differentiation observed in individual tumors is a strong prognostic indicator, with less differentiation being associated with worse patient outcomes (Hou et al., 2015; Pai and Westra, 2009). These observations imply that aberrant program(s) enforcing stem-like regenerative proliferation and blocked differentiation are fundamental drivers of squamous tumors (Qian et al., 2015). However, the molecular underpinnings of this aberrant program remain to be elucidated.

Among the most frequent somatic alterations in HNSCC involve the p53 family of transcription factors. Inactivating mutation of *p53* (*TP53*) is the most common somatic genetic event observed in HNSCC (Agrawal et al., 2011; Stransky et al., 2011). Conversely, genomic amplification of *p63* (*TP63*) is observed in up to 30% of tumors, with overexpression observed in the vast majority of cases (Cancer Genome Atlas, 2015; Pickering et al., 2013). P63 is a master regulator of epithelial development and maintenance whose expression is high in normal basal epithelia but declines with progressive differentiation (Moll and Slade, 2004). Deletion of *p63* or specifically N-terminally truncated ΔNp63 isoforms during embryogenesis results in perinatal lethality and a dramatic absence of skin and limbs, owing to defects in regenerative proliferation and differentiation (Mills et al., 1999; Romano et al., 2012; Yang et al., 1999). Moreover, while *p63* deletion in the adult epithelium induces senescence only after a period of weeks to months, its deletion in established, autochthonous SCC induces dramatic tumor regression within a period of days (Keyes et al., 2005; Ramsey et al., 2013). Additionally forced expression of ΔNp63α, the major p63 isoform present in tumors and normal epithelia, is sufficient to bypass

senescence and drive stem-like proliferation and tumorigenesis (Ha et al., 2011; Keyes et al., 2011). Collectively, these findings speak to an exquisite dependence of tumors on high levels of ΔNp63α. Whether this profound p63-dependence reflects a quantitative versus qualitative difference in transcriptional regulation between tumor and normal cells is not known.

Recent work has uncovered disruption of ATP-dependent chromatin remodeling complexes as a pivotal event in cancer pathogenesis (Hohmann and Vakoc, 2014; Kadoch and Crabtree, 2015). For example, genes encoding the catalytic ATPase subunits of the SWI/SNF (BAF) complex, *SMARCA4* (*BRG1*) and *SMARCA2* (*BRM*), are silenced or mutated in many cancers. Additionally, other subunit genes of the SWI/SNF complex including *ARID1A* (*BAF250*) are mutated frequently in certain carcinomas (Lawrence et al., 2014). While these data support a tumor suppressor role for this complex in some contexts, other data point to a potential oncogenic function for deregulated chromatin remodeling. Most notably, the SWI/SNF subunit gene *SS18* is involved in a chromosomal translocation with *SSX*, creating an SS18-SSX fusion protein that functions as an oncogenic driver in 100% of synovial sarcomas (Kadoch and Crabtree, 2013). Taken together, these observations suggest the importance of tumor and tissue-specific context for deregulation of the epigenome in cancer. By and large, however, the precise targets and mechanisms downstream of SWI/SNF deregulation have remained elusive.

Here we perform a genome-wide analysis of p63-mediated transcription in HNSCC to uncover programs regulated by p63 in tumors versus bulk normal epithelial cells. Based on integrated analysis we then sought to explore the mechanistic basis of transcription controlled collaboratively by p63 and the chromatin remodeling factor ACTL6A (BAF53A) in HNSCC, to define the downstream effector genes and programs controlled by these factors, and to test the potential contribution of ACTL6A as an oncogenic driver in this disease.

RESULTS

P63 Controls a Unique Transcriptome in Squamous Tumors

In order to establish the direct transcriptional regulatory mechanisms of p63 in HNSCC, we first assessed genome-wide p63 binding by chromatin immunoprecipitation/high-throughput sequencing (ChIP-seq) analysis of endogenous p63 in HNSCC cells. We then compared results of this analysis to p63 ChIP-seq studies performed in other SCC cell lines and in normal primary human foreskin keratinocytes (HFK) (McDade et al., 2014; Watanabe et al., 2014). Analysis of enriched loci (peaks) indicating p63 binding revealed, as expected, a large number of shared peaks among all p63-expressing cells (Figures 1A and S1A) (Zhang et al., 2008). Also as expected, the canonical p53 family DNA binding motif was the most highly enriched motif among p63 ChIP-seq peaks in all cases (Figure 1B). Remarkably however, distinct differences were consistently observed between tumor and normal cells, including both normal-specific and tumor-specific peaks shared by multiple samples (Figures 1A and 1C). Direct validation by ChIP of select differences in p63 binding in tumor and normal cells confirmed the distinct binding profiles of p63 (Figure 1D). To discern the direct gene regulatory programs controlled by p63 we ablated endogenous p63 in both HNSCC cells and untransformed keratinocytes (HaCaT) via lentiviral shRNA, and carried

out gene expression profiling (Figure S1B). Approximately one-third of significantly regulated genes were associated with ChIP-seq peaks in the respective tumor and normal cells, suggesting direct transcriptional regulation in these cases (Figure 1E). Yet less than 20% of these putative direct target genes were shared between tumor and normal cells, potentially suggesting a qualitatively different gene expression program mediated by p63 in tumors (Figure 1E).

To address this possibility we employed a series of biological pathway algorithms to determine the functional programs associated with the direct p63-regulated gene sets identified selectively in tumor and normal contexts (Chen et al., 2013). Most notably, we found that the distinct pathways identified in normal versus cancer contexts reflected the respective p63-dependent phenotypes observed in these contexts. For example, among the top functional programs identified in normal cells were senescence and autophagy, and indeed the major phenotype associated with p63 ablation in vivo is senescence (Figure 1F) (Keyes et al., 2005). In contrast, direct p63-dependent programs in tumor cells involved growth factor signaling, in keeping with established roles for p63 in these pathways in SCC (Figure 1F) (Ramsey et al., 2013; Yang et al., 2011). We then validated p63-dependent regulation of key genes within these pathways in HNSCC cells (Figure S1C). Together these findings suggest a distinct transcriptional program underpinning p63 oncogenic function in SCC.

ACTL6A and SWI/SNF Components are Highly Up-Regulated Together with p63 in HNSCC

In order to uncover the molecular mechanisms controlling p63-dependent transcription in HNSCC we further analyzed our ChIP-seq data to define additional DNA binding motifs that were associated with p63 peaks. We interrogated the Cistrome database that contains motifs associated with both direct DNA binding factors and other chromatin regulators (Liu et al., 2011). We observed that multiple motifs associated with subunits of the SWI/SNF (BAF) chromatin remodeling complex were enriched at the p63 binding sites in HNSCC (Figure 2A). This finding was notable as we had previously identified a key subunit of the SWI/SNF complex, ACTL6A (BAF53A), as physically associated with ΔNp63α to mediate p53-independent transcription (Gallant-Behm et al., 2012). These results were also provocative because ACTL6A has recently been demonstrated to be essential for maintaining a stem-like state of regenerative proliferation among multiple normal cellular progenitor populations, including in neural, hematopoietic and epidermal lineages (Bao et al., 2013; Krasteva et al., 2012; Yoo et al., 2009). Collectively, these findings suggested that ACTL6A and p63 might collaborate in this tumor-specific context.

We thus tested for co-expression of ACTL6A and p63 in normal human epidermis and HNSCC. Highest expression of p63 in normal epithelium is known to be present in basal and supra-basal cells (Figure 2B) (Koster, 2010). Unlike p63, we found that ACTL6A was expressed at low levels primarily throughout the supra-basal layers of the normal stratified epithelium (Figure 2B). In primary HNSCC tumors, however, the situation was strikingly different, as both ACTL6A and p63 were expressed at uniformly high levels in virtually all tumor cells (Figure 2B). In keeping with these findings, quantitative RT-PCR (qRT-PCR) analysis of primary uncultured epidermis and primary HNSCC tumors showed dramatic up-

regulation of ACTL6A expression in tumors (>50-fold), together with the expected tumor-specific up-regulation of p63 (Figure 2C) (Moll and Slade, 2004). Thus, ACTL6A and p63 are rarely co-expressed in normal epithelium but are highly expressed together in SCC tumors.

Analysis of genomic copy number data from TCGA provided a genetic mechanism for high-level ACTL6A and p63 co-expression in HNSCC. A substantial proportion of these tumors (nearly 20%) exhibit genomic co-amplification of the *p63* and *ACTL6A* loci, which are located approximately 10MB apart on chromosome 3q (Figure 2D) (Cancer Genome Atlas, 2015). Importantly, *ACTL6A* mRNA expression was correlated with its copy number (Figure S2A), and *ACTL6A* and *p63* mRNA were highly expressed and correlated (r= 0.305; *p*= 1.1e -12) across the entire set of HNSCC tumors in TCGA (Figure 2E). To ensure the validity of this finding we established an independent cohort of primary HNSCC specimens and tested them for *ACTL6A* and *p63* expression by qRT-PCR, confirming a highly significant correlation between these two factors (r >0.9) (Figure S2B). Furthermore, high levels of the respective proteins were corroborated by analysis of a panel of human SCC-derived cell lines (Figure S2C). As anticipated, immunoprecipitation for ACTL6A demonstrated a robust physical interaction between endogenous ACTL6A and p63 in HNSCC cells (Figure 2F), and we further confirmed the specificity of this interaction by employing epitope-tagged ΔNp63α to pull down endogenous ACTL6A (Figure S2D).

We then examined RNA expression of other SWI/SNF components in primary HNSCC specimens from the TCGA. Comparably high expression and statistically robust correlations were observed between *p63* and multiple SWI/SNF complex components in these tumors (Figures 2E and S2E). Correspondingly, protein levels of these other SWI/SNF subunits were also high in SCC cell lines (Figure S2C). Most notably, immunodepletion of ACTL6A in lysates from HNSCC cells induced quantitative depletion of other SWI/SNF subunits, supporting the stoichiometric association of ACTL6A with an intact SWI/SNF complex in HNSCC (Figure 2G). Thus, p63 is highly overexpressed together with an ACTL6A-containing SWI/SNF complex in HNSCC.

ACTL6A/p63 Control a Stem-Like Program of Regeneration in HNSCC

As a first step toward uncovering the functional contribution of ACTL6A in HNSCC, we defined the endogenous ACTL6A-regulated transcriptome using lentiviral shRNA knockdown followed by gene expression profiling in tumor cells. Gene Set Enrichment Analysis (GSEA) of this dataset revealed that a top signature associated with endogenous ACTL6A-dependent transcription in HNSCC is embryonic stem cell function (Figure 3A) (Wong et al., 2008). We next interrogated RNAseq data from human HNSCC in TCGA, using GSEA to identify genes and signatures enriched in tumors with high-level *ACTL6A* expression. In this in vivo context an embryonic stem cell signature was highly statistically enriched among tumors with high *ACTL6A* expression (Figure 3B) (Ben-Porath et al., 2008). We then asked whether p63 collaborated with ACTL6A for transcription of this stem-like program in HNSCC. We indeed observed a substantial and highly statistically significant overlap between genes regulated by endogenous p63 and ACTL6A in tumor cells (*p*= 7.3 e-53, Figure 3C). Most notable among these was prominent repression of a set of

genes associated with terminal differentiation in the stratified epithelium, which was verified upon GSEA analysis of ACTL6A-regulated genes in tumor cells (Figure S3A). Consistent with these findings, we observed significant induction of differentiation-associated cytokeratins, as well as loricrin and involucrin following knockdown of either p63 or ACTL6A in HNSCC cells (Figure 3D). Furthermore, loss of endogenous ACTL6A resulted in accumulation of cells in G1/G0 upon BrdU/propidium iodide labeling, and dramatically suppressed colony-forming ability in both 2-dimensional clonogenic assays and soft agar (Figures S3B-S3D). A more rigorous assessment of regenerative potential is provided by the oncosphere assay, in which cells are grown in defined serum-free medium under ultra-low attachment conditions (Justilien et al., 2014). Oncosphere formation is established to correlate with expression of stem cell genes and enhanced in vivo tumorigenicity (Justilien et al., 2014). Accordingly, ACTL6A knockdown powerfully suppressed clonogenic capacity in the oncosphere assay (Figure 3E). Taken as a whole, these findings suggest a central role for ACTL6A together with p63 in the maintenance of an undifferentiated, regenerative population in squamous epithelial tumors.

The ACTL6A Program Promotes the Undifferentiated, Proliferative State and Determines Patient Survival in HNSCC

In order to test the contribution of ACTL6A in a physiologic tumor context we developed a xenograft model of squamous cell carcinoma, employing HNSCC cells expressing two independent doxycycline-inducible shRNAs targeting ACTL6A. We then generated tumors and their stromal microenvironment by co-injecting these cells together with fibroblasts into immunodeficient mice (Figure 4A). Remarkably, loss of ACTL6A consistently blocked progression of these established squamous tumors with high statistical significance compared to controls (Figures S4A and 4B).

We then investigated the cellular basis for this effect. We observed no effect on cell survival following loss of ACTL6A in vivo, as assessed by staining for activated Caspase 3 (Figure S4B). In contrast, Ki67 staining revealed a significant and consistent loss of proliferation in these tumors (Figure 4C). Based on our results in vitro (Figure 3) we assessed the effect of ACTL6A loss on differentiation by staining tumors for Keratin 13 (K13), an acidic keratin and specific marker of differentiation in mucosal epithelial cells (Moll et al., 2008). While the proliferating control tumors showed only weak background staining for K13, ACTL6A-ablated tumors showed intense filamentous cytoplasmic staining (Figure 4D). Thus, ACTL6A is required to promote the proliferative, undifferentiated state of squamous tumors, and its loss triggers rapid arrest and differentiation in vivo.

In order to establish the relevance of these findings to human HNSCC we asked whether expression of ACTL6A was a determinant of clinical outcomes in this disease. Indeed, high levels of ACTL6A expression conferred significantly shorter survival among HNSCC patients (Figure 4E). Notably, the effect of ACTL6A expression on survival in HNSCC was more significant than that for either PIK3CA or SOX2, two adjacent genes localized to the 3q26 amplicon (not shown). This finding indicates that the survival effect is not driven by these potentially co-amplified factors, but instead by ACTL6A itself. Thus the

undifferentiated, proliferative state driven by ACTL6A in HNSCC is associated with aggressive disease and poor patient survival.

ACTL6A/p63 Mediate Direct repression of the Hippo/YAP regulator *WWC1* in Squamous Carcinomas

We next sought to unveil the key, proximal transcriptional targets and mechanisms underpinning the fundamental contribution of ACTL6A/p63 in SCC. Our approach exploited the convergence among several datasets we had generated to define ACTL6A and p63-regulated target genes. These data included not only gene expression profiling following endogenous p63 and ACTL6A knockdown in multiple tumor-derived cell lines, but also in vivo gene expression analysis following conditional genetic deletion of endogenous *p63* in an autochthonous mouse model of SCC we developed for this purpose (Figure 5A) (Ramsey et al., 2013). Numerous genes were consistently regulated by both p63 and ACTL6A in all these analyses, and the majority of these genes were repressed, showing increased expression following ACTL6A and p63 loss-of-function (Figure 5A). We then independently validated a subset of these genes as co-regulated by endogenous ACTL6A and p63 in SCC cell lines. These included previously identified direct transcriptional targets of p63, such as the p63-repressed cell cycle regulatory gene *CDKN1A* and the p63-activated EGF family ligand *NRG1* (Figures 5B and S5A, respectively) (Forster et al., 2014; Westfall et al., 2003). Multiple novel transcriptional targets were also identified through this approach. Most notably, among the top genes regulated in all these analyses was *WWC1* (*KIBRA*) which encodes a cytosolic phosphoprotein that has recently been shown to function as a tumor suppressor in *Drosophila* and potent regulator of the Hippo pathway in both flies and mammalian cells (Yu et al., 2010; Zhang et al., 2014). This finding was particularly notable in the HNSCC context because we recently demonstrated that the Hippo pathway effector YAP (yes-associated protein 1) is epistatic to p63 in the normal airway epithelium, functioning to regulate differentiation, self-renewal and stem cell identity (Zhao et al., 2014). We then confirmed by direct qRT-PCR that *WWC1* was induced following knockdown of either endogenous ACTL6A or p63 in multiple HNSCC cell lines (Figures 5B and S5B). Furthermore, we verified the specificity of this effect by showing that expression of an shRNA-resistant murine ACTL6A was sufficient to block induction of *WWC1* and the associated activation of cellular differentiation following ablation of endogenous ACTL6A (Figure S5C).

WWC1 is commonly silenced in B cell leukemias through hypermethylation of a well-defined CpG island associated with its promoter (Hill et al., 2011). However, no such hypermethylation has been observed in epithelial cancers. Our ChIP-seq data for p63 binding in SCC showed a single peak upstream of the transcription start site for *WWC1*, suggesting direct transcriptional repression of this locus by ACTL6A/p63 (Figure 5C). Indeed, direct ChIP analysis demonstrated high enrichment for both ACTL6A- and p63-bound sequences at this site (Figure 5D). Most importantly, knockdown of p63 abrogated ACTL6A binding to background levels, suggesting the presence of an ACT6A/p63 complex at this site (Figure 5D). We then performed a test for chromatin remodeling activity at this locus by determining nucleosome accessibility using micrococcal nuclease (MNase). Enzymatic digestion of DNA by MNase occurs selectively between nucleosomes and is

inhibited with chromatin compaction as occurs in the setting of transcriptional repression (Rao et al., 2001). We developed a qPCR assay to detect uncut DNA at the p63-bound locus and observed, as anticipated, that knockdown of ACTL6A or p63 significantly increased accessibility compared to a control shRNA (Figure 5E). Accessibility was not similarly affected following loss of function for p63 at more distant loci (Figure 5E). As anticipated, loss of ACTL6A induced broader peak of accessibility, consistent with the fact that SWI/SNF typically binds over a span of 2 to 4 kb (Figure 5E) (Ho et al., 2009). Thus, an ACTL6A/p63 complex controls chromatin accessibility and functions as a direct transcriptional repressor of the Hippo regulator *WWC1* in SCC.

In order to test directly the potential contribution of WWC1 in HNSCC we first performed gain of function experiments. Overexpression of the repressed WWC1 in HNSCC cells phenocopied the induction of cellular differentiation observed following loss of endogenous ACTL6A/p63 (Figure 5F). Furthermore, WWC1 overexpression potently suppressed both soft agar colony and oncophere formation, recapitulating the effect observed with ACTL6A/p63 knockdown (Figures S5D and 5G, respectively). Next we performed a phenotypic rescue experiment, asking whether a failure to induce endogenous *WWC1* would abrogate effects of ACTL6A loss. Indeed, ablation of WWC1 expression had no effect on ACTL6A levels, but it blocked induction of cellular differentiation hallmarks including Keratin 10 expression following knockdown of ACTL6A (Figure S5E). These data provide direct evidence that repression of *WWC1* by ACTL6A/p63 is required for suppression of the differentiation program in HNSCC. We also found that overexpression of the putative lung tumor suppressor GPRC5A, like WWC1, was sufficient to induce cellular differentiation and suppress colony formation in this context (Figures S5D and S5F) (Zhong et al., 2015). Taken together, these findings imply that regulation of WWC1 and potentially other key genes regulated by ACTL6A/p63 acts to oppose differentiation and promote regenerative proliferation in HNSCC.

Activation of YAP in Squamous Carcinoma Cells and Tumors is Controlled by ACTL6A/p63

In human cells WWC1 suppresses YAP, a key oncogenic downstream Hippo effector, through effects on the LATS1/2 kinases (Yu et al., 2010; Zhang et al., 2014). YAP suppression by WWC1 is achieved through inhibitory phosphorylation on serine 127 (S127), which results in 14-3-3 binding and YAP cytoplasmic retention (Zhao et al., 2007). To determine whether loss of p63/ACTL6A and the resulting transcriptional activation of *WWC1* were associated with YAP regulation in HNSCC we first assessed YAP localization. Immunofluorescence staining for total YAP in HNSCC cells showed marked punctate nuclear localization at baseline, indicating activated YAP (Varelas, 2014). Remarkably, p63 knockdown abolished nuclear YAP staining to background levels (Figure 6A). Concurrently, we observed a striking increase in cytoplasmic phosphorylated YAP-S127 following loss of p63 in these cells (Figure 6B). Essentially the same effects were observed following ACTL6A knockdown, confirming that the control of YAP occurs through ACTL6A/p63 (Figure 6A, B). We also analyzed YAP localization and phosphorylation by western analysis of fractionated lysates from SCC cells. Strong nuclear YAP expression was evident at baseline, while p63 knockdown resulted in a decrease in nuclear YAP and a dramatic accumulation of phosphorylated YAP-S127 in the cytosol (Figure 6C). Notably, the same

effect on YAP-S127 phosphorylation was observed following treatment of HNSCC cells with cisplatin, which is established to induce degradation of endogenous p63 in HNSCC (Figure S6A) (Chatterjee et al., 2008). Furthermore, we found that overexpression of WWC1 itself was sufficient to induce YAP phosphorylation in HNSCC cells (Figure S6B). This finding, together with the requirement for WWC1 to mediate the other effects of p63/ACTL6A loss (Figure 5), collectively supports the link between p63/ACTL6A-dependent YAP regulation and WWC1. Interestingly, these experiments also revealed a decrease in total YAP protein expression following loss of p63, which we found was due to decreased *YAP* mRNA, suggesting that multiple mechanisms contribute to YAP regulation by ACTL6A/p63 (Figure S6C).

Our findings imply that ACTL6A/p63 activate YAP-dependent transcription in HNSCC. Indeed, GSEA analysis of RNA profiles following endogenous ACTL6A knockdown in HNSCC cells revealed significant enrichment for a published gene expression signature of YAP-dependent transcription in oral HNSCC cells (Hiemer et al., 2015) (Figure S6D). Importantly, this signature was also enriched among human HNSCC tumors in TCGA exhibiting high vs. low ACTL6A expression (Figure S6D). We then sought to directly confirm that ACTL6A was in fact an endogenous regulator of YAP-dependent genes. Knockdown of ACTL6A and YAP confirmed coordinate regulation of multiple YAP targets identified in HNSCC, including *CYR61*, a YAP-induced factor linked to angiogenesis and tumor progression in multiple cancer types (Figure 6D) (Dhar and Ray, 2010; Hsu et al., 2015). Furthermore, we found that YAP, like ACTL6A and p63, was required to sustain oncosphere formation by HNSCC cells (Figure 6E). In keeping with these data, a recent report demonstrates that activated YAP is required for in vivo tumorigenesis in cutaneous squamous cell carcinoma (Jia et al., 2016). Collectively, these data reveal that ACTL6A/p63 are required to induce and sustain YAP localization and activity in HNSCC.

ACTL6A Control of YAP Activity in HNSCC Tumors Mediates Poor Patient Survival

Finally, we sought to directly test the ability of ACTL6A to promote tumorigenesis in vivo, and to determine whether this effect was associated with activated YAP. Ectopic ΔNp63α expression in primary keratinocytes is sufficient to bypass RAS-induced senescence and induce squamous tumorigenesis following injection into immunodeficient mice (Ha et al., 2011; Keyes et al., 2011). We therefore tested the ability of ACTL6A to induce tumors in this setting. As controls, we compared the effects to expression of a control vector, ΔNp63α alone, or both ACTL6A and ΔNp63α, each in the context of activated RAS (H-RAS-G12V) (Figure S7A). As anticipated, RAS expression alone did not induce progressive tumors in these mice after more than seven weeks, and as previously demonstrated, RAS/ΔNp63α-expressing cells formed slowly-growing tumors (Figure 7A). Most remarkably, however, expression of ACTL6A resulted in larger and more rapidly growing tumors whose histologic features included cells with large nuclei and prominent nucleoli characteristic of squamous carcinomas (Figures 7A and 7B). Co-expression of ACTL6A and ΔNp63α together produced tumors at a rate similar to ACTL6A expression alone (not shown), most likely because primary squamous epithelial cells already express substantial levels of p63 (Figure 2B). Significantly, an activated YAP pathway was evidenced in the ACTL6A-expressing

tumors by high levels of nuclear YAP protein (Figure 7B), which was not observed in tumors induced by expression of ΔNp63α alone (Figure S7B).

In order to further credential in human squamous tumors the potent control of Hippo-YAP signaling mediated by ACTL6A/p63, we analyzed proteomic data available from a subset of primary HNSCC specimens (Cancer Genome Atlas, 2015). Lysates from tumors were subjected to reverse-phase protein array (RPPA) analysis in order to assess levels of 190 key proteins and phospho-proteins (Li et al., 2013). Not surprisingly, we noted that p63 was among the top proteins most positively correlated with *ACTL6A* expression in these primary tumors (Figure S7C). Most importantly, among the top four proteins showing an inverse correlation with *ACTL6A* expression in HNSCC was phosphorylated YAP-S127 ($r = -0.343$; $p = 3.15 e -07$) (Figure 7C). Notably, the ACTL6A-repressed gene *WWC1* was positively correlated with YAP-S127 in HNSCC (Figure S7D). Further supporting the relevance of YAP in this setting, we observed dramatic re-localization of nuclear YAP to the cytosol in vivo, following endogenous ACTL6A ablation in our xenograft HNSCC model (Figure 7D). These results provide further compelling evidence that ACTL6A controls YAP phosphorylation in human HNSCC, in part through WWC1.

We lastly asked whether the YAP/CYR61 program controlled by ACTL6A was a mediator of overall survival of patients with HNSCC. Indeed, we found that a gene expression signature associated with Hippo activation in breast cancer was statistically associated with poor survival for patients with HNSCC (Figure S7E) (Cordenonsi et al., 2011). Furthermore, increased expression of *CYR61* itself in primary HNSCC was a determinant of poor survival for patients with this disease (Figure 7E). Collectively, these data demonstrate that the ACTL6A chromatin remodeler, together with p63, functions through regulation of WWC1 and potentially other factors as a potent activator of the Hippo-YAP signaling pathway in HNSCC, driving an undifferentiated, regenerative state that is associated with an aggressive disease course and poor survival (Figure 8).

DISCUSSION

Here, we have revealed the SWI/SNF subunit ACTL6A as a central oncogenic driver in head and neck squamous cell carcinomas (HNSCCs). Recent systematic genomic analyses have uncovered mutations in chromatin remodeling factors, particularly subunits of the SWI/SNF complex, as among the most frequent genomic alterations in human cancer (Kadoch et al., 2013). As noted, most of these studies have identified loss-of-function mutations within key subunit genes in a variety of cancers, leading to the paradigm of the “SWI/SNF tumor suppressor complex” (Lu and Roberts, 2013). In contrast, *ACTL6A* is commonly subject to genomic amplification and overexpression in SCCs, particularly HNSCC (Cancer Genome Atlas, 2015). We demonstrate that ACTL6A collaborates with p63, a long-suspected tumor driver in this setting, to direct a transcriptional program of blocked differentiation and regenerative proliferation in these tumors. Mechanistically, the seeming paradox regarding a tumor suppressive versus oncogenic function of SWI/SNF and ACTL6A can be reconciled in this tissue context by recent data demonstrating a role for ACTL6A in opposing SMARCA4/SMARCA2-dependent induction of differentiation specifically in progenitor cells of the normal epidermis (Bao et al., 2013). Similarly, other recent work points to p63 as

a prominent contributor to SWI/SNF-dependent function in the normal epidermis, in keeping with our findings and with its established role as a master epidermal transcription factor (Bao et al., 2015; Senoo et al., 2007). In HNSCC, *ACTL6A* and *p63* are co-amplified, and we show that they are physically associated and highly co-expressed together with other subunits of the SWI/SNF complex. We demonstrate that high levels of ACTL6A and p63 are required to suppress differentiation and promote proliferation in this tumor context, in agreement with a proposed model whereby ACTL6A promotes the progenitor cell state in the normal epidermis by sequestering SMARCA4/SMARCA2-containing complexes that promote differentiation (Bao et al., 2013).

Previous work showed that conditional deletion of *Actl6a* in stratified epidermis using a Keratin 14-driven Cre transgene induces a skin phenotype with marked similarities to that resulting from deletion of either *p63* or *ΔNp63* isoforms, including profound hypoplasia and intact expression of terminal differentiation markers (Bao et al., 2013; Yang et al., 1999). Reflecting a dramatic co-option of their normal tissue-specific roles, we provide compelling evidence that ACTL6A and p63 are highly overexpressed and function together in a common pathway to drive a refractory cancer phenotype in HNSCC. Specifically, we demonstrate that ACTL6A and p63 co-regulate a key set of relevant genes and programs. We show that this transcriptional regulation is direct, as these two factors are co-localized to regulatory elements of a pivotal gene required for Hippo-YAP regulation, *WWC1*. Together, ACTL6A and p63 control chromatin accessibility to repress *WWC1* transcription at this locus. Loss of ACTL6A or p63 in HNSCC cells confers increased chromatin accessibility, again in agreement with a role for ACTL6A in opposing catalytic SWI/SNF function, as loss of the latter is associated largely with decreased accessibility in epidermal cells (Bao et al., 2015).

The Hippo-YAP pathway is emerging as a significant cancer driver, leading to diverse efforts aimed at extinguishing the activity of this pathway as a therapeutic strategy (Gomez et al., 2014; Plouffe et al., 2015). Nonetheless, data supporting the relevance of Hippo-YAP in HNSCC have been limited (Akervall et al., 2014; Ehsanian et al., 2010; Hiemer et al., 2015). By identifying *WWC1* and YAP as key targets controlled by ACTL6A and p63 in HNSCC, our findings forge a novel and unanticipated connection between p63/SWI/SNF function and the oncogenic Hippo pathway in this disease. As noted, selective pressure to silence *WWC1* transcription is also evident in B cell leukemias where repression occurs through a distinct mechanism involving promoter methylation (Hill et al., 2011). Additionally, in the mesenchymal context SOX2 has been shown to suppress *WWC1*, promote YAP activation and thereby maintain a stem-like population in osteosarcomas (Basu-Roy et al., 2015). WWC1 functions to regulate YAP activity through phosphorylation at serine 127 (Yu et al., 2010; Zhang et al., 2014). Accordingly, we demonstrate that repression of *WWC1* by ACTL6A and p63 is associated with strikingly parallel control of oncogenic YAP phosphorylation, localization and activity by these two factors. Importantly, we reveal that expression of WWC1 itself in HNSCC is sufficient to recapitulate the effects of ACTL6a/p63 loss on YAP phosphorylation, tumor cell growth and differentiation. Taken together, these observations strongly support the collaboration of ACTL6A and p63 to regulate Hippo-YAP signaling in HNSCC through genes including *WWC1*.

Emerging evidence from in vivo models and human tumors further supports the relevance of an ACTL6A/p63/YAP network in the epithelium. We recently reported that in the bronchial airway epithelium YAP mediates proliferation, blocked differentiation and stem cell identity, and that it is epistatic to *p63* in this role (Zhao et al., 2014). Here, we demonstrate using a RAS-dependent squamous carcinoma model that ACTL6A overexpression in p63-expressing primary keratinocytes is sufficient to drive tumorigenesis associated with YAP nuclear translocation. Conversely, loss of endogenous ACTL6A arrests tumor grown and induces YAP nuclear export in vivo. Accordingly, in human primary HNSCC, ACTL6A expression is strongly inversely correlated with YAP-S127 phosphorylation, and both ACTL6A and a YAP-regulated transcriptional program are significant determinants of poor overall survival for patients with this disease. We speculate that a stem-like state promoted by ACTL6A/p63/YAP underlies this effect on patient outcomes, given substantial evidence that a regenerative sub-population underlies the treatment-refractory nature of many HNSCC tumors (Qian et al., 2015). Consequently, the rational application of inhibitors of the pathway could well target the cell population of most interest in this heterogenous disease. By identifying herein the activation of YAP downstream of ACTL6A/p63, and defining its regulation and contribution to outcomes in HNSCC, we provide a framework for testing nascent therapeutic approaches targeting YAP activity in this disease.

EXPERIMENTAL PROCEDURES

Detailed information on cell culture and fractionation, ChIP and ChIP-seq, chromatin analysis, lentiviral transduction, qRT-PCR, western analysis, immunohistochemistry and immunofluorescence, oligonucleotide sequences and antibodies used are provided in Supplemental Experimental Procedures. All animals were housed and treated in accordance with protocols approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital. Local primary human tumor collection and analysis were approved by the Harvard Cancer Center IRB with waiver of consent for collection of existing clinical specimens.

Oncosphere assay

Oncosphere assay was performed as described in (Justilien et al., 2014). Briefly 10,000 cells/well were seeded in a 6 well low adherent plate in serum-free medium containing 50 μg/mL Insulin, 20 μg/mL EGF, 10μg/mL FGF, B-27, N-2 and grown for 2 weeks and re-plated after pelleting and trypsinization and grown for 2 weeks in low adherent plates in the oncosphere medium.

Statistics

P values were determined using the student's unpaired *t* test unless indicated otherwise. Statistical analysis of ChIP-seq, RNAseq, and gene expression data are described in Supplemental Experimental Procedures

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SIGNIFICANCE

HNSCC is an aggressive malignancy driven by a regenerative, stem-like population. While p63 has been linked to regenerative proliferation in normal epithelia, its contribution and underlying mechanisms in SCC remain obscure. Here, we show that ACTL6A is an important partner in p63-driven tumorigenesis. ACTL6A and p63 coordinately regulate key genes, including *WWC1*, to dictate oncogenic YAP activity and patient outcomes in HNSCC. These findings establish a mechanism for p63 to alter epigenetic states, and they uncover a critical oncogenic function for SWI/SNF, which has been largely viewed as a tumor suppressor in human cancer. Furthermore, by revealing the Hippo-YAP pathway as a major target of ACTL6A/p63 linked to tumor behavior, this work suggests potential therapeutic approaches for refractory HNSCC cases.

HIGHLIGHTS

- ACTL6A is co-amplified, co-expressed and physically associated with p63 in HNSCC.
- ACTL6A/p63-mediated transcription drives undifferentiated regenerative proliferation.
- An ACTL6A/p63 complex suppresses *WWC1* to activate YAP and promote tumorigenesis.
- ACTL6A and an activated YAP pathway confer poor prognosis in primary HNSCC.

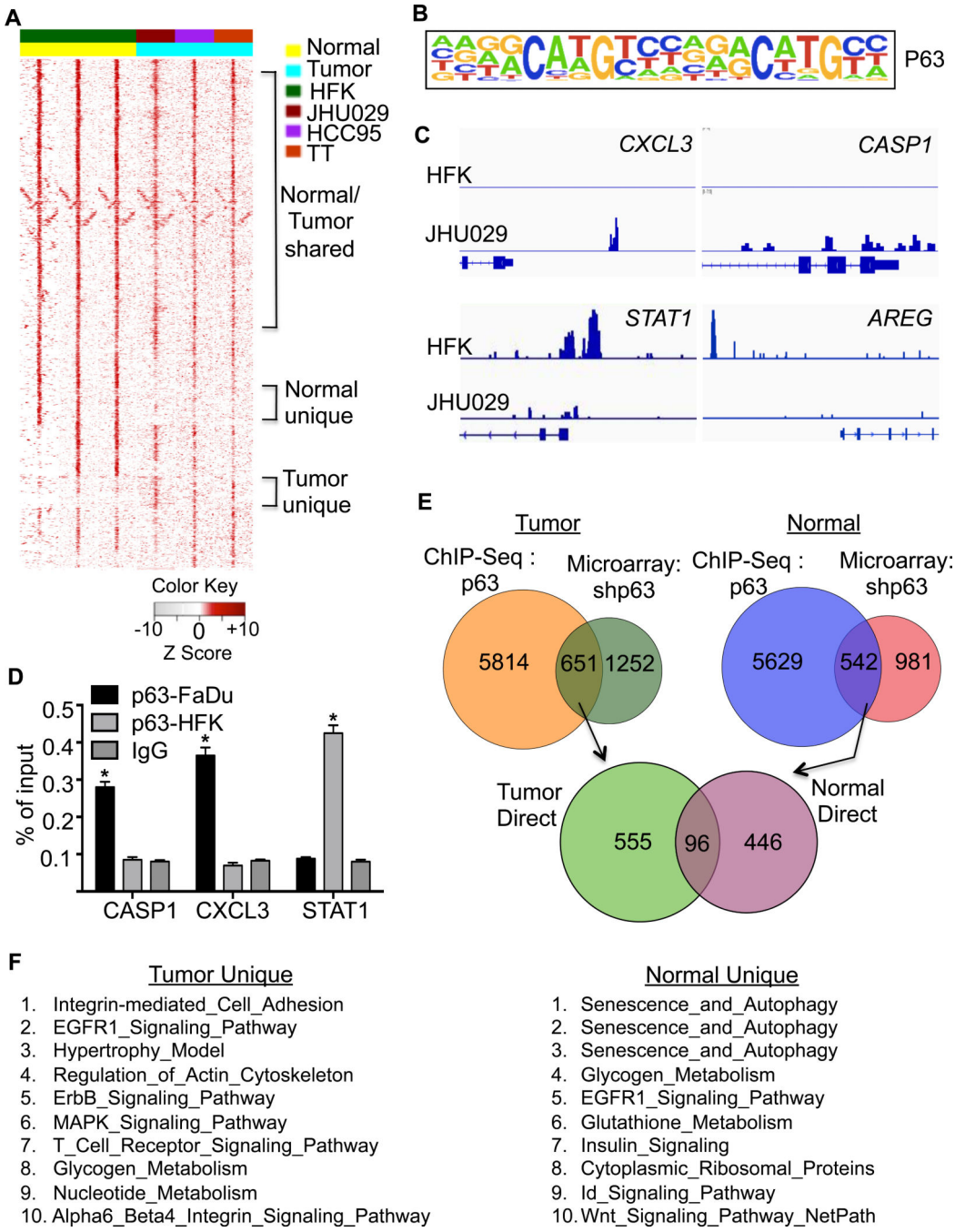


Figure 1. P63 Exhibits Distinct Chromatin Binding and Transcriptional Regulation in Normal Cells and Squamous Carcinomas

(A) Heat map summarizing ChIP-seq data for endogenous p63, comparing JHU-029 SCC cells, 2 additional SCC lines, and 3 keratinocyte (HFK) studies. Profiles are centered on p63 binding peaks and depict enrichment Z-scores in a +/− 2kb window.

(B) P63 binding motif corresponding to p63 peaks in JHU-029 ChIP-seq data as produced by MDSeqPos analysis of the Cistrome platform.

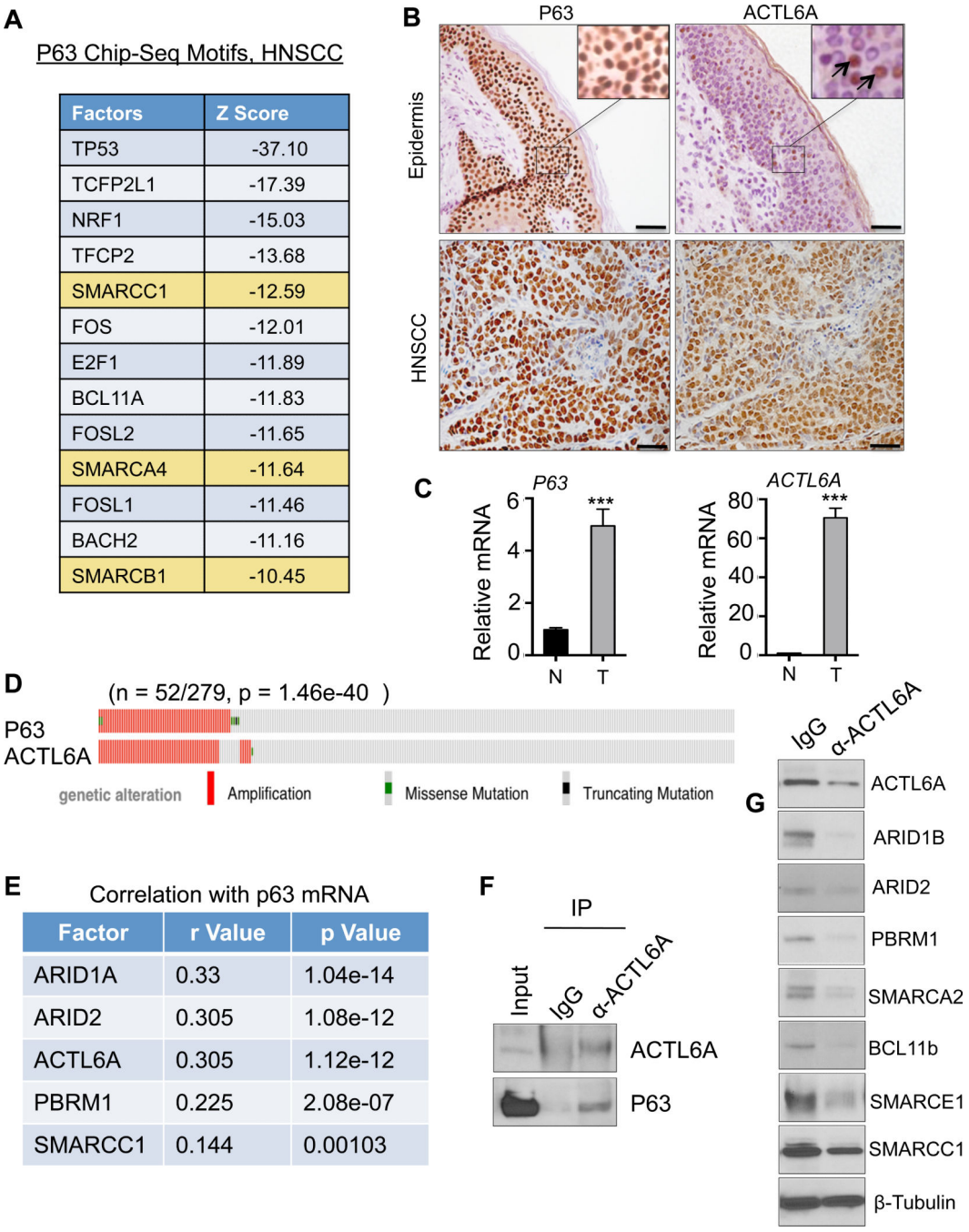
(C) Representative ChIP-seq traces for the indicated genes in HFK and JHU-029 cells, demonstrating distinct binding patterns. The respective transcription units are shown below each trace.

(D) Direct ChIP for endogenous p63 in HFK and FaDu followed by qPCR for the loci identified by ChIP, confirming ChIP-seq findings. Shown are mean values from technical triplicates in a representative experiment, performed three times. * $p<0.05$. Error bars indicate SD.

(E) Direct transcriptional targets of p63 in tumor (SCC) and normal cells, identified as the intersection of the ChIP-seq peaks (tumor, JHU-029; normal, HFK) with the respective regulated genes determined by microarray analysis ($p<0.05$ by t-test) following knockdown of p63 in JHU-029 and FaDu SCC cells (tumor) and HaCat cells (normal). Below, limited overlap between direct p63 targets in tumor and normal cells.

(F) Top-ranked functional pathways associated with 575 “tumor direct” and 451 “normal direct” p63 transcriptional targets from (E), identified by WIKI pathway analysis. FDR<0.05, $p<0.0001$ for all pathways shown.

See also Figure S1.



Factors	Z Score
TP53	-37.10
TCFP2L1	-17.39
NRF1	-15.03
TFCP2	-13.68
SMARCC1	-12.59
FOS	-12.01
E2F1	-11.89
BCL11A	-11.83
FOSL2	-11.65
SMARCA4	-11.64
FOSL1	-11.46
BACH2	-11.16
SMARCB1	-10.45

Factor	r Value	p Value
ARID1A	0.33	1.04e-14
ARID2	0.305	1.08e-12
ACTL6A	0.305	1.12e-12
PBRM1	0.225	2.08e-07
SMARCC1	0.144	0.00103

Figure 2. ACTL6A is Co-Amplified, Overexpressed and Physically Associated with p63 and Other SWI/SNF Subunits in HNSCC

(A) Top enriched motifs associated with binding of the indicated transcription and chromatin regulatory factors, identified by analysis of ChIP-seq data from JHU-029 using MDSeqPos on the Cistrome platform. SWI/SNF subunits are indicated in yellow.

(B) Immunohistochemical staining for p63 and ACTL6A in human skin (top) and a representative HNSCC tumor (bottom). High-power insets show scattered ACTL6A positive cells (arrows), contrasting with diffuse staining in tumor. Scale bars represent 50μm.

(C) Overexpression of *p63* and *ACTL6A* in tumors, assessed by qRT-PCR from normal foreskin epidermis (N, n=5) or primary HNSCC tumors (T, n=29). Shown are mean values from all specimens measured in triplicate; error bars indicate SD.
(D) Gene copy number and mutation data from TCGA for HNSCC, showing frequent co-amplification of *p63* and *ACTL6A*. P value by Fisher's exact test.
(E) Positive correlation of *p63* with mRNA expression of the indicated SWI/SNF subunit genes, obtained from analysis of RNAseqV2 data from >500 HNSCC cases in TCGA.
(F) Physical association of endogenous ACTL6A with p63 in FaDu whole cell extracts, assessed by immunoprecipitation/western analysis. IgG serves as a control.
(G) ACTL6A is stoichiometrically bound to SWI/SNF subunits in HNSCC cells. Shown are immunodepleted lysate following ACTL6A immunoprecipitation. IgG serves as a specificity control, and β-tubulin as a loading control.
See also Figure S2.

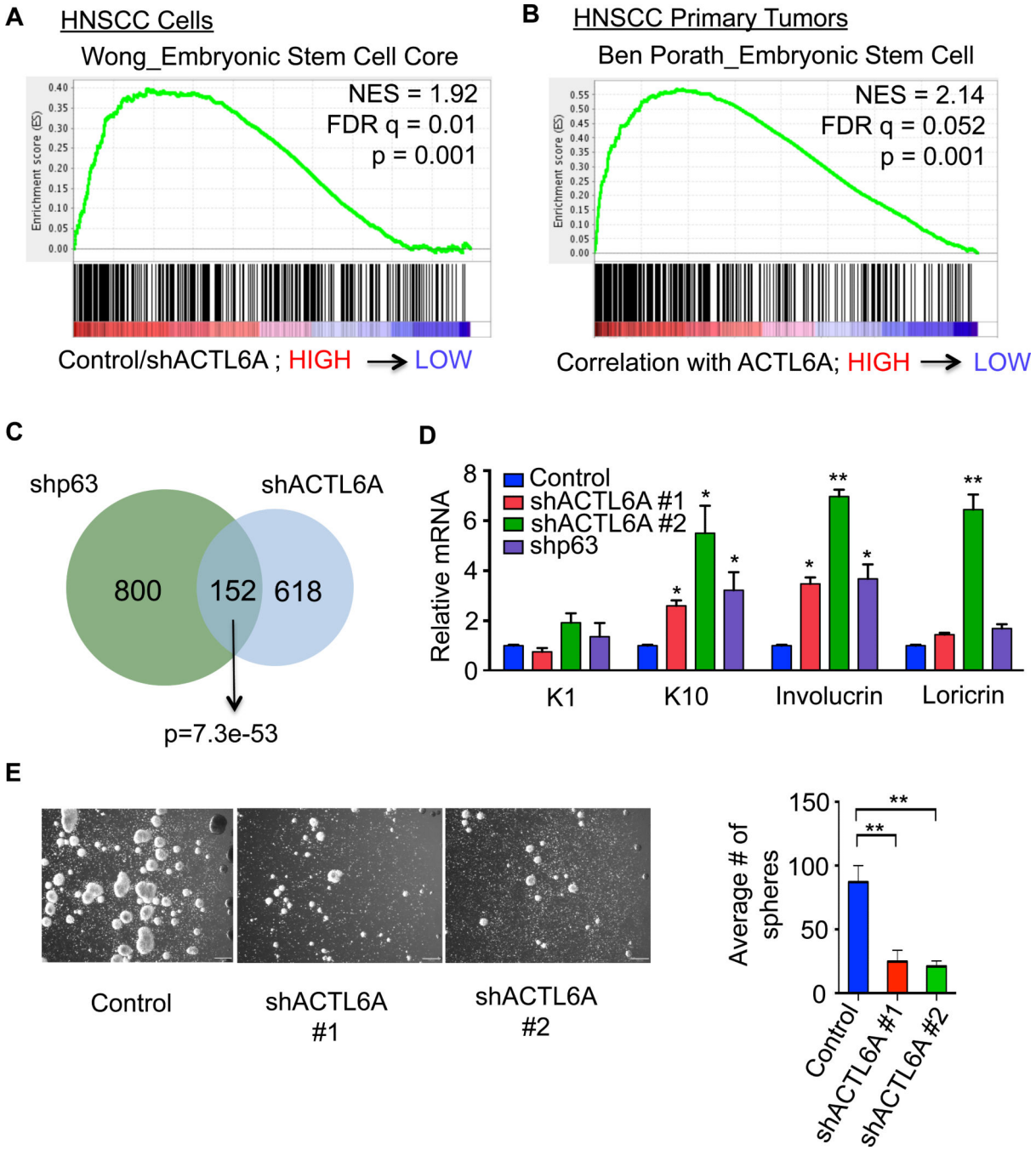


Figure 3. ACTL6A and p63 Control a Stem-Like Program of Regenerative Proliferation in HNSCC

(A) Enrichment of a stem cell signature in GSEA analysis of genes regulated following knockdown of endogenous ACTL6A via lentiviral shRNA in HNSCC cells. NES, normalized enrichment score; FDR, false-discovery rate.

(B) *ACTL6A* expression in primary HNSCC is associated with a stem cell signature. RNAseq data from 424 HNSCC cases in TCGA were analyzed with GSEA to identify genes and gene sets correlated with *ACTL6A* expression levels.

(C) Significant overlap of genes regulated by endogenous p63 and ACTL6A in HNSCC cells, identified by microarray analysis 72h following knockdown via lentiviral shRNA (fold change ≥ 1.5; $p<0.05$ by t-test).

(D) Induction of terminal differentiation genes following knockdown of either ACTL6A or p63 in HNSCC cells, determined by qRT-PCR analysis. Shown are mean values from two experiments performed in triplicate, *$p<0.05$; **$p<0.01$. Error bars indicate SD.

(E) Following endogenous ACTL6A knockdown or control, cells were plated in serum-free defined medium under ultra-low attachment conditions for 14 days. Summary graph at right shows mean values from two experiments performed in triplicate. **$p<0.01$. Error bars indicate SD.

See also Figure S3.

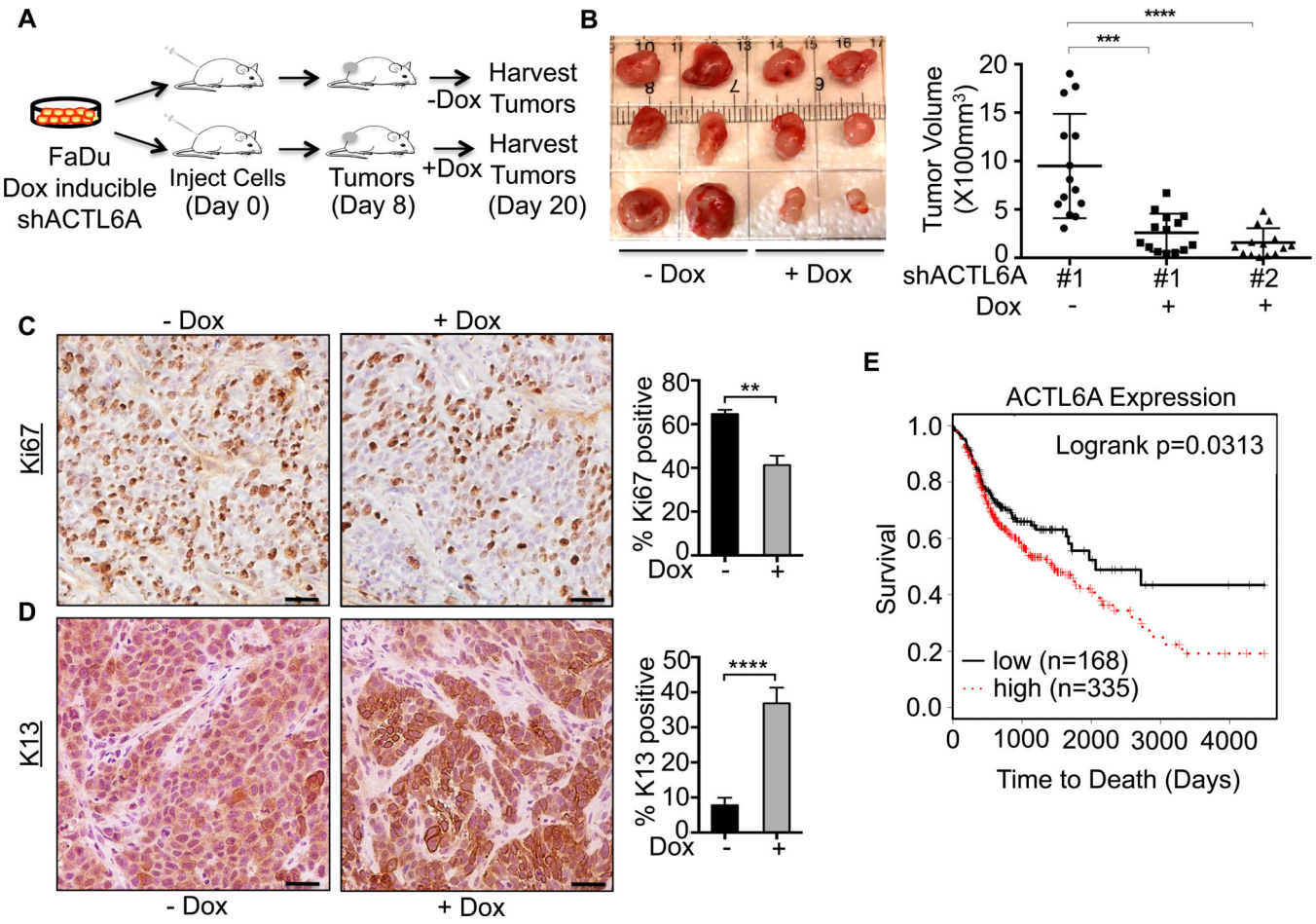


Figure 4. ACTL6A Drives Endogenous Tumor Progression and Poor Clinical Outcomes in HNSCC in vivo

(A) Schematic showing xenograft experiment. HNSCC cells (1×10^6) expressing two independent ACTL6A-directed, doxycycline (Dox)-regulated shRNAs were injected with stromal cells (2×10^6) and matrigel into nude mice. When tumors reached 100 mm^3 mice were fed Dox or control for 12 days prior to sacrifice.

(B) Left, representative tumors; right, summary of mean tumor volumes in Dox-treated and control tumors measured at time of sacrifice. ***p<0.001; ****p<0.0001.

(C) Ki67 and (D) Keratin 13 (K13) immunohistochemical staining from representative Dox-treated and control tumors. Right, summary data obtained by counting >20 high-powered fields/tumor X6 tumors in each group. Note dense filamentous K13 staining in Dox-treated versus control tumors. **p<0.01; ****p<0.0001. Error bars indicate SD. Scale bars represent 50μm.

(E) Kaplan-Meier analysis of RNAseq V2 data and outcomes from 503 HNSCC cases in TCGA, showing *ACTL6A* expression (highest versus lowest tertile) is associated with decreased overall survival. P value by log-rank test.

See also Figure S4.

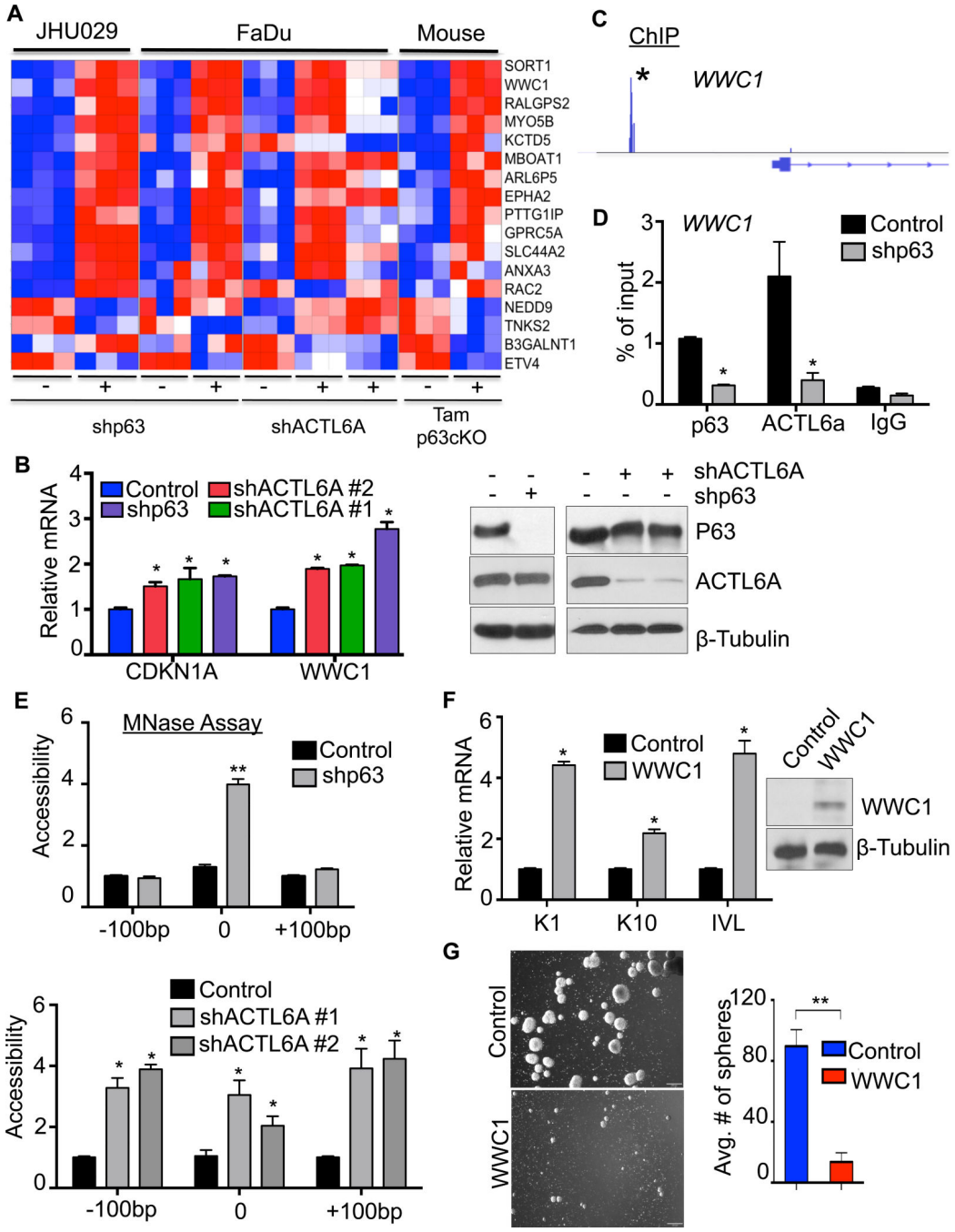


Figure 5. ACTL6A and p63 are Direct Transcriptional Suppressors of the Hippo Regulator *WWC1*

(A) Heat map showing shared p63- and ACTL6A-regulated genes in HNSCC cells (JHU-029, FaDu) and autochthonous SCC tumors, identified in each case by microarray analysis. Red shows high, and blue low gene expression. *P63-cKO* squamous tumors were established by a chemical carcinogenesis protocol in $p63^{lox/lox};K14\text{-}CreER;p53^{+/-}$ mice, and treated with tamoxifen (Tam) or vehicle to excise endogenous *p63*.

(B) Direct confirmation of shared ACTL6A- and p63-regulated gene expression, using qRT-PCR following knockdown of the respective genes via lentiviral shRNA at 72h. Shown are

mean of two experiments performed in triplicate. *p<0.05. Error bars indicate SD. Western blots verifying knockdown are shown at right.

(C) Trace from p63 ChIP-seq in HNSCC cells showing a single binding peak upstream of the transcriptional start of *WWC1.*

(D) ChIP for endogenous p63 or ACTL6A in HNSCC cells, followed by qPCR at the locus identified in (C). Knockdown of p63 72h prior to ChIP confirms specificity and demonstrates p63-dependent binding of ACTL6A at this site. Shown are means of technical triplicates in a representative experiment, performed twice. *p<0.05. Error bars indicate SD.

(E) Chromatin accessibility is controlled by p63 (top) and ACTL6A (bottom) at the *WWC1* locus in HNSCC cells, assessed by micrococcal nuclease (MNase) digestion (3 U/ml for 15 min) following knockdown of the respective genes via lentiviral shRNA. X-axes indicate distance from p63 ChIP-seq peak. Shown are means of technical triplicates in a representative experiment, performed twice. **p<0.01. Error bars indicate SD.

(F) Expression of WWC1 via retrovirus induces cellular differentiation in HNSCC cells. Left, qRT-PCR for the indicated genes at 72h; right, western blot for WWC1, essentially undetectable at baseline. Shown are means of technical triplicates in a representative experiment, performed twice. *p<0.05. Error bars indicate SD.

(G) WWC1 expression suppresses oncosphere formation in HNSCC cells, assayed as described in 3E. Summary graph at right shows mean values from two experiments performed in triplicate. **p<0.01. Error bars indicate SD.

See also Figure S5.

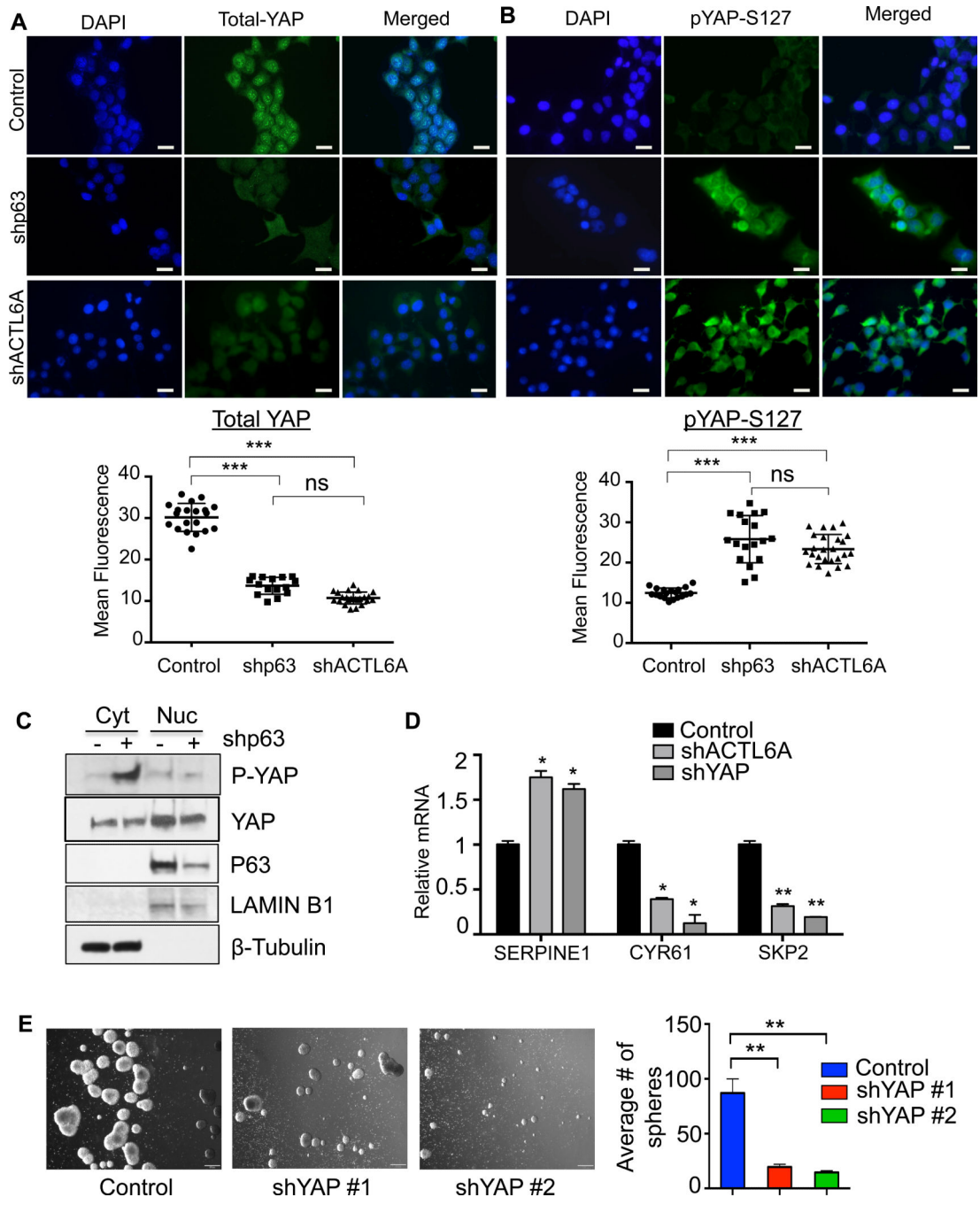


Figure 6. ACTL6A and p63 Control Endogenous YAP Localization and Activity in HNSCC Cells

(A, B) Immunofluorescence staining for total YAP (A) or phosphorylated YAP-S127 (pYAP-S127) (B) in HNSCC cells, showing reduced nuclear staining (A) and increased cytoplasmic staining (B) following knockdown of either ACTL6A or p63 at 72h. Graphs below show mean fluorescence of individual cells in a representative field (5 fields counted per condition) assessed by ImageJ analysis. Experiment was performed three times. ***p<0.001. Scale bars represent 20μm.

(C) YAP expression and phosphorylation are controlled by p63, evidenced by western blots in fractionated lysates of HNSCC cells following endogenous p63 knockdown at 72h. Lamin B1 and β-tubulin serve as loading and fractionation controls for nuclear and cytosolic fractions, respectively. Shown is a representative experiment performed three times.
(D) ACTL6A and YAP function in a common pathway, evidenced by co-regulation of established YAP transcriptional targets following knockdown of endogenous ACTL6A or YAP at 72h in HNSCC cells. Shown are means from qRT-PCR data from two experiments performed in triplicate. *p<0.05; **p<0.01. Error bars indicate SD.
(E) Following endogenous YAP knockdown or control, cells were plated in serum-free defined medium under ultra-low attachment conditions for 14 days. Summary graph at right shows mean values from two experiments performed in triplicate. **p<0.01. Error bars indicate SD.
See also Figure S6.

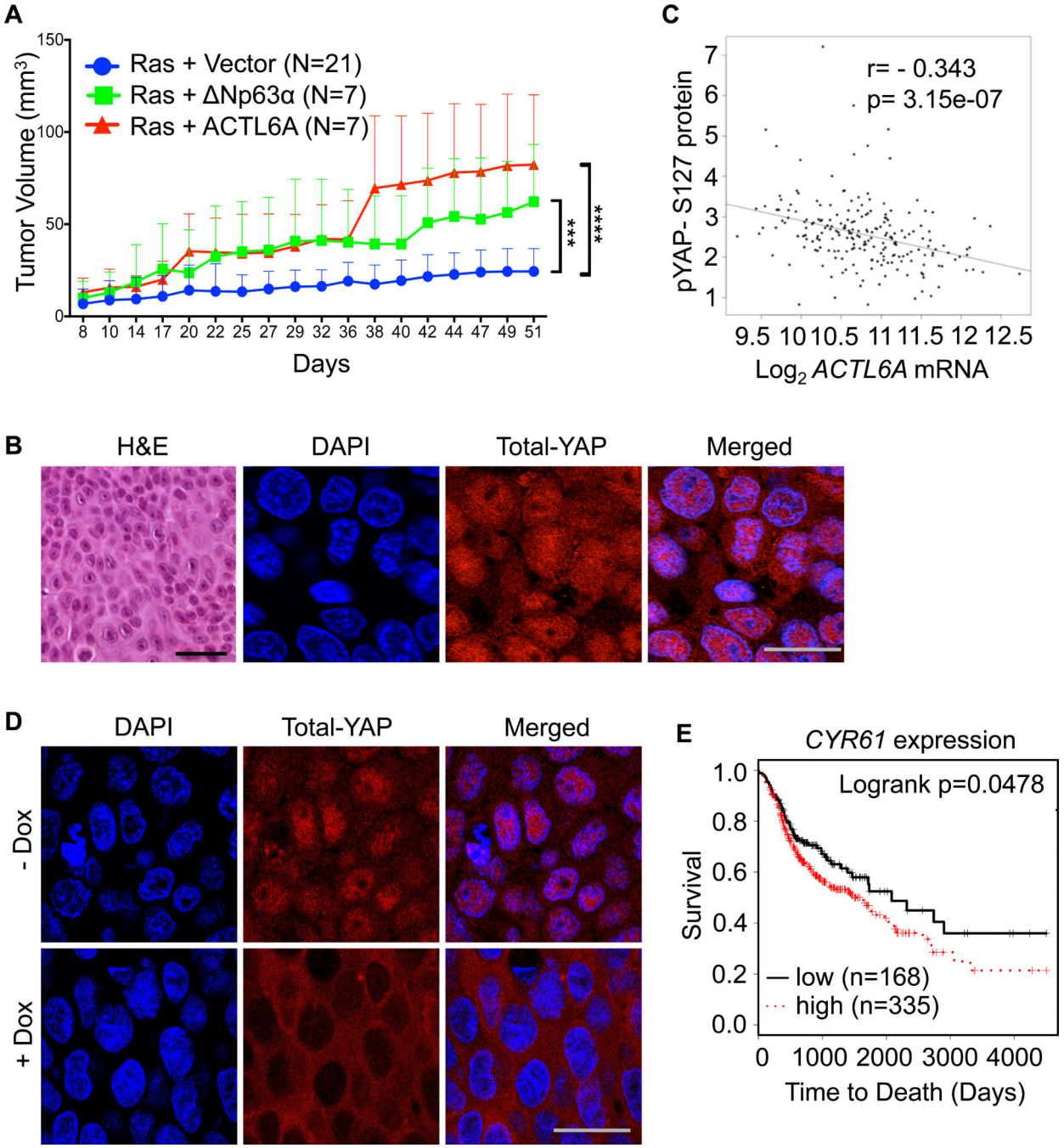


Figure 7. ACTL6A Regulates YAP Activity in vivo to Drive Poor Prognosis in HNSCC

(A) ACTL6A expression promotes tumorigenic growth of primary keratinocytes. Cells were transduced with retroviral activated RAS (HRAS-G12V, 24h), followed by retroviral control vector, ΔNp63α or ACTL6A (5days), then cells (3×10^5) plus stroma (6×10^5) were subcutaneously injected with matrigel into nude mice. P values by multiple measures ANOVA. ***p<.001; ***p<.0001.

(B) Histological staining (left panel) and confocal images following immunofluorescence staining for total YAP in a representative ACTL6A-induced squamous tumor, demonstrating

nuclear YAP in tumor cells. Black and grey scale bars represent 50μm and 20μm, respectively.

(C) Scatter plot representing analysis of the 212 primary HNSCC tumors from TCGA with reverse-phase protein array, showing inverse correlation between log2 *ACTL6A* expression by RNASeqV2 and phosphorylated (inhibited) YAP. Pearson correlation is represented by r.

(D) Confocal images following immunofluorescence staining of tumors from the HNSCC xenograft model (Figure 4) for total YAP, demonstrating nuclear YAP exclusion following doxycycline (Dox)-induced knockdown of endogenous ACTL6A. Scale bar represents 20μm.

(E) Kaplan-Meier analysis of RNAseqV2 data and outcomes from >500 HNSCC cases in TCGA, showing that expression (highest versus lowest tertile) of the YAP target *CYR61*, like *ACTL6A*, is associated with decreased overall survival. P value by log-rank test.

See also Figure S7.

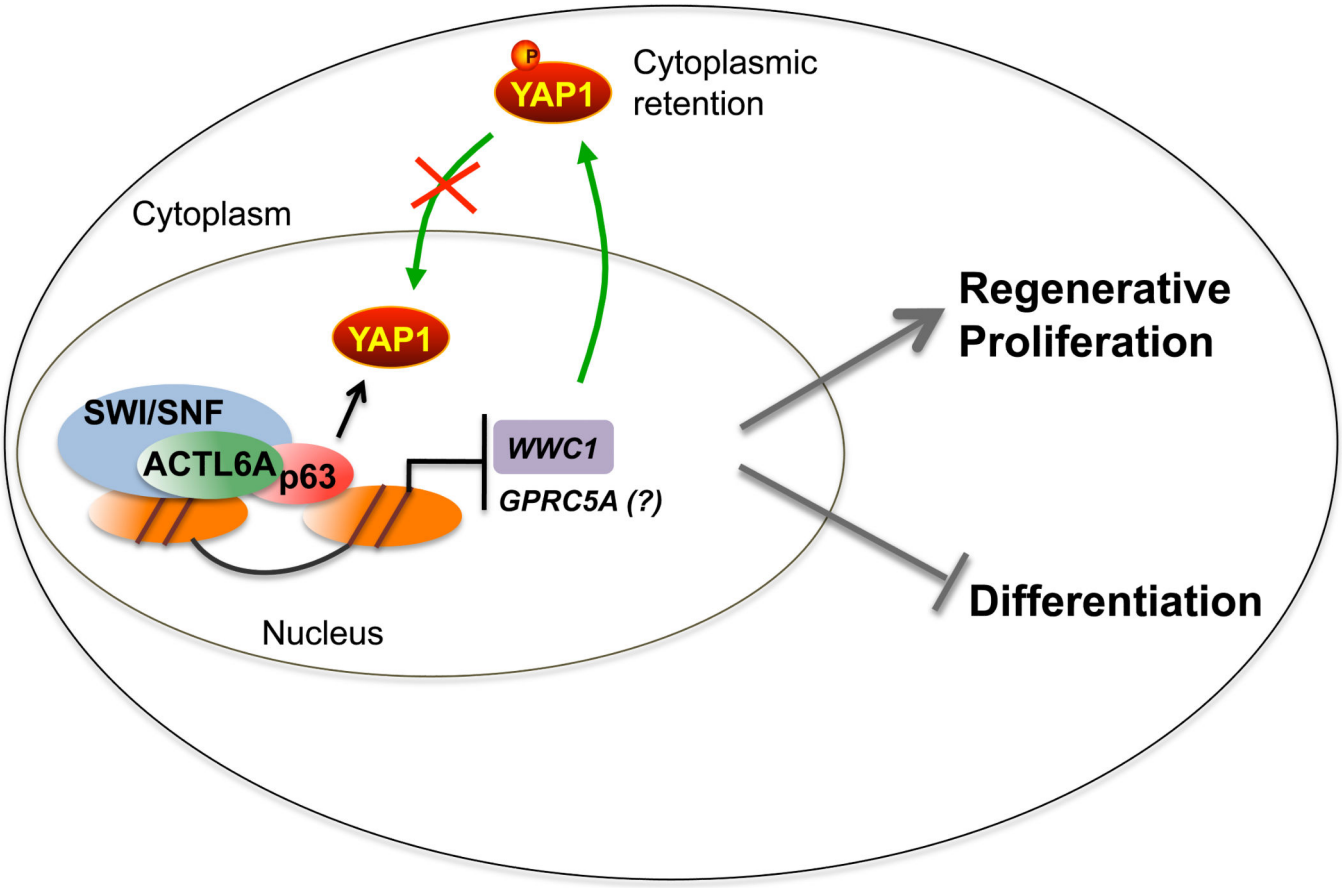


Figure 8. Model for proposed role for ACTL6A/p63 in HNSCC

Deregulated ACTL6A and p63 remodel chromatin to induce a regenerative state in HNSCC through regulation of *WWC1, GPRC5A*, and YAP expression and activity, to control proliferation, differentiation, and clinical outcomes in this disease.