Assembly and activation of the Hippo signalome by FAT1 tumor suppressor

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Dysregulation of the Hippo signaling pathway and the consequent YAP1 activation is a frequent event in human malignancies, yet the underlying molecular mechanisms are still poorly understood. A pancancer analysis of core Hippo kinases and their candidate regulating molecules revealed few alterations in the canonical Hippo pathway, but very frequent genetic alterations in the FAT family of atypical cadherins. By focusing on head and neck squamous cell carcinoma (HNSCC), which displays frequent FAT1 alterations (29.8%), we provide evidence that FAT1 functional loss results in YAP1 activation. Mechanistically, we found that FAT1 assembles a multimeric Hippo signaling complex (signalome), resulting in activation of core Hippo kinases by TAOKs and consequent YAP1 inactivation. We also show that unrestrained YAP1 acts as an oncogenic driver in HNSCC, and that targeting YAP1 may represent an attractive precision therapeutic option for cancers harboring genomic alterations in the FAT1 tumor suppressor genes.

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Persistent activation of YAP1 and its paralog WWTR1 (also known as TAZ), is a hallmark of multiple human malignancies1–3. However, the molecular mechanisms driving YAP1 activation in cancer are still poorly defined. Genetic analysis in Drosophila revealed that the activity of Yorkie (Yki), the Drosophila YAP1 ortholog, is controlled by an intricate molecular network collectively known as the Hippo pathway4. Mammalian cells, however, appear to have evolved to fine tune the activity of YAP1 by multiple signals under physiological conditions, including growth promoting and inhibitory factors, matrix composition, cell–cell contact, cell density, mechanical perturbation, and metabolic conditions, to name but a few5. The highly conserved core Hippo kinase cascade is initiated by the activation of the mammalian Hippo orthologs, MST1 and MST2 (MST1/2), which are associated with the adaptor protein WW45/SAV1. MST1/2 phosphorylates and activates LATS1/2 kinases, referred to herein as LATS, in complex with MOB. In turn, active LATS phosphorylates and inhibits the mammalian transcription co-activator YAP1 and its related protein TAZ, which are degraded or excluded from the nucleus, thereby preventing their association with their target transcription factors, including TEAD family members6. In light of the crucial role of MST1/2 and LATS in YAP1 regulation, there are surprisingly few recent alterations in these core Hippo pathway components in cancer1. Indeed, there are only a few examples of known YAP1 regulating genes altered in cancer, which include LATS2 and an upstream Hippo pathway component, NF2, in malignant mesothelioma (35% and 50%, respectively)7, and inherited NF2 mutations and microdeletions in neurofibromatosis type 28, overall accounting for a small fraction of human malignancies displaying YAP1 hyperactivity.

Here, we identify the alteration of FAT1 as a recurrent event in human cancer acting in coordination with other YAP1 activating mechanisms. We found that in normal conditions, FAT1 enables the assembly of a signaling complex including the canonical Hippo signaling components leading to phosphorylation and inactivation of YAP1. Gene deletions or truncating mutations of FAT1 result in impaired regulation of YAP1 activity. The high prevalence of these alterations underscore the crucial role of this oncogenic mechanism in human malignancies. Finally, we show that targeting unrestrained YAP1 may represent an attractive precision therapeutic option for cancers harboring genomic alterations in the FAT1 tumor suppressor genes.

Results
Widespread alterations in FAT1 in cancer. As an approach to explore the molecular mechanisms resulting in tumor-associated YAP1 activation, we investigated the presence of genomic alterations in all human orthologs of Drosophila Hippo pathway components in a large panel of 38 distinct cancers sequenced by The Cancer Gene Atlas consortium (TCGA, 14729 neoplastic lesions, Supplementary Fig. 1a)9. Among these genes, a recently developed mutation significance method (MutSigCV), which provides a statistical metric to identify driver candidates in cancer with respect to the gene nucleotide length and the background mutation rate of each cancer analyzed10, recognized only FAT1 to be significantly mutated when conducting a pancancer analysis (Supplementary Fig. 1a and b, and see below, Fig. 1a). Of interest, some members of the canonical Hippo pathway also achieved statistical significance when analyzing each cancer type individually (Supplementary Fig. 1c), suggesting their potential role in YAP activation in these specific cases. In addition to mutations, we also studied somatic copy number alterations predicted by the GISTIC2.0 method11. We found many known or candidate YAP1 and FAT regulators or associated transcription factors to be significantly amplified (MST2/STK3, TEAD4, TAZ/WWTR1, and YAP1) or deleted (DCHS2, FAT1, FAT4, LATS1/2, TEAD1, TEAD2, and KIBRA/WWC1) (Supplementary Fig. 1b). However, most of these copy number variations involve large genomic segments containing additional genes, thus precluding the identification of these Hippo pathway genes as candidate onco-drivers. Indeed, only the deletion of FAT1 and WWC1 and amplification of YAP1 appeared to be highly significant and focal, likely reflecting the specificity and biological impact of their gene copy variations.

Remarkably, both analyses converged in the recurrent and highly significant alterations of FAT1, such as somatic mutations and focal gene deletions, in multiple human malignancies (Fig. 1a, b). Some of these frequent genomic alterations in FAT1 have been recently well documented12,13. However, whether FAT1 controls the Hippo pathway is unclear, as its deficiency has been proposed to result in aberrant WNT signaling by increasing the availability of cytosolic β-catenin, similar to classical cadherins14,15. In this regard, we found that FAT1 mutations and gene copy loss occur in many cancers, such as squamous cell carcinoma of the head and neck (29.8%), lung (18.5%), and cervix (9.9%) (Fig. 1b), which are known to display expression of classical E-cadherin, hence making it improbable that loss of FAT1 will solely contribute to cell–cell contact mediated regulation of β-catenin.

Coordinated alteration of YAP1 and FAT1 in HNSCC. To begin addressing FAT1 functions in cancer, we focused on HNSCC, the cancer type in which FAT1 is most frequently mutated (Fig. 1b) and which displays characteristic Hippo signaling dependency, YAP1 overactivity, and frequent YAP1 gene amplification9,15. We posit that these findings may provide an opportunity to investigate the mechanism by which FAT1 regulates YAP in a biologically relevant cancer. The vast majority of FAT1 mutations in HNSCC are inactivating by virtue of resulting in early termination and likely truncated gene products (Fig. 1c). Moreover, expression of FAT3 and FAT4 is limited in most normal oral tissues and HNSCC lesions (Supplementary Fig. 1c). This simplified our analysis by reducing the chance of potential functional compensation between FAT family members, making HNSCC the ideal tumor type to investigate FAT1 function. Remarkably, the use of our recently developed REVEALER16 computational approach identified FAT1 truncating mutations as the top genomic abnormality complementary of YAPI gene amplification significantly associated with YAP1 activity (p = 0.01), as measured by CTGF and CYR61 overexpression, out of a total of 1589 candidate genomic abnormalities in the large cohort (n = 504) of HNSCC (Fig. 1d). This unbiased approach supported the potential role of FAT1 in the regulation of YAP1, and also identified NOTCH1 mutations and CYC1 amplification, which achieved nearly statistical significance, as additional candidate YAP1 regulating events in HNSCC.

To begin dissecting the molecular mechanisms controlled by FAT1 we next studied the gene expression profiles of 264 HNSCC tumors from the TCGA. We stratified tumor cases containing FAT1 and FAT2 alterations, including mutations, copy loss alterations or loss of expression (n = 188) and compared them to those exhibiting unaltered FAT1 and FAT2 (n = 76). Analysis of 366 differentially expressed genes between these groups (Supplementary data 1) revealed that the genes upregulated in the FAT altered group were enriched for genes containing TEAD2 and TCF4 binding sites in their promoter regions (Fig. 1e and Supplementary data 2), and thus potentially regulated by YAP1 or β-catenin, the latter consistent with prior reports13,14. We further confirmed the status of YAP1 protein activation in a panel of 111 primary HNSCC lesions and 5 normal tissues, and observed that the number of cells displaying the active, nuclear localized form
**Figure 1** Frequent alterations of FAT1 are linked to YAP1 overactivity in cancer. **a** Analysis of FAT1 alterations in human malignancies. The significance of FAT1 alterations in a panel of common human malignancies was analyzed by the MUTSIG and GISTIC methods. NS: not significant. See abbreviations and number of cases analyzed for each cancer type in Supplementary Fig. 1. **b** Graphical representation of cancer types in which FAT1 is most frequently altered. c Analysis of FAT1 mutations in 279 fully characterized HNSCC samples from TCGA. d REVEALER analysis on the TCGA HNSCC RNASeq dataset (n = 504) was used to identify genomic abnormalities that negatively correlate with YAP1 amplification and increased expression of the YAP1 targets CTGF and CYR61. e Enrichment analysis of transcription factor binding sites on upregulated genes in HNSCC samples harboring FAT1 and FAT2 alterations. f YAP1 immunohistochemistry depicting the increase of expression levels and nuclear localization (activation) during HNSCC disease progression. Scalebar upper panel, 100 μ, lower panel, 50 μ. g YAP1 staining quantification. WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated. Bars represent average plus standard error of the mean (SEM). **P < 0.01, ***P < 0.001 (One-way ANOVA)

YAP1 activation is generalized (Fig. 1f, g). This trend is similar to that observed in other tumor types such as lung squamous carcinoma, skin melanoma, and cervical cancer (Supplementary Fig. 2), whereas YAP1 activation is absent in most sarcomas and lymphoid tumors, which, coincidentally, display low alteration rates in FAT genes (<5%).

**FAT1 regulates YAP1 in mammals.** We next explored the potential link between FAT1 alterations and YAP1 activation using HEK293 cells, a widely used model system to study Hippo signaling and YAP1 function. We began investigating the impact of siRNA-mediated FAT1 and FAT2 knockdown, mimicking FAT1/2 inactivation, and observed a robust YAP1 nuclear translocation (Fig. 2a). This resulted in the YAP1-dependent increased expression of the YAP1 target genes CTGF and CYR61,
**Fig. 2** FAT1 regulates YAP1 nuclear localization and activity. 

a. siRNA-mediated knockdown of FAT1 and FAT2 in HEK293 cells induces the accumulation of nuclear YAP1 as depicted by immunofluorescence and its associated quantification. In green (Alexa 488), YAP1 staining and in blue, the nuclear counterstain DAPI. A representative experiment is shown. Scale bar, 25 µm.

b. Knockdown of FAT1 and FAT2 induces the upregulation of the YAP1 targets CTGF and CYR61 in HEK293 cells in a YAP1-dependent manner. Gene expression analysis of HEK293 cells transiently transfected with siRNAs against control (scrambled), YAP1, FAT1, and FAT2 as indicated. mRNA levels were evaluated by quantitative PCR (qPCR). Bars represent the GAPDH-normalized mean ± SEM (N = 3).

c. Schematic representation of the CD4-FAT1-ICD chimeric constructs.

d. Expression and correct plasma membrane localization of the CD4 chimeras by western blot and FACS. Arrows indicate the specific CD4-chimera bands. Predicted molecular weights CD4ext 46 kDa and both CD4-FAT1 91 kDa.

e. TEAD-Luciferase reporter assay in HEK293 cells stably transfected with the CD4-FAT1 chimeric constructs. Luciferase expression was evaluated in exponentially growing cultures 36 h after transfection. Bars represent mean Renilla-normalized luciferase expression ± SEM (N = 4).

f. Transient overexpression of CD4ext and CD4-FAT1-TM/ICD induces YAP1 nuclear exclusion in HEK293 cells 24 h after transfection. A representative immunofluorescence is shown. The nuclear or cytoplasmatic localization of YAP1 was visually evaluated in at least 100 transfected CD4 positive cells from three independent experiments and their quantification is shown in the right panel. Bars represent mean proportion ± SEM of the nuclear and cytoplasmatic localization of YAP1. Scale bar, 20 µm.

g. Quantitative PCR depicting gene expression levels of the YAP1 transcriptional targets CTGF and CYR61 in HEK293 stably expressing CD4-FAT1 ICD constructs. Bars represent the GAPDH-normalized mean ± SEM (N = 3). *P < 0.05, **P < 0.01, ***P < 0.001 (One-way ANOVA)
as it was blunted by YAP1 knockdown (Fig. 2b). In contrast, FAT1/2 knockdown in these cells did not induce the expression of the β-catenin target AXIN2. Knockdown of FAT1/2 resulted in Hippo pathway inactivation, as judged by a marked decrease in the levels of phospho-MST1 (pMST1) and phospho-YAP1 (pYAP1) (Supplementary Fig. 3a). We confirmed these findings in human oral keratinocytes (NHOK) isolated from healthy volunteers (Supplementary Fig. 3b–d). FAT1/FAT2 knockdown resulted in increased YAP1 nuclear accumulation and increased expression of CTGF and to a lesser extent AXIN2, supporting a role of FAT1/2 in the regulation of both YAP1 and WNT/β-catenin pathways in these normal cells. FAT1/2 knockdown led to increased growth of these normal epithelial cells, as reflected by their nearly doubled proliferation rate, which was blunted by the concomitant YAP1 knockdown (Supplementary Fig. 3d).

As a complementary approach to knocking down FAT1/2 expression, we next studied the impact of FAT1 overexpression in HEK293 cells. To overcome the limitation of the sheer length of the FAT1 gene, as previously noted,12,13 we used as a molecular tool the expression of the FAT1 intracellular domain (ICD) as this domain has been shown in Drosophila to mediate most of its signaling activity.19 We engineered chimeric constructs in which the very large extracellular portion of FAT1 was replaced by the extracellular domain of the human CD4 molecule, in which the transmembrane and juxta-membrane regions were provided by either FAT1 (CD4-FAT1-TM/ICD) or CD4 (CD4-FAT1-ICD) (Fig. 2c). These chimeric constructs were correctly expressed and membrane-localized in HEK293 cells, as judged by western blot and FACS analysis (Fig. 2d, and see Fig. 2f). Strikingly, when overexpressed in HEK293 cells, both FAT1 chimeras but not CD4 respectively control were sufficient to induce the downregulation of a YAP1-regulated TEAD luciferase reporter (Fig. 2e) and nuclear exclusion of YAP1 (Fig. 2f), which is consistently nuclear localized in proliferating, non-confluent HEK293 cells. The inactivation of YAP1 by expression of the CD4-FAT1 chimeric constructs was further confirmed by qPCR analysis of CTGF and CYR61 (Fig. 2g).

FAT1 forms a molecular complex with Hippo pathway members. Because YAP1 nuclear localization is regulated by the activity of the Hippo signaling pathway, we studied the regulation by FAT1 of the phosphorylation status of the Hippo pathway core components. As shown in Fig. 3a, expression of CD4-FAT1-TM/ICD in HEK293 induced the robust phosphorylation of the kinases MST1 and LATS1, as well as MOB1A and YAP1 without seemingly altering total YAP1 levels, therefore ruling out the degradation of YAP1 as a mechanism by which FAT1 regulates YAP1 activity. To dissect the underlying molecular mechanism, we used a panel of CRISPR/Cas9 engineered HEK293 cells lacking the expression of the critical Hippo pathway components LATS1/2, NF2, and MST1/2. As shown in Fig. 3b, the ability of FAT1 to stimulate YAP1 is strictly dependent on the presence of a completely functional Hippo signaling complex, as removal of any one of its components resulted in the inhibition of pYAP1 accumulation caused by the FAT-1 ICD. While MST1 activation may result from autophosphorylation24, recent reports implicating the TAOKI/2/3 kinases in the initiation of the Hippo kinase cascade in Drosophila22,23 prompted us to investigate if these kinases play a role in the FAT1-initiated pathway. As shown in Fig. 3c, the triple knockdown of TAOKI/2/3 completely impaired YAP1 phosphorylation induced by CD4-FAT1-TM/ICD expression in HEK293 cells, indicating that TAO kinases are strictly required for Hippo pathway activation triggered by FAT1.

We then investigated the ability of FAT1 ICD to physically interact with members of the Hippo pathway by using GST-FAT1-ICD fusion protein purified from bacteria (Supplementary Fig. 4a) to perform pulldown experiments using cellular lysates as indicated in Fig. 3d, using GST-LATS1 as a positive control. The ICD of FAT1 strongly interacted with MST1, SAV1, NF2, AMOT, and PATJ, which are all key Hippo regulatory components, whereas it did not interact with YAP1, and TAOK-1 (Supplementary Fig. 4b) and had only a very weak interaction with TAZ. The direct interaction of FAT1-ICD with LATS1 was not evaluated in this experiment due to cross-reactivity of the LATS1 antibody with bacterially expressed GST-FAT1-ICD; however, this interaction was demonstrated in cells in vivo (see below). Of interest, FAT1-ICD binds to total and phosphorylated MST1, but GST-LATS1 failed to interact with pMST1. Deletion mutagenesis analysis of the FAT1-ICD showed that the presence of the first 58 amino acids were sufficient to bind strongly to MST1/2, whereas the last 186 amino acids were able to interact with MST1 independently as well, albeit to a much lesser degree (Fig. 3e). LATS1 and FAT1-ICD associated equally well with SAV1, NF2 and PATJ; however in the case of AMOT, it interacted much more strongly with FAT1-ICD. Finally, unlike FAT1-ICD, LATS1 interacted strongly with YAP1 and TAZ, and at least a portion of YAP1 was phosphorylated YAP1. These results indicate that FAT1-ICD can associate with a multimeric complex containing core Hippo signaling components.

To further investigate how the formation of the FAT1-Hippo signaling complex is coordinated, we performed siRNA studies to determine their molecular hierarchy. We successfully knocked down the expression of several key Hippo pathway components (Supplementary Fig. 4c) and studied its impact on complex assembly. As shown in Fig. 3f, MST1 knockdown prevented the efficient interaction of FAT1-ICD with all other members of the complex. On the other hand, LATS1/2 knockdown partially prevented the interaction of FAT1-ICD with SAV1, AMOT and NF2, while AMOT knockdown only affected NF2 binding. Therefore, we concluded that MST1 is an essential component of the complex, likely mediating the direct interaction with FAT1-ICD and promoting the assembly of a multiprotein complex including also LATS, SAV1, NF2, AMOT1, and PATJ (Fig. 3d).

We next sought to confirm the physical interaction of MST1 with FAT1 in vivo. We were unable to consistently detect interaction of CD4-FAT1-TM/ICD with members of the Hippo cascade by standard co-immunoprecipitation and western blotting. We suspected that the failure of FAT1-TM/ICD to co-immunoprecipitate Hippo pathway components was likely due to the labile nature of the FAT-Hippo signaling complexes and their disruption in the presence of the detergent concentrations required for achieving the efficient extraction of CD4-FAT1-TM/ICD from the plasma membrane. Indeed, we readily visualized the interaction of CD4-FAT1-TM/ICD with LATS, NF2, MST1, SAV, and MOB1A upon pretreatment of the cells with a reversible cross linker, diethiobis succinimidyl propionate (DSP), prior to CD4-mediated immunoprecipitation, as shown in Supplementary Fig. 5a. Aligned with our in vitro data, this interaction is dependent on MST1/2, as siRNA-mediated knockdown of MST1/2 readily abolished the formation of immunoprecipitable complexes. Moreover, taking advantage of a monoclonal antibody recognizing FAT1 extracellular region,24 we observed that endogenous FAT1 associates with the core-Hippo kinase complex, as judged by its co-immunoprecipitation with FAT1 in DSP cross-linked cells, but not after FAT1 knockdown (Fig. 3g). Supporting the role of FAT1 in Hippo complex assembly, endogenous MST1 co-immunoprecipitates with NF2 and LATS/SAV primarily in CD4-FAT1-TM/ICD expressing cells, whereas MST1-MOB1A complex formation does
not require FAT1 (Supplementary Fig. 5b). Taken together, these results indicate that the FAT1 ICD interacts with and facilitates the assembly of the core Hippo signaling complex, and that this interaction may induce the activation of Hippo kinases by TAOKs, thereby providing a direct molecular mechanism linking FAT1 to YAP1 phosphorylation and inactivation.

**YAP1 as a molecular therapeutic target in HNSCC.** To explore whether FAT1 alterations and its associated downstream molecular signaling play a role in tumorigenesis we used representative HNSCC-derived cells displaying prototypical FAT1 alterations as evidenced by exome sequencing of a large cell panel\(^2\), and identified SCC25 (FAT1 copy loss), CAL27 (heterozygous FAT1 copy

![Diagram](image-url)

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decreased YAP1 activity as depicted by reporter assay (Fig. 4c) and displayed reduced cell proliferation (Supplementary Fig. 6a) in vitro. Remarkably, expression of CD4-FAT1 ICD, but not its controls, normal FAT1 levels (Fig. 4a) but harbor YAP1 gene copy gain25. expression using tetracycline-inducible YAP1 shRNA lentiviruses dependent on YAP1 function, we next knocked down YAP1 regulated by FAT1.

Fig. 6c). These transcriptional and biological effects of CD4-FAT1-mutant that cannot be phosphorylated by LATS (Fig. 4f and Supplementary Fig. 8a) and FSTL, while YAP1 mRNA levels remained unaffected and hence served as a control (Fig. 5b and Supplementary Fig. 8e), supporting the idea that VP causes the inhibition of YAP1 transcriptional activity. Moreover, VP reduced the proliferation of HNSCC and tumor spheroid formation in vitro (Fig. 5c, e, and Supplementary Fig. 8b, and 8f) and caused apoptosis in HNSCC cells in vitro (Fig. 5d and Supplementary Fig. 8c). We then evaluated the impact of VP in vivo in HNSCC cells in flank and oral orthotopic xenograft models. In both cases, VP reduced tumor growth and cancer cell proliferation when administered daily (Fig. 5f–k), suggesting that YAP1 is an attractive therapeutic target in human HNSCC and likely other human malignancies.

Discussion

The recent advance on massively parallel sequencing techniques has afforded an unprecedented wealth of information about the genetic makeup of human cancer. The emerging information confirmed the preponderant roles of many classical tumor suppressors and oncogenes while some other less studied molecules have been brought under the spotlight. Using HNSCC as an example, this cancer type displays widespread genetic alterations that include inactivating mutations of the tumor suppressor TP53 (70% of the cases) and mutations, homozygous deletions and promoter methylation of CDKN2A (49%), which are believed to be prerequisites for tumor formation27. This requirement of tumor suppressor inactivation can be bypassed by HPV infection27,28. Recurrent mutation and amplification of PIK3CA (35%) and to a lesser extent HRAS (4%) mutations have emerged as the most frequent oncogenic drivers in both HPV- and HPV + HNSCC, with other potential oncogenic events including EGFR, CCND1 overexpression, or PTEN inactivation27,28. Here, we provide evidence that inactivation mutations and genomic alterations in FAT1 results in YAP1 activation via inactivation of the Hippo signaling pathway, thereby contributing to the progression of HNSCC and likely multiple other human malignancies displaying unrestrained YAP1 function.

Our analysis identified genomic alterations in members of the canonical Hippo pathway, including LATS1 and NF2 in some cancers, whose potential role in YAP activation warrants further investigation in each individual cancer type. Remarkably, FAT1 mutations and focal gene deletions resulting in the loss of its protein product appear to be widespread. However, whether human FAT1 regulates the Hippo pathway has been much less clear, and if so its underlying mechanism was not known. Most
prior studies on YAP1 regulation in mammals have focused on the role of \textit{FAT4} due to its genetic relatedness to drosophila \textit{ft}19, but the role of \textit{FAT1} and other \textit{FAT} members in mammals during development is less dramatic than \textit{ft} in drosophila. In this regard, it is now evident that the regulation of the Hippo pathway has diverged from arthropods to vertebrates, likely to accommodate the unique developmental, physical and physiological features of both phyla5. \textit{FAT1} regulates the development and function of certain organs such as the kidney, while knockdown experiments in mice and rat suggest that \textit{FAT1} has an important...
role in the development of the central nervous system (CNS)\textsuperscript{29}, and acts upstream of TAZ in neural differentiation\textsuperscript{30}. Because alterations in \textit{FAT1} KO mice occur with a variable penetrance, it is possible that other \textit{FAT} family members may compensate for \textit{FAT1} loss, particularly in tissues such as the CNS during development, in which all \textit{FAT} family members are consistently expressed\textsuperscript{13}, likely precluding the display of stronger phenotypes. Indeed, our analysis identified MST/Hippo binding sites in \textit{FAT1} ICD in areas that are conserved in other \textit{FAT} family members, thus providing a potential structural basis for their redundancy. As FAT expression levels drop in adult tissues\textsuperscript{32}, it is possible to hypothesize that this mechanistic compensatory effect might be reduced in cancers arising during adulthood. For example, disruption of \textit{FAT1} function could be sufficient to dysregulate Hippo signaling in the squamous epithelium, as this tissue expresses \textit{FAT3} and \textit{FAT4} poorly and \textit{FAT2} might not be sufficient to compensate \textit{FAT1} deficiency fully.

Overall, our oncogenic and functional analyses support the role of \textit{FAT1} in cancer as a tumor suppressor acting upstream of YAP1. Of interest, this raises the possibility that \textit{FAT1} may control \(\beta\)-catenin, as previously reported\textsuperscript{13}, and \textit{YAP1} independently. Alternatively, \textit{FAT1} deficiency may promote \(\beta\)-catenin activation indirectly through a recently described mechanism downstream from \textit{YAP1}\textsuperscript{33}. Further studies re-expressing full length \textit{FAT1} and its deletion mutants in \textit{FAT1} defective cells may facilitate addressing these exciting possibilities, as well as extend our analysis using \textit{FAT1} ICD chimeras. Mechanistically, we show that chimeric and endogenous \textit{FAT1} signals to \textit{YAP1} by its direct interaction with \textit{MST1} and the consequent association with a multimeric signaling complex including the core Hippo pathway kinases and their regulatory and scaffolding molecules, such as \textit{AMOT} and NF2. This, in turn, may result in the activation of \textit{MST1} by \textit{TAOKs} and the subsequent phosphorylation of \textit{LATS} and \textit{YAP1} (Fig. 5I). How precisely \textit{FAT1} association to \textit{MST1} may lead to its phosphorylation by \textit{TAOKs} is not fully understood. We can speculate that the physical interaction between \textit{LATS1} and \textit{FAT1} and the assembly of the Hippo kinase complex at the plasma membrane may relieve \textit{MST1} from an auto inhibitory mechanism\textsuperscript{21}, thereby enabling its recognition and phosphorylation by \textit{TAOKs}, ultimately leading to the activation of the Hippo kinase cascade (Fig. 5I). This and other possibilities by which \textit{FAT1} may regulate \textit{YAP1}, including the recruitment of \textit{TAOKs} to the proximity of \textit{MST1} by additional yet to be identified Hippo signaling complexes, or \textit{FAT1} and \textit{TAOK} acting on other Hippo pathway components warrant further investigation.

The high frequency of alterations in the \textit{FAT} gene family can now provide a widespread mechanism contributing to the recurrent activation of \textit{YAP1}/TAZ in human malignancies, in addition to other more restricted processes in other tumor types\textsuperscript{34–36} (Fig. 5I). In turn, targeting \textit{YAP1}/TAZ function or their relevant downstream targets poses a very attractive therapeutic venue that also justifies further investigation. In this regard, while VP cannot be considered a specific \textit{YAP1} inhibitor and is likely to impact several other molecular targets in addition to blocking TEAD-YAP1 interactions\textsuperscript{37,38}, this FDA-approved agent decreased the expression of \textit{YAP1}-regulated genes and its administration resulted in increased apoptosis, and decreased proliferation and HNSCC tumorigenesis in vivo, mimicking \textit{YAP1} knockdown in these cells. These observations provide further support of the potential benefits of the use of pharmacological inhibitors of \textit{YAP1} in cancer. Specifically for HNSCC, our results indicate that the frequent alteration of \textit{FAT1} might result in defective Hippo signaling and unrestrained \textit{YAP1} activity, thus raising the possibility that molecular therapies targeting \textit{YAP1} or its downstream targets may represent an attractive precision medicine therapeutic option for the treatment of this and other malignancies harboring genomic activation in the \textit{FAT} family of tumor suppressor genes.

**Methods**

**Antibodies and reagents.** Antibodies against \textit{YAP1}, p\textit{MST1/2}, \textit{MST1}, \textit{MOB1}, p\textit{LATS1/2}, LATS1, LATS2, AMOT, NF2, SAV1, \textit{PATJ}, p\textit{YAP1}, p\textit{YAP539} (1:2000), and Tubulin-HRP (1:10000) were purchased from Cell Signaling Technology (Beverly, MA). A monoclonal antibody against \textit{FAT1} (NDTD1, 1:1000) against the \(n\)-terminal region has been recently described\textsuperscript{14}. Cytokeratin 10 rabbit polyclonal antibody was purchased from Covance (Princeton, NJ) (1:10000), anti-BrDU (Bromodeoxyuridine) Rat monoclonal antibody was from Accurate (Westbury, NY). Anti-\textit{TAZ} antibody was purchased from Santa Cruz (Santa Cruz, CA)(1:2000). Antibodies anti-CD4 (OKT4) was from eBioScience (San Diego, CA)(1:2000). Verteportin (VP) was purchased from USP (Rockville, MD).

**Cell lines, culture conditions and transfections.** \textit{CAL27}, \textit{CAL33}, and \textit{HN12} cell lines were obtained from the NIDCR Oral and Pharyngeal Cancer Branch cell collection\textsuperscript{25}. Their identity was confirmed by STR profiling and were tested free of mycoplasma infection. HEK293 were purchased from ATCC (Manassas, VA). \textit{CAL27}, \textit{CAL33}, \textit{HN12}, and \textit{HEK293} cell lines were cultured on DMEM (D-6429, Sigma-Aldrich, St. Louis, MO), 10% fetal bovine serum, 5% CO\textsubscript{2}, at 37 °C. Normal Human Oral Keratinocytes (HOKN) lines were prepared as described before\textsuperscript{39} and grown on Defined Keratinocyte-SFM with supplements and antibiotics (Life Technologies, Carlsbad, CA). \textit{LATS1/-2}, \textit{MST1/-2}, or \textit{NF2-deficient HEK293A} cells were created through the CRISPR/Cas9 system as described before\textsuperscript{40}. \textit{SIRNAs SMARTpool ON-TARGETplus for FAT1, FAT2, SAV1, NF2, non-targeting control, and \textit{YAP1} inducible lentiviral shRNAs, clones V2THS_h05509 and V2THS_247011 were from GE Healthcare (Lafayette, CO). \textit{SIRNAs} for \textit{MST1} were purchased from IDT (DsiRNA Duplexes human MST5-AGGUGUCGAACUUAAACA 5'-AGGUCAUGUUAAGCCT, IDT, Corvalle, IA). \textit{SIRNAs} for \textit{LATS1} and \textit{LATS2} were from Sigma (MISSION siRNA human LATS1 SASI_Hs01_00046128, siRNA human LATS2 SASI_Hs01_00158083)\textsuperscript{40}.

![Fig. 4](image-url) **YAP1** is required for HNSCC survival and proliferation in vitro and in vivo. **a** Analysis of YAP1 expression in a panel of epithelial cells, including HNSCC. **b** Expression of CD4ext and CD4-FAT1-TM/ICD chimera in the HNSCC cell line \textit{CAL33} by CD4 FACS analysis. **c** TEAD-Luciferase reporter assay in \textit{CAL33} cells transiently transfected with the \textit{CD4-FAT1} chimeric reporter. Luciferase expression was evaluated in exponentially growing cultures 36 h after transfection. Bars represent mean Renilla-normalized luciferase expression ± SEM (\(N = 4\)). **d** Quantitative PCR depicting gene expression levels of the \textit{YAP1} transcriptional targets \textit{CTGF} and \textit{CYR61} in \textit{CAL33} stably expressing the \textit{CD4-FAT1} ICD constructs. Bars represent the GAPDH-normalized mean ± SEM (\(N = 3\)). **e** In vivo xenograft assay. One million \textit{CAL33} cells expressing indicated constructs were injected in \textit{nu}/\textit{nu} mice. Data points represent mean volume (\(N = 10\) tumors per group) ± SEM. **f** \textit{YAP1}-rescue experiments. In vivo flank xenograft assay as in (e) using \textit{CAL33} expressing \textit{CD4-FAT1-TM/ICD} and control or the indicated \textit{YAP1} expression vector. Data points represent mean volume (\(N = 10\) tumors per group) ± SEM. **g** Spheroid formation assay of stable \textit{CAL33} shRNA control and \textit{YAPI} shRNA cell lines. Representative pictures are shown on top and diameter quantifications (>200 colonies per group) are shown below. Black lines represent mean ± SEM. **h** \textit{CAL33} stably expressing control and \textit{YAPI} shRNAs were stimulated with doxycycline for five days (1\(\mu\)g/ml) and then transfected with a \(8\times\)TEAD-luciferase reporter. Renilla-normalized reporter activity is shown as % of control. Bars represent mean ± SEM (\(N = 4\)). **i** Apoptosis assay by propidium iodide staining of \textit{CAL33} cells lines expressing control or \textit{YAPI} shRNAs after 5d of Doxycycline stimulation. Bars represent mean ± SEM (\(N = 4\)). **j** In vivo xenograft assay. One million cells were injected in \textit{nu}/\textit{nu} mice. Animals were fed Doxycycline food (5\% Kgd) ad libitum 24 h after tumor cell injection for the duration of the experiment. Data points represent mean volume per group (\(N = 10\) tumors) ± SEM. **k** Representative immunohistochemical staining of \textit{CAL33} cells tumors from panel (j). In the Cytokeratin 10 (CK10) panels the dotted red line delimits the proliferating front of the tumor. Scale bar, 100 μm. **l** Automated histological quantification of stainings in (k), bars represent mean ± SEM (\(N = 3\)). **P < 0.01, ***P < 0.001 (One-way ANOVA).
**Immunohistochemistry.** H&E stained paraffin sections were used for histopathological evaluation. For immunohistochemistry, 5-μm unstained paraffin sections were deparaffinized in three changes of SafeClear II (ThermoFisher Scientific, Waltham, MA), 5 min each, and the hydrated with graded alcohols (100°, 95°, 70°), two changes each, 5 min each. The endogenous peroxidase was blocked by incubating for 30 min in 3% H2O2 in 70° ethanol. Antigens were retrieved with 10 mM citric acid (2.1 g/L) in a microwave oven, 2 min at 100% power, followed by 18 min at 20%. The slides were allowed to cool for 15 min and washed extensively with distilled water, followed by three changes of PBS, 5 min each. After blocking with 2.5% BSA in PBS at room temperature, for 30 min, the slides were incubated overnight at 4 °C with the appropriate primary antibodies diluted in 2.5% BSA in PBS. The slides were then washed with PBS, 3 × for 5 min, and successively incubated biotinylated anti-rabbit/rat immunoglobulins, 1:400 in blocking buffer at room temperature, for minutes, washed with PBS 3 × for 5 min each, and incubated with ABC complex (Vector Labs, Burlingame, CA), 30 min at room temperature. The slides were extensively washed with PBS; the reaction was developed with

![Viability assay](image)

![Gene expression CAL33](image)

![Cell proliferation CAL33](image)

![Apoptosis CAL33](image)

![Orosphere assay CAL33](image)

![Tumor growth CAL33](image)

![Tumor xenografts](image)

![Orthotopic tumor growth CAL33](image)

![Orthotopic xenografts](image)

![Normal cell](image)

![Cancer cell (FAT1 defective)](image)
3.3'-Diaminobenzidine under microscopic control and stopped with distilled water. The slides were the counterstained with Hematoxylin and washed 15 min in running water. They were then dehydrated in ethanol and cleared in SafeClear II and mounted in Permount mounting medium (ThermoFisher Scientific). The histological slides were processed and developed at the same time to minimize inter-assay variability. All stained slides were scanned at ×40 using an Aperio CS Scanscope (Aperio, Vista, CA) and quantified using the available Aperio algorithms.

**Immunofluorescence and image quantification.** NOK were seeded on the coverslips coated with collagen, 293 cells were seeded on coverslips coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO). For immunofluorescence, the cells were washed with ice-cold PBS and fixed with 3.2% paraformaldehyde in PBS. Then the cells were washed three times with PBS and permeabilized with Triton X-100 0.1% in glycerine 200 mM in PBS. Nonspecific binding was blocked with 3% of bovine serum albumin (BSA) in PBS for 1 h. Fixed cells were incubated with the primary antibody overnight at 4 °C, followed by 1.5 h incubation with the secondary antibody. Nuclei were stained with Hoechst 33342 (Life Technologies). Images were taken using Zeiss Axio Imager Z1 microscope equipped with an Apotome device (Carl Zeiss, Peabody, MA) and a motorized stage. Final images were bright contrast adjusted with Zen 2012 (Carl Zeiss) or PowerPoint. Image quantifications were performed with ImageJ with the MBI Image bundle (http://www.macrophotoinc.ca/image/installing_image.htm). For nuclear YAP the intensity per nucleus was quantified by drawing a region of interest around nuclei and calculating the average gray value per nucleus.

**Quantitative PCR.** RNA was extracted from exponentially growing cultures by the Trizol method following manufacturer’s recommendations (Life Technologies). Samples were treated with RNase-free DNase (Fermentas) and transferred to microtubes with miliQ water after processing was poor (RIN < 7). One microgram total RNA was converted to cDNA using the Superscript III kit (Invitrogen). One microgram cDNA was used as input for the PCR reactions. GAPDH was used for normalization.

**Western blotting.** Exponentially growing cells were washed in cold PBS, lysed on ice in RIPA buffer (0.5% NaDOC, 0.1% SDS, 25 mM Heps pH 7.5, 100 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1% Triton X-100, 20 mM β-glycerophosphate, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1× Complete Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN), and cell extracts collected, sonicated, and centrifuged to remove the cellular debris. Supernatants containing the solubilized proteins were quantified using the detergent compatible PC protein estimation kit (Bio-Rad, Hercules, CA); equal amounts by mass were separated by SDS-PAGE, and transferred to PVDF membranes (Millipore Corporation, Billerica MA). Equivalent loading was confirmed with Ponceau-S staining. For immunodetection, membranes were blocked for 1 h at room temperature in 5% non-fat dry milk in T-TBS buffer (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.1% [v/v] CHAPS, 1.5 mM MgCl2, 1 mM EGTA, 100 mM NaF, 500 μM sodium orthovanadate, 10 μg/mL aprotinin, and 10 μg/mL leupeptin and 1 μM PMSF). The pull-down was performed by adding 20 μL of beads (or 10 μg of protein) to 1 mL of cell lysate and incubated at 4 °C for 2 h with their corresponding GST beads (Amersham, Piscataway, NJ). Beads were then washed three times with pulldown buffer, resuspended in Laemmli sample buffer and resolved by SDS-PAGE.

**Immunoprecipitation, kinase assay, and crosslinking.** Exponentially growing cultures in 150 mm plates were lysed in CHAPS buffer (1 mL of 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 6 mM CHAPS, 1.5 mM MgCl2, 1 mM EGTA, 100 mM NaF, 500 μM sodium orthovanadate, 10 μg/mL aprotinin, and 10 μg/mL leupeptin and 1 μM PMSF). A volume of 15 μL of anti-CDC4 antibody (OKT4 clone, ebioscience) and 20 μL of Gambambid G Sepharose beads (GE Healthcare) were premixed in 500 μL CHAPS buffer and then combined with the extract and incubated on a rocker for 2 h at 4 °C. Beads were pelleted by centrifugation, the supernatant was washed twice with CHAPS buffer, once in wash buffer (40 mM HEPES, 200 mM NaCl), and once in kinase assay buffer (30 mM HEPES, 50 mM potassium acetate, 5 mM MgCl2). The immunoprecipitate was subjected to a kinase assay for 30 min at 30 °C in the presence of 200 μM ATP and 100 ng of GST-MOB1 A beads expressed and purified in E. coli BL21 cells. Kinase reactions were stopped by resuspension in Laemmli sample buffer and resolved by SDS-PAGE. Crosslinking experiments were performed as described elsewhere, differing in the immunoprecipitation step that was performed above.

**Cytometry.** For apoptosis determination, the cells were trypsinized, washed once with PBS, and fixed in 50% ethanol for 30 min at 4 °C. After fixation, the cells were centrifuged, resuspended in a 1:125% sodium citrate, and 10 μg/mL RNase A in PBS and incubated at 37 °C for 30 min. Propidium iodide (PI) was added to a final concentration of 50 μg/mL 5 min before the analysis. Samples were analyzed in a FACScalibur cytometer (Becton Dickinson, Franklin Lakes, NJ). For anti-CDC4 staining, the cells were collected by incubation on PBS/EDTA/FBS (2 mM EDTA and 0.005% FBS in PBS) until detached. The cells were washed once in PBS/BSA (3% BSA in PBS), resuspended in blocking buffer (3% mouse serum in PBS) and incubated for 20 min on ice. FITC-conjugated anti Human CD4 antibody (BD Biosciences, San Jose, CA) was added (1:100) and incubated for 30 min on ice. The cells were washed once with PBS/BSA and immediately analyzed in and FACS-calibur cytometer.

**Reporter assays.** HEK293 cells were transfected in 24-well plates with 1 μg the CD4 chimeric expression vectors in combination with 100 ng of a 8 × TEAD Firefly luciferase reporter (Addgene ID 34615, Addgene, Cambridge, MA). 100 ng of a Pol III-driven Renilla Reniformis Luciferase for normalization (pRL U6) and 100 ng of pCEFL mcerry, an EF-1a-driven mCherry expression vector to monitor transfection efficiency. Luciferase activity was measured 24 h after transfection in a Biotech Synergy Neo luminometer (Winooski, VT).

**GST Pulldowns.** GST-LATS1-142-742 was obtained by PCR amplification from the vector p2xFlag CMV2 LATS1 (a gift from Marius Suldol) and cloned into pgEX4T3 with adapters for BamHI and NotI. GST-FAT1-ICD was generated by subcloning FAT1-ICD from pCEFL CD4-FAT1-ICD into the BamHI and NotI sites of pGE4T3. Both GST fusion proteins, and GST as a control, were expressed in BL21 E.coli transfected with each pgEX4T3 construct and coupled to GST- Sheperose beads. To incubate the GST beads with mammalian cell extracts, HEK293 cells were cultured in 15 cm plates and, upon reaching confluency, they were quickly washed twice in ice-cold PBS and then lysed in pulldown buffer (1 mL of 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 0.3% (w/v) CHAPS, 1.5 mM MgCl2, 1 mM EGTA, 100 mM NaF, 500 μM sodium orthovanadate, 10 μg/mL aprotinin, and 10 μg/mL leupeptin and 1 mM PMSF). The pull-down was performed by adding 20 μL of beads (or 10 μg of protein) to 1 mL of cell lysate and incubated at 4 °C for 2 h with their corresponding GST beads (Amersham, Piscataway, NJ). Beads were then washed three times with pulldown buffer, resuspended in Laemmli sample buffer and resolved by SDS-PAGE.
**Spheroid formation.** Spheroid (orosphere) assays were performed in 24-well plates. Briefly, the wells were coated with 250 μL, 1.5% agarose in DMEM. Ten thousand cells were resuspended in 0.5 mL warm DMEM, 10% FBS, and 0.1% agarose containing the appropriate treatment and layered on top. Agarose was allowed to solidify at room temperature for 15 min before adding an additional 0.5 mL of DMEM, 10% FBS containing the same treatment as above, and returning the cells to the incubator. The cells were allowed to grow for two weeks, replacing the liquid media every three days before spheroid formation was analyzed. For quantification, the wells were photographed using an automatic wide well scan mode in a Zeiss Axiosvert 200 M microscope. Colony counting and diameter measurements were performed manually using the Zeiss Axiovision 4.8 software.

**Animal work.** All animal studies were carried out according to NIH-approved protocols, in compliance with the Guide for the Care and Use of Laboratory Animals (http://www.niaid.nih.gov) and approved by the University of California San Diego (UCSD) Institutional Animal Care and Use Committee (IACUC). All handling, transplantation, and infection procedures were conducted in a laminar-flow biosafety hood. Female athymic (nu/nu) nude mice (Harlan Sprague-Dawley, Frederick, MD), 5–6 weeks of age and weighing 18–20 g, were used in the study, and housed in appropriate sterile filter-capped cages and fed and watered ad libitum. No randomization was used prior to experimental group assignment except when indicated. Briefly, exponentially growing HNSCC cultures were harvested, washed, resuspended in DMEM, and 1 × 10^6 viable cells were transplanted s.c. into both flanks of the athymic mice. For orthotopic injections, SCID/NOD 5–6-weeks of age female mice were used. Briefly, 1 × 10^6 resuspended in 50 μL of DMEM were used and injected in the tongue from the ventral aspect of the tongue following a posterior to anterior trajectory of the needle, so that the cells accumulate toward the tip of the tongue. The investigators were not blinded to allocation of samples during experiments and outcome assessment. For tumor growth analysis, tumor volume was determined, with volume (V) = ((LW^2)/2); were L, whereby tumor volume (Vunits); and W represent the length and the width of the tumor) was converted to weight (mg) assuming unit density. No statistical method was used to estimate animal sample size. The animals were monitored twice weekly for tumor development. Results of animal experiments were expressed as mean ± SEM, and unpaired Student’s t-test was used to determine the difference between experimental and control groups for each of the transplanted cell lines. p < 0.05 was considered to be statistically significant.

**Proliferation and viability.** Population doubling was calculated as described elsewhere. For viability assays, cells in either 96 well plates were treated as indicated and supplemented with 1/10 of the volume of culture of AlamarBlue (Invitrogen, Carlsbad, CA) reagent for the last 6 h of the treatment. Absorbances were recorded at 570 nm in a Biotek Synergy Neo microplate reader. Custom python code used during the TCGA analysis is available upon request.

**REVEALER analysis.** The REVEALER analysis finds mutually exclusive genetic variants that correlate with a “functional” phenotype such as the activity of YAP1. We used mutations and copy number alterations from the level 3 TCGA HNSCC data from the Fire Hose Portal (https://gdc.broadinstitute.org/). The REVEALER analysis included pre-filtered variants from the mutation and copy number HNSCC TCGA datasets (a total of 1589 candidate genomic abnormalities). The functional target for REVEALER was the mean normalized expression of CTGF and Cyr61 from the TCGA HNSCC RNAseq dataset. As the input “seed” to REVEALER we used the amplification status of YAP1 (YAP1_AMP).

**Statistical analysis.** No statistical method was used to predict sample size. The investigators were not blinded to allocation of samples during experiments and outcome assessment. All analyses were performed in triplicate or greater and the means obtained were used for ANOVA or independent, two-tailed, unequal t-tests. Statistical analysis for variance estimation and validation of treatment effects were carried out using the Prism 6 statistical analysis program (GraphPad). Asterisks denote statistical significance (non-significant or N.S., p > 0.05; *p < 0.05; **p < 0.01; and ***p < 0.001). All data are reported as mean ± standard error of the mean (S.E.M.).

**Data availability.** All data supporting the findings in this study are available from the corresponding author upon reasonable request.

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Appendix

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Author contributions
D.M., B.S., and J.S.G. initiated the study; D.M. and J.S.G. designed the study and experiments; D.M. performed the genomic analyses; D.M., M.S.D., and J.L.C., performed in vitro interaction experiments, D.M., L.V.C., and Z.W. performed the in vivo studies; R.I.B performed FAT1 knockdown and immunohistochemistry experiments; X.F. performed quantitative gene expression analyses; M.Z. performed the spheroid assays; H.Y., V.M., and P.T. performed TCGA and REVEALER analyses; T.M. performed the TAOK K.O. and interaction analyses; D.M. and J.S.G. prepared the manuscript, S.C.K., Y.S., R.T., K.L.G., and J.G.L. provided antibodies, tissue samples, biological reagents, and advice. S.M.L. provided critical advice and guidance with the study design and manuscript writing, and valuable resources. All authors discussed the results and reviewed the manuscript.

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