NF2 loss promotes oncogenic RAS-induced thyroid cancers via YAP-dependent transactivation of RAS proteins and sensitizes them to MEK inhibition.

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Running title: NF2 loss transactivates oncogenic RAS through YAP.
Abstract

Ch22q LOH is preferentially associated with RAS mutations in papillary and in poorly differentiated thyroid cancer (PDTC). The 22q tumor suppressor NF2, encoding merlin, is implicated in this interaction because of its frequent loss of function in human thyroid cancer cell lines. Nf2 deletion or Hras mutation are insufficient for transformation, whereas their combined disruption leads to murine PDTC with increased MAPK signaling. Merlin loss induces RAS signaling in part through inactivation of Hippo, which activates a YAP-TEAD transcriptional program. We find that the three RAS genes are themselves YAP-TEAD1 transcriptional targets, providing a novel mechanism of promotion of RAS-induced tumorigenesis. Moreover, pharmacological disruption of YAP-TEAD with verteporfin blocks RAS transcription and signaling, and inhibits cell growth. The increased MAPK output generated by NF2 loss in RAS-mutant cancers may inform therapeutic strategies, as it generates greater dependency on the MAPK pathway for viability.

Significance:

Intensification of mutant Ras signaling through copy-number imbalances is commonly associated with transformation. We show that NF2/merlin inactivation augments mutant RAS signaling by promoting YAP/TEAD-driven transcription of oncogenic and wild-type RAS, resulting in greater MAPK output and increased sensitivity to MEK inhibitors.
Introduction

Neurofibromatosis type 2 is an autosomal dominant syndrome caused by germline heterozygous mutations of NF2, which encodes Merlin. It is characterized by tumors of the nervous system such as schwannomas, meningiomas and ependymomas. The associated neoplasias often harbor loss of heterozygosity (LOH) of the chromosome 22q region encompassing the NF2 gene (1). Other malignancies associated with NF2 defects include mesotheliomas, melanomas and clear cell renal cancers.

Merlin inhibits cell growth in response to cell contact. It interacts with multiple partners to modulate distinct pathways including Hippo (2), receptor tyrosine kinases (RTK) (3, 4), Rac/Cdc42/p21-activated kinases (PAK) (5-7) and mTOR (8, 9). Merlin also has a nuclear function by inhibiting the CRL4<sup>DCAF1</sup> E3 ubiquitin ligase (10). Hippo is an evolutionarily conserved kinase cascade that suppresses tissue overgrowth through phosphorylation of YAP, leading to its sequestration in the cytoplasm and disrupting its ability to promote transcriptional enhancer activation domain (TEAD)-dependent transcription of genes involved in proliferation and survival (11-14). Despite the critical role of the Hippo pathway in growth control, NF2 is the only commonly mutated cancer gene in this pathway(15). The lineage-specific properties and the genetic repertoire intrinsic to different cancer types may predispose NF2-deficient cells to be preferentially addicted to distinct pathways. For instance, Merlin loss activates effectors of mTOR in meningiomas, schwannomas and mesotheliomas and confers sensitivity to rapamycin (8, 9, 16). In glial cells, merlin loss induces cell growth in an Erbb2-dependent manner (17). By contrast, hepatocellular carcinomas in mice with hepatocyte-targeted deletion
of Nf2 have been variously reported to be dependent on Hippo (18) or on EGFR signaling (19).

Papillary thyroid cancers (PTC) are indolent tumors associated with mutually exclusive mutations of BRAF, RAS and of fusion RTK oncogenes, such as RET, NTRK1 and NTRK3 (20). The driver frequency is different in poorly differentiated (PDTC) and anaplastic thyroid cancers (ATC), in that the latter are enriched for RAS mutations (21-23). Here we show that NF2 is a novel thyroid tumor suppressor, preferentially associated with RAS mutations. Although loss of Nf2 or Ras activation is insufficient to independently induce thyroid cancers in mice, their combination is highly tumorigenic. NF2 loss cooperates with mutant RAS to increase signaling via MAPK, acting in part through YAP-induced transcriptional activation of oncogenic and wild-type RAS, providing a novel mechanism of promotion of RAS-induced tumorigenesis. This has therapeutic implications, as these and other inputs resulting from merlin deficiency converge to confer preferential sensitivity to selective MEK inhibitors in vitro and in mouse genetic models of the disease. In addition, pharmacological disruption of the YAP-TEAD transcriptional complex decreases expression of oncogenic and wild-type RAS and inhibits tumor cell growth.
Results

Loss of chromosome 22q in PTC, advanced thyroid cancers and thyroid cancer cell lines: The Cancer Genome Atlas recently completed an analysis of ~ 400 PTCs, which showed a high frequency of ch22q loss in RAS-mutant PTC (45% had 22q LOH) (24) (Supplementary Table S1). The association was particularly striking for HRAS: 10/14 (71%) (p<5 x E-6; OR >10). ch22q is the only region of copy number variation in the genomes of most of these cancers, which are otherwise diploid, suggesting that one or more tumor suppressors on 22q play an important role in tumorigenesis. We found that PDTCs also had a high frequency of 22q LOH (14/63; 22%) as determined by SNP-CGH and/or copy number analysis of sequence reads derived from an exon capture NGS panel of 341 cancer genes, 6 of which mapped to Ch22q (Supplementary Table S1; Supplementary Fig. S1A-D). Although most tumors had LOH of all 6 genes, spanning the majority of the chromosome arm, CHEK2, NF2 and EP300 were consistently lost. As was the case in PTC, Ch22q LOH in PDTCs was seen preferentially in association with RAS (8/16; 50%) as compared to BRAF-mutant tumors (0/26). Five of the 20 ATCs were RAS mutant, one of which had 22q LOH (Supplementary Table S1).

Of the cancer genes mapping to Ch22q, we focused in greater detail on NF2 because 3/40 thyroid cancer cell lines had homozygous nonsense mutations of this gene (Cal62: c.643G>T, pE215*; 8505c: c.385G>T, p.E129* and TCO-1: c.303T>A, p.Y101*). In addition, the KHM-5M ATC cell line had a homozygous deletion of exon 4 of NF2 that disrupts the central FERM domain of merlin, previously reported in neurofibromatosis.
patients (25) (Supplementary Fig. S2). Consistent with the low frequency of homozygous NF2 inactivation in cell lines, there are limited data supporting biallelic NF2 inactivation in primary thyroid cancers. Indeed, NF2 mutations in tumor samples were rare other than for one ATC with a somatic G>A substitution at the −1 position of the intron 14/exon 15 boundary (splice donor site), which removes exon 15 and impairs the biological effects of merlin (26). As ATCs are heavily infiltrated with macrophages, which decrease sensitivity of genomic profiling, we expanded the analysis of NF2 copy number by performing FISH on ATC tissue microarrays, which showed that 10/16 had NF2 LOH, one of which had a homozygous deletion (Supplementary Fig. S3A,B).

Several thyroid cancer cell lines that were wild-type or hemizygous for NF2 had very low or absent merlin mRNA and/or protein levels (Supplementary Fig. S4A, B). Despite lower NF2 mRNA, we did not detect aberrant methylation patterns of CpG islands in the promoter of NF2 in cell lines or tumors (not shown). Interestingly, the Hth74 ATC cell line had a markedly decreased NF2 mRNA half-life (Supplementary Fig. S4C). Hence, as reported in other lineages, loss of merlin in thyroid cancers occurs through diverse mechanisms (Supplementary Fig. S4D): LOH or intragenic deletions, somatic base substitutions as well as posttranscriptional events (27-30).

Mice with thyroid-specific activation of Hras<sup>G12V</sup> and Nf2 loss develop PDTC: In view of the strong association between NF2 and RAS in human thyroid cancers, we next explored the potential biological significance of this interaction in mouse models. Endogenous expression of Hras<sup>G12V</sup> in thyroid cells was achieved by activating the
latent FR-Hras\textsuperscript{G12V} allele by crosses with TPO-Cre mice (31) (Fig. 1A). None of the TPO-Cre/FR-Hras\textsuperscript{G12V} heterozygous or homozygous animals developed thyroid cancer. Although ~ 65% of mice with thyrocyte-specific homozygous deletion of Nf2 (TPO-Cre/Nf2\textsuperscript{floxed}) exhibited mild nodular hyperplasia after 18 months, none developed cancer (Supplementary Table S2). By contrast, TPO-Cre/FR-Hras\textsuperscript{G12V}/Nf2\textsuperscript{floxed} mice developed large thyroid cancers with high penetrance (Fig. 1B). Most of these were PDTCs, and were associated with a marked increase in pERK (Fig. 1C).

**Enforced merlin expression inhibits growth of human NF2-null thyroid cancer cells and attenuates RAS-induced signaling:** We next investigated mechanisms accounting for the NF2-RAS interactions seen in vivo in RAS-mutant human thyroid cancer cell lines. Re-expression of wild-type (WT) merlin in RAS-mutant / NF2-null Cal62 and Hth83 cells decreased growth and colony formation in soft agar, whereas the loss-of-function mutant NF2-L64P (32) was without effect (Fig. 2A). NF2, but not NF2-L64P also decreased pMEK and pERK in these cells (Fig. 2B). Conversely, knockdown of merlin in HRAS-mutant /NF2-WT C643 cells enhanced growth and MAPK signaling (Fig. 2C).

Merlin loss enables sequestration of Rich1, a GTPase activating protein, by a tight junction protein complex, which de-represses Rac1, leading to activation of Raf-MEK signaling (33). We found that this pathway was also operative in the setting of constitutively activated RAS (Supplementary Fig. S5). Expression of merlin suppressed Rac1-GTP as well as phosphorylation of its effector p21-activated kinase (PAK) at S141.
and T423. This in turn decreased CRAF and MEK phosphorylation at S298, a PAK substrate. In a reciprocal experiment, merlin silencing was associated with increased pPAK-T423 (Supplementary Fig. S5A, B).

Expression of a dominant negative PAK construct (PAK1 83-149) modestly suppressed growth of KRAS$^{G12R}$/NF2 null Cal62 cells. As predicted, this was associated with decreased phosphorylation of the PAK substrate S298-MEK. However, pS217/p221-MEK and pERK were not significantly reduced, suggesting that, in the context of mutant RAS, merlin loss augments MAPK signaling through alternative mechanisms (Supplementary Fig. S5C). Consistent with this, the effects of merlin on PAK signaling were apparent 24h after dox-induction (Supplementary Fig. S5B), whereas pS217/p221-MEK and pERK were seen to decrease beginning at 48h (Fig. 2B). The ATP-competitive PAK kinase inhibitor FRAX597 also preferentially suppressed PAK phosphorylation in merlin-silenced C643 cells, with no apparent effects on pERK (Supplementary Fig. S5D). It also inhibited cell growth after merlin knockdown, although it was toxic at higher concentrations, possibly through off-target effects.

Upon cell-cell contact merlin reportedly prevents internalization and signaling of the epidermal growth factor receptor (EGFR) by sequestering it into an insoluble membrane compartment (3, 4). The impairment of EGFR signaling by merlin also manifests in RAS-mutant thyroid cancer cell lines. Although merlin loss augments EGFR signaling, this does not appear to contribute significantly to growth regulation (Supplementary Fig. S6).
NF2 loss leads to increased expression of mutant and wild-type RAS through YAP-dependent transcriptional activation: To explore the possible contribution of other inputs upstream of RAS to MAPK activation, we measured RAS-GTP levels prior to and 72h after merlin expression. Unexpectedly, merlin inhibited both WT and mutant RAS-GTP, which was associated with decreased protein and mRNA levels of all RAS isoforms (Fig. 3A, B). Conversely, knockdown of NF2 increased mutant and wild type RAS mRNA and protein levels in NF2-WT cells (Fig. 3C). Strikingly, this was also the case in the mouse models of the disease. Thus, protein levels of the three Ras genes were markedly increased in thyroid tissues with conditional deletion of Nf2. This was associated with increased pERK and pMEK in TPO-Cre/HrasG12V/Nf2flox2 mice, presumably because of overexpression of the oncogenic Hras allele (Fig. 3D). Copy number abnormalities of Hras were found in a subset of these tumors (Supplementary Fig. S7). However, this does not account for the consistent increase in Hras in all tumors we examined, or the higher expression of the wild-type Ras proteins.

Consistent with the known interaction of merlin with the Hippo pathway, expression of WT-merlin in NF2-null RAS-mutant cell lines resulted in YAP phosphorylation and retention in the cytoplasm, with reciprocal changes seen after knockdown of merlin in NF2 wild-type cells (Fig. 4A, B). YAP is a required component of a transcriptional regulatory complex that includes its close homologue TAZ, and TEAD. Silencing of YAP in Cal62 (KRASG12R, NF2 null) cells decreased mRNA levels of all RAS isoforms, as well as of the canonical YAP-transcriptional target CTGF (Fig. 4C). This was associated with a profound decrease of all three RAS proteins (Fig. 4D). YAP
knockdown also prevented the induction of RAS protein and of MAPK signaling by merlin silencing in C643 (HRAS\textsuperscript{G13R}, NF2-WT) cells (Fig. 4E) and inhibited growth of NF2-null cell lines (Fig. 4F). Consistent with this, expression of constitutively active YAP\textsuperscript{S127A} in C643 cells (NF2-WT) induced RAS gene expression (Fig 4G) and promoted cell growth (Fig. 4H). YAP\textsuperscript{S127A} also rescued the growth inhibition (Fig 4I) and the suppression of RAS mRNA levels by merlin in Cal62 cells (Fig. 4J). In silico analysis identified consensus TEAD binding sites in the promoters of the three RAS genes (Fig. 5A). The functional relevance of this prediction is supported by ChIP-PCR with antibodies to either YAP or TEAD1 (TEAD1 is the most abundant TEAD isoform in thyroid cancer cells – see Supplementary Fig. S8A). This showed that merlin expression markedly diminished occupancy by YAP and TEAD1 of TEAD consensus motifs in the promoters of the three RAS genes (Fig. 5B and Supplementary Fig S8B).

Verteporfin (VP) is an FDA-approved drug used as a photosensitizer for photodynamic ablation of abnormal blood vessels in patients with macular degeneration (34). VP was identified in a drug screen for compounds that could disrupt YAP-TEAD driven transcription, and shown to inhibit YAP-dependent growth (14). We found that VP inhibited growth of NF2-null Cal62 (Fig. 5C) and Hth83 cells (Supplementary Fig. S8C), an effect that was dampened by merlin expression (Supplementary Fig S8D). VP decreased YAP and TEAD, lowered Ras protein abundance and inhibited MAPK signaling. Reciprocal findings were seen in C643 cells after merlin silencing (Supplementary Fig. S8E). VP treatment inhibited TEAD occupancy of the oncogenic KRAS gene promoter in Cal62 cells (Fig. 5D). Hence, genetic and pharmacological
disruption of YAP-TEAD leads to decreased oncogenic RAS gene expression, MAPK signaling and growth.

**NF2/Merlin deficiency sensitizes RAS-mutant cancer cells and murine PDTC to MEK inhibition:** We next explored whether the increased MAPK signaling seen in RAS mutant/NF2-deficient thyroid cancer cell lines rendered them more dependent on this pathway for growth. This was explored in isogenic lines derived from C643 cells (HRAS^{G13R}, NF2-WT) modified to stably express sh-NF2 (Fig. 6A). Although expression of oncogenic HRAS, as well as baseline pMEK and pERK, were markedly higher in the merlin-depleted shNF2-M4 line, the inhibition of the pathway by the MEK inhibitor was comparable. Despite this, growth suppression by AZD6244 was greater in cells depleted of merlin. We also tested a set of 7 RAS-mutant thyroid cancer cell lines for growth response to MEK inhibition. There was a trend for greater sensitivity to AZD6244 in RAS mutant thyroid cancer cell lines that were merlin null or low as compared to merlin wild-type cell lines (Supplementary Fig. S9A). To explore the contribution of distinct inputs into MEK (i.e. Rac1-PAK vs canonical Ras-RAF), we explored growth in response to AZD6244, FRAX597 or their combination in C643 cells with or without merlin silencing. Consistent with the data shown in Fig. 6, C643-shNF2.M2 cells are exquisitely sensitive to the MEK inhibitor. Although knockdown of merlin also sensitizes cells to growth inhibition by the PAK inhibitor, the effects were comparatively modest. Combined inhibition showed only modest additive effects (Supplementary Fig. S9B).
We also examined the effects of AZD6244 in three models of Hras\(^{G12V}\)-driven mouse poorly differentiated thyroid cancers, arising in the context of \(\text{Nf2, Pten}\) or \(\text{p53}\) homozygous loss. All of these gave rise to PDTCs, defined based on the presence of necrosis and a high mitotic rate on histology. These tumors differed, however, in tumor doubling time (\(\text{Pten=Nf2}>\text{p53}\)) (Supplementary Fig S10A), and overall survival (Supplementary Fig S10B). \(\text{Hras/Nf2}\) and \(\text{Hras/p53}\) tumors stained intensely for pERK, whereas \(\text{Hras/Pten}\) tumors had low pERK and increased pAKT staining (Fig 6B).

Treatment of mice with \(\text{Hras}^{G12V}/\text{Nf2-null}\) thyroid cancers with AZD6244 resulted in a greater and more consistent reduction of tumor size than in cancers arising in \(\text{Hras}^{G12V}/\text{Pten-null}\) or \(\text{Hras}^{G12V}/\text{p53-null}\) mice (Fig. 6C). IHC of treated tumors showed marked reduction of pERK staining in all three tumor types, whereas pAKT IHC remained very high in \(\text{Hras/Pten}\) tumors, possibly accounting for their lack of response to therapy (Fig. 6D).

Discussion

The striking tissue overgrowth phenotypes induced by genetically disabling the core components of the Hippo pathway in \(\text{Drosophila melanogaster}\) prompted exploration of its role in mammalian cell proliferation and cancer development (13). Hippo inactivation converges to regulate gene expression through the transcriptional coactivator YAP and/or its close homolog TAZ. This raises the question of how the YAP/TEAD transcriptional targets may impact the biology of cancers driven by oncogenes, such as RAS, which nominally operate through a distinct canonical signaling network. Our
finding that RAS genes are themselves transcriptionally regulated by YAP may have profound implications for RAS-driven tumors.

The importance of Ras mutant allele gene dosage for transformation is well established. A requirement for intensification of mutant Ras signaling through copy-number imbalances is supported by studies in Hras mutant fibroblasts, in which transformation is strongly associated with amplification of the mutant allele (35). Increased mutant allele copy number is an obligate early event in HrasG12V-induced papilloma development (31, 36, 37). Similarly, the aggressiveness of myeloproliferative neoplasms expressing NrasG12D is augmented when the mutant allele is homozygous (38). Moreover, the expression of oncogenic RAS in human cancer cell lines is consistently higher than that of the other wild type RAS proteins (38). Although HRAS gene amplification has been reported in thyroid cancer (39), the overall frequency, at least in PTC, appears to be low (24). Despite the critical significance of mutant Ras protein abundance on its signaling and transforming properties, there is so far limited information on how other oncogenic inputs may alter its expression. The 3'UTRs of human RAS genes contain multiple complementary sites to the let-7 family of microRNAs, which regulate RAS expression by translational inhibition and/or by decreasing mRNA stability (40, 41). Loss of let-7 miRs increases Ras protein levels, and conversely let-7 overexpression decreases them and attenuates oncogenic Ras-induced tumorigenesis (41, 42).
Regulation of mutant RAS gene transcription as a functional consequence of disrupting a parallel oncogenic pathway has, to our knowledge, not been previously implicated as a mechanism of tumor promotion. The YAP-TEAD complex modulates expression of a diverse array of transcriptional targets (12). However, comparatively little is known about the specific YAP-regulated genes responsible for mediating the effects of Hippo inactivation on growth, invasiveness, metastases or senescence (43-46). CTGF is a prototypical target of YAP-TEAD, and has been functionally implicated in hepatocellular growth and tumorigenesis (47). YAP1 was recently reported to rescue viability of pancreatic cancer cells conditionally expressing mutant Kras after oncoprotein withdrawal in mice. The authors implicated genes co-regulated by YAP1 and E2F in this process, many of which were cell cycle regulators (48). In a screen for genes promoting survival of KRAS-mutant cells following oncogene silencing in vitro, YAP1 was the most prominent hit among those that could also activate either MAPK or PI3K signaling. YAP1 was found to regulate a set of immediate response genes that were also activated by KRAS, ultimately converging on the transcription factor FOS, which also rescued cells after KRAS silencing (49). YAP is also required for KrasG12D-induced pancreatic transformation in mice, by inducing expression of genes encoding secreted factors that promote cancer cell growth and the establishment of a tumorigenic stromal microenvironment (50). We do not yet know whether YAP1-mediated transcriptional activation of RAS mRNAs is ubiquitous or context dependent, or if this may have contributed to some of the observations in the lineages and models described above. Our analysis of previously published genome-wide Chip-Seq data in mouse embryonic stem cells points to clear YAP-binding peaks in all three RAS genes ((51); not shown),
suggesting that these are likely to be regulated via Hippo inactivation in other cell types as well. However, tissue overgrowth induced by Hippo silencing likely requires the concerted regulation of genes involved at multiple key steps of the process. As RAS proteins are critical signaling nodes, modulation of their expression would impact the signal amplitude emanating from a wide array of upstream inputs.

The studies described above implicate YAP as an important effector in RAS-mutant cancer. However, the mechanisms causing YAP activation in these contexts is not known. In the case of RAS-mutant thyroid cancer, merlin loss-of-function is a key event (Fig. 7A,B). Although germline or somatic NF2 mutations are primarily implicated in the pathogenesis of nervous system tumors, mesotheliomas, melanomas and renal cell carcinomas, mice with heterozygous Nf2 mutation develop a broader range of tumors, which lose the wild-type allele (52). Loss-of function of merlin also occurs in the absence of homozygous deletion or mutation, through aberrant splicing, mRNA loss, calpain-mediated proteolysis, proteasomal degradation, or phosphorylation (27-30, 53). Consistent with this, several thyroid cancer cell lines that were merlin-null or low did not have homozygous genomic NF2 mutations. This is particularly relevant in advanced thyroid cancer, as intragenic NF2 mutations are rare in tumors with 22q LOH. The extent by which NF2 haploinsufficiency is associated with impaired expression of the wild type allele in human tumors is currently uncertain. Hence, sensitive protein-based assays may need to be developed to screen cancers for merlin loss of function, particularly if this proves to be an actionable event.
There are potential therapeutic opportunities resulting from Hippo pathway inactivation in the context of RAS-induced tumorigenesis. We found that these tumors hyperactivate MAPK signaling, and consequently generate increased dependency on this pathway for viability. Thus, although merlin loss leads to a global increase in YAP-TEAD transcriptional output, the genes that augment susceptibility to MEK inhibition are paramount in this setting. Moreover, as illustrated by our data with VP, targeting of YAP may prove to be viable strategy for thyroid, and perhaps other cancers, driven by oncogenic RAS.

Experimental Procedures

Sample collection and DNA isolation
A total of 83 advanced thyroid tumors, including 20 anaplastic (ATC) and 63 poorly differentiated thyroid cancers (PDTC) were assessed. Both frozen (n=37) and formalin-fixed paraffin-embedded (FFPE, n=46) specimens were included. Forty-three thyroid cancer-derived cell lines, previously authenticated using short tandem repeat and single nucleotide polymorphism array analysis (54), were also evaluated. Genomic DNA was extracted from all specimens using the DNeasy Tissue kit (Qiagen). Patient samples were studied under a protocol approved by the Memorial Sloan Kettering IRB.

Targeted sequencing and copy number assessment
Two hundred and fifty nanograms of genomic DNA derived from all 126 thyroid samples and matched normal tissues (when available) were subjected to deep-coverage targeted sequencing with MSK-IMPACT™ (Integrated Mutation Profiling of Actionable
Cancer Targets), an exon-capture next generation sequencing platform covering the entire coding sequence and intron-exon boundaries of 341 cancer genes(55). Sequence data were analyzed to identify three classes of somatic alterations: single-nucleotide variants, small insertions/deletions (indels), and copy number alterations (CNAs). We focused in greater detail on the six genes mapping on chromosome 22q included in the platform: CRKL, MAPK1, SMARCB1, CHEK2, NF2 and EP300.

To confirm the ability of IMPACT to accurately call CNAs, we subjected our subset of 37 frozen tumors to array comparative genomic hybridization (aCGH). Briefly, 3 µg of DNA was digested and labeled by random priming using Cy3 or Cy5-dUTP labeled primers (Invitrogen). Labeled tumor DNA was co-hybridized to Agilent aCGH microarrays with a pool of reference normal DNA for 40 h at 60 °C. After washing, the slides were scanned and images were quantified using Feature Extraction 9.1 (Agilent Technologies). Raw copy-number estimates were normalized and segmented with Circular Binary Segmentation (56). Data were also analyzed using the RAE algorithm(57). IMPACT-sequencing reads and segmented copy-number data from both platforms was visualized in the Integrative Genomics Viewer (58), and all genome coordinates were standardized to NCBI build 37 (hg19) of the reference human genome.

FISH

FISH analysis for NF2 status was performed by hybridizing a tissue microarray containing 16 ATC specimens with BAC probes for NF2 probe (RP11-551L12, 22q12.2, red) and BCR (22q11.2, green). Deletion of NF2 was defined as tumors with more than
25% of cells with single or no NF2 hybridization signals (a minimum of 200 cells were scored for each tumor). This threshold was selected because of the high admixture of stromal cells, particularly tumor-associated macrophages, in these cancers.

Mouse genetic models

TPO-Cre mice express Cre recombinase under the control of the thyroid peroxidase gene promoter, which is active only in thyroid follicular cells beginning at E14.5 (59). FR-Hras<sup>G12V</sup> mice conditionally express a latent Hras<sup>G12V</sup> allele under the regulatory control of its endogenous gene promoter (31). To generate triple transgenic mice TPO-Cre/FR-Hras mice were bred with Nf2<sup>flox2</sup> (60), Trp53<sup>flox2</sup> (61), or Pten<sup>flox2</sup> (62). Both male and female mice were used. Mice were in the following backgrounds: TPO-Cre/FR-Hras<sup>G12V/Nf2</sup> ~7% 129sv, 56% C57bl/6, 12% swiss black, 25% FVB/n; TPO-Cre/FR-Hras<sup>G12V/p53</sup> ~56% 129sv, 7% C57bl/6, 12% swiss black and 25% FVB/n. TPO-Cre/FR-Hras<sup>G12V/Pten</sup> mice were backcrossed 5 generations into 129sv. TPO-Cre/FR-Hras<sup>G12V/Nf2</sup> and TPO-Cre/FR-Hras<sup>G12V/Pten</sup> mice were treated 5 days a week for 4 weeks with AZD6244 (25 mg/kg/BID) dissolved in 0.1% Tween 80 +0.5% methylcellulose or vehicle. TPO-Cre/FR-Hras<sup>G12V/p53</sup> mice were treated for 7 days a week for 2 weeks. Animal care and all procedures were approved by the MSKCC Institutional Animal Care and Use Committee.

Ultrasound imaging

Mice were anesthetized by inhalation of 2–3% isoflurane with 1% O<sub>2</sub>, neck hair removed with defoliating agent and placed on the heated stage. An aqueous ultrasonic gel was
applied to the skin overlying the thyroid glands. Thyroid tumors were imaged with the VisualSonics Vevo™ 770 In Vivo High-Resolution Micro-Imaging System (VisualSonics Inc, Toronto, Ontario, Canada). Using the Vevo™ 770 scan module the entire thyroid bed was imaged with captures every 250 microns. Using the instrument’s software the volume was calculated by manually tracing the margin of the tumor every 250 microns. The instrument is calibrated to allow measurements to be determined accurately.

Animals were included in the study if they had tumor present as demonstrated by ultrasound and at a size that could be accurately measured by the ultrasound probe.

Histology and IHC

Thyroid tissues were immediately placed in 4% paraformaldehyde and incubated overnight at 4°C. The next day, tissue was washed twice with PBS for 30 minutes followed by a single 30-minute 50% ethanol wash. The fixed tissue was then placed in 70% ethanol, paraffin embedded, and sectioned into 4-μm paraffin sections. H&E-stained slides were evaluated by a board-certified pathologist (R. Ghossein). Mouse thyroid sections were deparaffinized and immunostained with an antibody to p-ERK (#9101; Cell Signaling) or pPAK_S473 (#4051S; Cell Signaling) at the Memorial Sloan-Kettering Cancer Center Molecular Cytology Core Facility.

Thyroid cancer cell lines

Cancer cell lines were maintained at 37°C and 5% CO₂ in humidified atmosphere and grown in RPMI-1640 (C643, Hth83 and Cal62), DMEM (Hth7, ACT1) or DMEM:RPMI (ASH3, KMH2) supplemented with 10% of FBS, 2 mmoL/l glutamine, 50 U/mL penicillin
(GIBCO), and 50 μg/mL streptomycin. Cell line C643, Hth7 and Hth83 were obtained from Dr. Nils-Erik Heldin on 12/2006, 9/2007 and 12/2006, respectively. Cal62 cells were obtained from Dr. Jeanine Gioanni on 12/2006. The ACT1 line was obtained from Dr. Onoda Osaka in April 2006. ASH3 and KMH2 were obtained from JCRB in April of 2010. All thyroid cancer cell lines used in this study were authenticated using short tandem repeat and single nucleotide polymorphism array analysis between 2006 and 2010 (54). For cell growth assays, cells were plated in triplicate into 6-well plates at 50,000 cells per well, and incubated for 24h. The cells were treated with vehicle or concentrations of the indicated drug in media with 1% of FBS. For transient transfections, 20,000 cells/well were plated into 24-well plates in media containing 10% FBS without antibiotics. 24h after the transfection was performed as indicated in the “Gene Expression and Silencing” Cells were collected by trypsinization and counted in a Vi-Cell series Cell Viability Analyzer (Beckman Coulter) at times indicated. IC50 values were calculated by nonlinear regression using Prism v5.04 (GraphPad Software).

Colony formation assay

Cells were seeded in triplicate at 50,000 cells per 35mm dish. Dishes were first coated with a bottom layer of 0.4% agar in RPMI. Cells were resuspended in a top layer of 0.2% agar in RPMI with 10% FBS, and then fed every other day by adding drops of media onto the top layer. After 9 days the colonies were stained with crystal violet and counted in a GelCount™ colony counter (Oxford OPTRONIX). Minimum diameter of the colonies was 50µm.
Gene Expression and Silencing

PCR-amplified full length cDNAs of human NF2 wt and NF2-L64P were cloned into the pLVX-Tight-puro vector (Clontech) using the following primers containing BamHI and EcoRI restriction sites: 5’- GAGAGGATCCTCACCATGGCCGGAGCCATC-3’; 5’- GAGAGAATTCTTCGAACCGCGGGCCCTCTA-3’). Cal62 and Hth83 thyroid cancer cells were co-infected with pLVX-Tight-puro-NF2 or NF2-L64P and with pLVX-tet-on Advanced vector (Clontech). C643 were infected with pLKO.1 and 5 different hairpins for NF2 (M1#TRCN0000039973; M2#TRCN0000039975; M3#TRCN0000039977; M4#TRCN0000010397; M5#TRCN0000018338 from Open Biosystems). For infection, cells were incubated with infectious particles in the presence of 10 ng/ml hexadimethrine bromide (Sigma) overnight. After recovery in complete medium for 24 hours cells were placed under selection in 1µg/ml puromycin or 300µg/µL G418 for Cal62 and Hth83 or 1µg/ml puromycin for C643. The production of viral particles was performed with Mission Lentiviral Packing mix from SIGMA. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) or Amaxa Nucleofector System (Lonza). Dominant negative of PAK (PAK1 83-149) and YAP1-S127A were purchased from Addgene (#17790, #12214 respectively) (63, 64). Short interfering RNAs for YAP were from ORIGENE (SR307060). The media (10% FBS without antibiotics) was changed 4-6 hours after transfection. Efficiency of knockdown or overexpression was verified by immunoblotting.

RNA isolation, cDNA synthesis and qPCR
Total mRNA from snap frozen thyroid tissue was extracted with the PrepEase Kit (USB Corporation); total mRNA from cells was isolated by Trizol (Invitrogen). Equal amounts of isolated RNA (2μg) were subjected to DNase I Amplification Grade (Invitrogen) and subsequently reverse transcribed into cDNA using the SuperScript® III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. qPCR was then performed with the Power SYBR Green PCR Master Mix (Applied Biosystems). See Supplemental Table 3 for list of primers used. Expression was analyzed with the ΔΔCt method. The Ct values of the target genes were normalized to that of the housekeeping genes beta actin or GAPDH.

Hras allelic imbalance analysis
Genomic DNA from mouse thyroid tissues was used as template for PCR amplification with primers that distinguish mutant (668 bp product due to insertion of loxp) from WT-Hras alleles (622 bp). The primers and PCR conditions were previously described (31).

mRNA stability
Cells were treated with 1 µM actinomycin D for 1, 3, 6, 12 and 24 hours prior to harvesting. The mRNA levels were measured by quantitative RT-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems).

Immunoblotting
Cells were washed twice with cold PBS and lysed in lysis buffer (containing 125mM HEPES, pH7.5, 750mM NaCl, 5% Igepal CA-630, 50mM MgCl₂, 5mM EDTA and 10%
glycerol) supplemented with proteinase and phosphatase inhibitors (Roche). Protein concentration was determined using the BCA kit (Thermo Scientific). Western blots were conducted on 20 μg protein separated by 4-12% Bis-Tris SDS-PAGE gels (Invitrogen), transferred to PVDF membranes, and immunoblotted after blocking with 5% skim milk with the corresponding primary antibodies (listed below) in 5% BSA (SIGMA). This was followed by incubation for 1h with secondary antibodies conjugated with goat anti-rabbit HRP-conjugated antibody (1:5,000; Santa Cruz sc-2054) or goat anti-mouse HRP-conjugated antibody (1:5,000; Santa Cruz sc-2005). Bound antibodies were detected by chemiluminescence with the ECL detection system (GE Healthcare Biosciences).

RAS-GTP or RAC1-GTP immunoprecipitation was conducted using the RAS or RAC1 activation assay kits, respectively, from Millipore, according to the manufacturer’s protocol and subjected to Western blotting with the indicated antibodies. Cells were treated during 72h with dox in media with 1% of FBS.

Subcellular fractions were prepared using the Subcellular Protein Fractionation Kit for cultured cells following the manufacturer’s instructions (Thermo Fisher Scientific). Fractions were subjected to Western blotting with the indicated antibodies.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) lysates isolated from Cal62, Hth83 and C643 cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature, and stopped by the addition of glycine to a final concentration of 125 mmol/L followed by 2
washes with PBS. The cell pellet was resuspended in Lysis buffer (ChIP-IT® Express Enzymatic kit -Active Motif) supplemented with PMSF and protease inhibitors and incubated on ice for 30 minutes. After 20 strokes with a homogenizer and a centrifugation for 10 minutes at 5000rpm, the pellets were digested with an enzymatic shearing cocktail for 7 minutes at 37°C, to produce chromatin fragments of 200–500 bp on average. Equal amounts of cross-linked DNA were immunoprecipitated overnight at 4°C with antibodies against YAP (sc-15407, Santa Cruz) or TEAD1 (#8526, Cell Signaling). Immunoprecipitates were incubated with proteinase K overnight, and the DNA recovered by purification (QIAquick PCR purification kit from Quiagen) prior to real time PCR with primers bracketing the indicated promoter motifs. The ChIP ratio was calculated as enrichment over noise normalized to input.

Additional reagents used in vitro

Doxycycline (2µg/mL), hEGF (5ng/mL) and verteporfin were from Sigma. AZD6244 was from AstraZeneca. FRAX597 was from Selleckchem.

Statistical Analysis

Statistical analyses of the results were performed by unpaired two tailed Student's t test or Mann-Whitney test according to assumptions of the test, using Prism v5.04 (GraphPad Software). Graphs represent mean value and error bars represents s.d. Similar variance between groups was tested by F Test; if different, Welch’s correction was applied. The P values are presented in figure legends where p< 5 x E-2 was considered statistically significant. No randomization or blinding was used.
Antibodies

pMEK S217/221 (9121L), pERK (#4376), CCND1 (#2978P), pYAP S127 (#4911), YAP/TAZ (#8418), TUBULIN (#2148), pPAK T423 (#2601), PAK (#2604S), pCRAF s338 (#9427), pMEK S298 (#9128), pEGFR Y1068 (#3777S), EGFR (#4267S), GST (#2622), TEAD1 (#8526), FLAG (#2368S) from Cell Signaling; NF2/MERLIN ((a-19) sc-331), HRAS (sc-520), KRAS (sc-30), NRAS (sc-31), CTGF (sc-14939), YAP FOR ChIP (sc-15407) from Santa Cruz; ACTIN (A2228), pPAK S141 (p7871) from Sigma; Na+K+ ATPase (ab818), TATA binding (ab7671) from Abcam.

Acknowledgements

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Grant support

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Reference List


(37) Chen X, Makarewicz JM, Knauf JA, Johnson LK, Fagin JA. Transformation by Hras is consistently associated with mutant allele copy gains and is reversed by farnesyl transferase inhibition. Oncogene 2013;10.


Legends:

**Figure 1:** TPO-Cre/FR-Hras^{G12V}/Nf2^{flox2} mice develop poorly differentiated thyroid cancers with strong activation of MAPK signaling.

A) *Left:* The *Hras* allele in *FR-Hras^{G12V}* mice is replaced with 2 tandem copies of the gene, the first encoding *WT Hras* flanked by loxP sites, and the second harboring a mutation encoding *Hras^{G12V}* which is only expressed after *Cre* excision of the floxed WT allele. *Right:* Exon 2 of *Nf2* is floxed in *Nf2^{flox2}* mice. Thyroid-specific expression of *Hras^{G12V}* and inactivation of *Nf2* was achieved by crosses with *TPO-Cre* mice.

B) Weight of thyroids in 18 month-old mice with the indicated genotype (p values calculated by unpaired t test with Welch’s correction).

C) H&E-stained and pERK IHC thyroid sections representative of each genotype. The histology of tumors in *Hras^{G12V}/Nf2^{flox2}* mice is consistent with PDTC.

The mouse age was from 74-85 weeks for *TPO-Cre/FR-Hras^{G12V}/Nf2^{flox2}* (hets: n=23; hom: n=20) and *TPO-Cre/FR-Hras^{G12V}* (hom: n=8). *TPO-Cre* (n=21) and *TPO-Cre/Nf2^{flox2}* (n=27) were 77-119 weeks old.

**Figure 2:** Merlin inhibits growth and MAPK signaling in RAS mutant thyroid cancer cells.

A) *Left:* Effects of dox-induced expression of WT NF2 or NF2-L64P on growth of Cal62 (KRAS^{G12R}) and Hth83 (HRAS^{Q61R}) cells (*p<4 \times 10^{-4}, dox-induced WT NF2 versus NF2-L64P; n=3, Student's t test). *Right:* Soft agar colony counts of Cal62 and Hth83 cells treated with or without dox for 20 days, with media changes every 2 days (*p< 2 \times 10^{-2}; **p< 1 \times 10^{-3}; n=3, Student's t test).
B) **Left:** Time course of MAPK signaling after dox-induced merlin expression in Cal62 cells. **Right:** Effect of dox-induced expression of wild type or L64P-NF2 after 72 h in Cal62 and Hth83 cells.

C) **Left:** Growth of C643 cells (HRAS$^{G13R}$, NF2-WT) stably expressing scrambled or 2 different NF2 shRNAs (M2 and M4) (*p< 1 x E-3; n=3, Student's t test). **Right:** Western blots for pMEK and pERK after NF2 knockdown.

**Figure 3:** Merlin decreases RAS gene expression and total and oncogenic RAS-GTP levels.

A) Total RAS-GTP and KRAS-GTP levels of Cal62 (KRAS$^{G12R}$) cells before and 72h after induction of merlin. Input lysates were blotted for the indicated proteins. No GTP-bound NRAS or HRAS was detected (not shown).

B) **Left:** Oncogenic and wild type RAS protein levels in Cal62 (KRAS$^{G12R}$) and Hth83 (HRAS$^{Q61R}$) cells after merlin expression. **Right:** Merlin decreases mRNA levels of the three RAS genes as measured by real time PCR (*p<2 x E-3; ** p<3 x E-4; Student's t test).

C) **Top:** shRNA knockdown of NF2 with two different hairpins (M2 and M4) increases RAS protein levels in C643 (HRAS$^{G13R}$-NF2 WT) cells. **Bottom:** Knockdown of NF2 (shNF2.M2) increases RAS mRNAs in C643 cells (*p<5 x E-2, **p<4 X E-4; Student's t test).

D) Western blots of thyroid tissues of TPO-Cre, TPO-Cre/Nf2$^{flox2}$, TPO-Cre/Hras$^{G12V/-}$ (heterozygous), TPO-Cre/Hras$^{G12V/+}$ (homozygous) and TPO-Cre/Hras$^{G12V/+}$/Nf2$^{flox2}$ mice probed with the indicated antibodies. Each lane contained pooled thyroids, except
where indicated. *TPO-Cre*: pool of 4 thyroids each from 12-15 week-old mice; *TPO-Cre/Nf2^{floxed}:* pools of two thyroids each from 101-115 week-old mice; *TPO-Cre/Hras^{G12V+/}:* pools of two thyroids each from 10 week-old mice; *TPO-Cre/Hras^{G12V+/+}:* single thyroids and *TPO-Cre/ Hras^{G12V}/Nf2^{floxed}:* pools of two thyroids, 66-79 weeks old.

**Figure 4**: Merlin loss increases RAS gene and protein expression through YAP.


**C)** RT-PCR for the indicated transcripts in Cal62 cells expressing three different siRNAs for YAP (1, 2 and 3) or a scrambled siRNA (siSC) (*p<2 x E-3; **p<1 x E-4; Holm-Sidak method). Bars represent the average of 4 independent experiments. **D)** Western blot of Cal62 cell lysates with the indicted antibodies 72 h after transfection with siSC or YAP siRNAs. The antibody to YAP recognizes both YAP and TAZ.

**E)** C643 cells stably expressing shNF2.M2 have increased expression of HRAS and higher pMEK and pERK, which is abolished in cells transfected with YAP siRNA.

**F)** Growth of Cal62 cells expressing scrambled or YAP siRNAs. Cells were incubated in 10% serum and counted 4 days after transfection (*p < 3 x E-3, **p < 1 x E-6; n=3, Student’s t test).
G) Yap1\textsuperscript{S127A} increases RAS mRNA levels in C643 cells (*p < 5 x E-2; ** p < 6 x E-4; Student's \textit{t} test).

H) Transient expression of constitutively active Yap1\textsuperscript{S127A} induces C643 cell growth. Bars represent cell counts at 6 days (p< 8 x E-3; Student's \textit{t} test).

I) Yap1\textsuperscript{S127A} rescues the inhibition of growth (p < 2 x E-3; Student's \textit{t} test) and \textbf{(J)} of RAS mRNA (* p < 5 x E-2; ** p < 3 x E-3; Student's \textit{t} test) by dox-induced merlin in Cal62 cells.

**Figure 5:** Merlin loss increases RAS gene expression through YAP/TEAD-dependent transcriptional activation.

\textbf{A)} Putative TEAD binding motifs in the three \textit{RAS} gene promoters. Highlighted in red are the binding sites shown in the ChiP figure.

\textbf{B)} Merlin decreases YAP and TEAD promoter occupancy of \textit{RAS} gene promoters. ChIP-PCR with antibodies to YAP and TEAD1 in Cal62 cells treated with or without dox for 72h (*p < 3 x E-2; ** p< 7 x E-3; *** p< 2 x E-4; n=3, three independent experiments, Student's \textit{t} test). Primers used bracketed consensus TEAD binding sites in the 3 \textit{RAS} genes (highlighted in red in panel A).

\textbf{C)} \textit{Left:} Dose-dependent decrease of YAP, pYAP and TEAD by 72h of incubation with verteporfin (VP) in Cal62 cells is associated with lower KRAS and HRAS, and decreased pMEK and pERK signaling. Control cells were treated with dox for 72h. \textit{Right:} VP decreases growth of Cal62 cells in a dose-dependent manner. Cells were counted 3 days after incubation with the indicated concentration of the compound (*p < 5 x E-2; ** p< 3 x E-3; *** p< 4 x E-4; n=3).
D) ChIP-PCR with TEAD antibody for KRAS in Cal62 cells treated with or without dox in the presence or absence of 0.5uM of VP for 72h (* p< 2 x E-3; ** p< 4 x E-4; n=3, three independent experiments, Student's t).

**Figure 6:** Merlin deficient RAS mutant cells and murine PDTCs are more sensitive to MEK inhibition.

A) *Left:* Western blots of C643 cells expressing scrambled or shNF2.M4 for pMEK, pERK and HRAS after incubation with AZD6244 for 1h. *Right:* Growth inhibitory effects of AZD6244 in C643 (HRAS<sup>G13R</sup>, NF2-WT) cells stably expressing scrambled or 2 different NF2 shRNAs (M2 and M4). Cells were counted at 6 days. (n=3). Average IC50 of two replicate experiments is shown. *p<2 x E-2.

B) H&E, pERK and pAKT<sub>S473</sub> IHC of representative thyroid cancer sections of each genotype.

C) Effect of *in vivo* treatment of thyroid cancers in TPO-Cre/FR-Hras<sup>G12V</sup>/NF2<sup>flox2</sup>, TPO-Cre/FR-Hras<sup>G12V</sup>/PTEN<sup>flox2</sup> and TPO-Cre/FR-Hras<sup>G12V</sup>/p53<sup>flox2</sup> mice for 4 weeks with 25 mg/kg/b.i.d. AZD6244. Tumor volume was determined pre and post-treatment by ultrasound. Bars represent the percent change in tumor volume from baseline in each mouse. AZD6244 reduced tumor size in Hras<sup>G12V</sup>/NF2<sup>flox2</sup> mice (AZD6244 vs vehicle p=3 x E-4); in Hras<sup>G12V</sup>/Pten<sup>flox2</sup> there was a paradoxical increase in tumor volume with the MEK inhibitor (p = 8 x E-4). AZD6244 had no significant effects on PDTC/ATC of Hras<sup>G12V</sup>/p53<sup>flox2</sup> mice (p=0.45). n=18 per group, Mann-Whitney test. *denotes ATC.

D) H&E, pERK and pAKT<sub>S473</sub> IHC of representative thyroid cancer sections of each genotype. H&E-stained, pERK and pAKT<sub>S473</sub> IHC thyroid cancer sections in TPO-
Cre/FR-Hras\textsuperscript{G12V}/NF\textsuperscript{flox2}, TPO-Cre/FR-Hras\textsuperscript{G12V}/PTEN\textsuperscript{flox2} and TPO-Cre/FR-Hras\textsuperscript{G12V}/p53\textsuperscript{flox2} mice treated with AZD6244 or vehicle.

**Figure 7:** Mechanisms by which merlin loss cooperates with oncogenic RAS to induce thyroid tumorigenesis.

**A)** In the presence of wild-type merlin, mutant RAS modestly increases MAPK signaling, which is insufficient to drive tumorigenesis (1). Wild-type merlin blocks RAC1 activation, decreasing stimulatory input of PAK into CRAF and pMEK (2). Merlin also activates the Hippo pathway, leading to phosphorylation of YAP and its retention in the cytoplasm. In the absence of nuclear YAP, TEAD is unable to induce transcription of wild-type and oncogenic RAS, restricting the magnitude of the oncogenic drive (3).

**B)** In Merlin-deficient cells the Hippo pathway is off (1), allowing YAP to form transcriptionally active complexes with TEAD, leading to increased oncogenic and wild-type RAS gene expression. This, in concert with engagement of RAC1-PAK signaling (2), leads to increased of MAPK output and promotion of tumorigenesis, while generating increased dependence on the pathway for viability.
Figure 1

A

B

C

Normal FR-Hras\textsuperscript{hom} TPO-Cre Nf2-flox2 FR-Hras\textsuperscript{het} TPO-Cre Nf2-flox2 FR-Hras\textsuperscript{hom} TPO-Cre
Figure 2

(A) Graph showing cell count over days for Cal62 (KRAS\textsuperscript{G12R}) and Hth83 (HRAS\textsuperscript{G61R}) with different conditions: WT - DOX, WT + DOX, L64P - DOX, L64P + DOX. Bars show number of colonies for WT and L64P conditions.

(B) Western blot analysis of MERLIN, pMEK, MEK, pERK, ERK, CYCLIN D1, and ACTIN after 72h treatment with DOX (0, 24, 48, 72h) in Cal62 (KRAS\textsuperscript{G12R}) and Hth83 (HRAS\textsuperscript{G61R}) cells.

(C) Graph showing cell count over days for M2 and M4 conditions with pLKO.1 and shNF2. Western blot analysis of MERLIN, pMEK, pERK, ERK, and ACTIN in C643 (HRAS\textsuperscript{G61R}) cells.
Figure 3

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Hth83 (HRASG61R)  Cal62 (KRASG12R)

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C643 (HRASG13R)

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Short exposure
Long exposure
Figure 5

(A) Schematic representation of the binding site for TEAD1 in the promoter region of HRAS, KRAS, and NRAS. The DNA sequence and the binding motifs are shown.

(B) Histogram showing the percentage of input for YAP and TEAD binding to HRAS, KRAS, and NRAS promoters in the absence and presence of DOX. The bars indicate significant differences with asterisks (*, **, ***).

(C) Western blot analysis of Cal62 (KRASG12R) cells treated with DOX and VP (uM) concentrations. The bands for pYAP, YAP, TEAD1, KRAS, HRAS, pMEK, and pERK are shown along with ACTIN as a loading control.

(D) Graph showing the cell count of Cal62 cells treated with DOX and different VP concentrations. The bars indicate significant differences with asterisks (*, **, ***).
Figure 6

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TPO-Cre

TPO-Cre/FR-Hras/Nf2<sup>lox2</sup>

TPO-Cre/FR-Hras/Pten<sup>lox</sup>

TPO-Cre/FR-Hras/p53<sup>lox</sup>

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TPO-Cre/FR-Hras/Nf2<sup>lox2</sup>

TPO-Cre/FR-Hras/Pten<sup>lox</sup>

TPO-Cre/FR-Hras/p53<sup>lox</sup>

Vehicle

AZD6244
NF2 loss promotes oncogenic RAS-induced thyroid cancers via YAP-dependent transactivation of RAS proteins and sensitizes them to MEK inhibition.

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