Inactivation of the Hippo tumor suppressor pathway promotes melanoma

Melanoma is commonly driven by activating mutations in the MAP kinase BRAF; however, oncogenic BRAF alone is insufficient to promote melanomagenesis. Instead, its expression induces a transient proliferative burst that ultimately ceases with the development of benign nevi comprised of growth-arrested melanocytes. The tumor suppressive mechanisms that restrain nevus melanocyte proliferation remain poorly understood. Here we utilize cell and murine models to demonstrate that oncogenic BRAF leads to activation of the Hippo tumor suppressor pathway, both in melanocytes in vitro and nevus melanocytes in vivo. Mechanistically, we show that oncogenic BRAF promotes both ERK-dependent alterations in the actin cytoskeleton and whole-genome doubling events, which independently reduce RhoA activity to promote Hippo activation. We also demonstrate that functional impairment of the Hippo pathway enables oncogenic BRAF-expressing melanocytes to bypass nevus formation and rapidly form melanomas. Our data reveal that the Hippo pathway enforces the stable arrest of nevus melanocytes and represents a critical barrier to melanoma development.
Cutaneous melanoma arises from the malignant transformation of melanocytes, which are neural crest-derived cells mainly localized to the basal layer of the epidermis. When locally resected, melanoma is highly curable; however, melanoma is the most aggressive of all skin cancers and distant-stage disease is associated with significant mortality. Unraveling the molecular features underlying the pathogenesis of cutaneous melanoma is essential for the development of preventative and therapeutic treatment strategies.

The vast majority of melanocytic neoplasms are initiated by oncogenic mutations in the mitogen-activated protein kinase (MAPK) pathway, with activating mutations in BRAF and NRAS occurring in ~50% and ~20% of cutaneous melanomas, respectively. Within BRAF-mutant melanomas, the most common activating mutation results from a single amino acid substitution from a valine to a glutamic acid generating the constitutively active mutant $BRAF^{V600E}$. Despite strongly inducing proliferative signaling, melanocyte-specific expression of $BRAF^{V600E}$ is insufficient to induce melanoma in multiple animal models; instead, $BRAF^{V600E}$ expression leads to the development of benign nevi (moles) comprised of growth-arrested melanocytes. This is corroborated by clinical evidence as melanocytes within benign human nevi also frequently contain $BRAF^{V600E}$ mutations and these melanocytic nevi rarely transform into melanoma (annual rate <0.0005%). Similarly, mutations within NRAS are commonly detected in congenital melanocytic nevi and oncogenic NRAS expression in melanocytes in vivo does not rapidly yield melanoma. Although the risk of any single melanocytic nevus transforming into melanoma is minimal, understanding how such transformations occur is paramount as roughly one-third of all melanomas co-exist with or arise from nevi.

These observations indicate that tumor suppression mechanisms restrain melanoma development following the acquisition of activating MAPK pathway mutations in melanocytes. A longstanding view is that strong oncogenic signals driven by mutations in MAPK pathway components lead to oncogene-induced cellular senescence (OIS), which safeguards against tumorigenesis. Supporting this view, it has been demonstrated that expression of $BRAF^{V600E}$ in primary melanocytes in vitro induces an immediate cell cycle arrest and that these arrested melanocytes exhibit all of the hallmarks of oncogene-induced senescence: they become large, flat, vacuolar, express p16INK4a, display senescence-associated β-galactosidase (SA-β-gal) activity, and have increased heterochromatic foci and DNA damage.

However, while it is clear that oncogene-induced senescence occurs in vitro, the extent to which this mechanism operates to ward off tumorigenesis in vivo remains unclear. Several pieces of evidence argue against OIS as being the predominant mechanism restraining the proliferation of melanocytes harboring oncogenic mutations in vivo (extensively reviewed in ref. 16). Most notably, oncogene expression (e.g., $BRAF^{V600E}$) in melanocytes does not induce an immediate proliferative block in vivo. Rather, these oncogenes initially induce proliferation, as evidenced by the clonal outgrowth of melanocytes that ultimately form a nevus, which requires many rounds of cell division. Furthermore, melanocytes lacking proteins known to enforce senescence, such as p16 and p53, retain the capacity to enter a growth-arrested state, as melanocytes in $Braf^{V600E}$/Cdkn2a−/− and $Braf^{V600E}$/Trp53−/− mouse models still primarily form nevi, with only a rare few melanocytes stochastically transforming into melanoma.

Collectively, these data suggest that additional tumor suppressive mechanisms have the capacity to restrain the proliferation of $Braf^{V600E}$-positive mouse melanocytes, independent of inducing senescence. Recent modeling studies have led to the postulation that the growth arrest of nevus melanocytes is not solely due to oncogene activation and OIS in individual cells, but rather due to cells sensing and responding to their collective overgrowth, similar to what occurs in normal tissues. This cell growth arrest is reminiscent of the arrest induced by activation of the Hippo tumor suppressor pathway, which is an evolutionarily conserved pathway known to regulate organ size. When the Hippo pathway is activated, the Hippo kinases LATS1/2 phosphorylate the transcriptional co-activators YAP (YAP1) and TAZ (WWTR1), resulting in their inactivation by nuclear exclusion and subsequent degradation. In contrast, when the Hippo pathway is inactivated, YAP and TAZ are active and form DNA-binding complexes with the TEAD family of transcription factors, which act synergistically with AP-1 complexes to stimulate the expression of genes mediating entry into the S-phase and cell proliferation.

It is not known if Hippo pathway activation contributes to the growth arrest of nevus melanocytes. Moreover, while Hippo pathway inactivation has been suggested to promote cutaneous melanoma growth and invasion, it remains unknown whether Hippo inactivation is sufficient to induce cutaneous melanoma initiation and/or progression. Here, we use a combination of in vitro and in vivo model systems to examine the role of the Hippo tumor-suppressor pathway in restraining melanoma development.

**Results**

$BRAF^{V600E}$ expression activates the Hippo tumor-suppressor pathway in vitro. We sought to examine if the expression of $BRAF^{V600E}$ is sufficient to induce activation of the Hippo tumor-suppressor pathway in cultured melanocytes. Previous studies using primary melanocytes have demonstrated that exogenous expression of oncogenic $BRAF^{V600E}$ leads to an immediate p53-dependent growth arrest. We, therefore, developed a system in which $BRAF^{V600E}$ expression could be induced without an immediate cell cycle arrest in an attempt to explore Hippo pathway activation over multiple cell cycles. To do so, we generated a doxycycline-inducible system to permit controlled $BRAF^{V600E}$ expression in non-transformed Simian Virus 40 (SV-40) immortalized melanocytes (Mel-ST cells). Expression of the SV-40 early region, which encodes the small and large T viral antigens, imparts immortalization to primary melanocytes via multiple mechanisms including impairment of the p53/Rb pathways. Induction of $BRAF^{V600E}$ expression in Mel-ST cells increased the phosphorylation levels of the downstream kinases ERK and RSK, indicating that the cell model successfully hyperactivates MAPK signaling upon the addition of doxycycline (dox) (Fig. 1a).

To determine if $BRAF^{V600E}$ activates the Hippo tumor-suppressor pathway in vitro, we induced $BRAF^{V600E}$ expression and examined the relative levels of active, phosphorylated LATS1/2 at the hydrophobic motif (T1079). We found a significant increase in LATS phosphorylation following expression of oncogenic $BRAF^{V600E}$ (Fig. 1b). We then assessed total YAP phosphorylation (p-YAP) via phos-tag gel electrophoresis. We observed that $BRAF^{V600E}$ induction promoted phosphorylation of YAP at multiple sites (Fig. 1c). Consequently, expression of $BRAF^{V600E}$ led to nuclear exclusion of YAP and a corresponding decrease in the expression of the YAP target genes CYR61 and AMOTL2 (Fig. 1d, e and Supplementary Fig. S1A). The observed effects on LATS and YAP activity were due to $BRAF^{V600E}$, as overexpression of wild-type $BRAF$ had no effect on LATS or YAP phosphorylation (Supplementary Fig. S1B–E). We further confirmed these results in multiple cell lines, including...
non-immortalized primary adult human melanocytes with an intact p53 pathway (Fig. 1f and Supplementary Fig. S1F). Importantly, the observed effects of Hippo pathway activation were not limited to the expression of BRAFV600E alone, as we also found that inducible expression of oncogenic NRASQ61R similarly activates the Hippo pathway (Supplementary Fig. S1G). Collectively, these data demonstrate that hyperstimulation of the MAPK signaling pathway through the expression of oncogenic BRAFV600E or NRASQ61R leads to activation of the Hippo tumor-suppressor pathway in vitro.

Growth-arrested melanocytes in benign nevi show evidence of Hippo pathway activation. It is well established that melanocyte-specific expression of BRAFV600E in animal models gives rise to benign nevi that harbor non-proliferating melanocytes. We hypothesized that these BRAFV600E-positive melanocytes may also demonstrate evidence of Hippo pathway activation, similar to our in vitro results. To test this possibility, we analyzed a single-cell RNA sequencing dataset of whole-skin extracts collected at postnatal days 30 (P30) and 50 (P50) from wild-type control mice and tamoxifen-painted
Tyr::CreER\textsuperscript{T2}/Braf\textsuperscript{CA} mice expressing active Braf\textsuperscript{V600E}25. We interrogated this dataset to examine if YAP/TAZ-dependent gene transcription was repressed in melanocytes expressing oncogenic Braf\textsuperscript{V600E}. Following dimensionality reduction and initial clustering, we identified the cluster representing melanocytes based on the expression of the melanocyte-lineage marker Dct as previously reported (Fig. 2a)\textsuperscript{25}. As expected, this cluster was found to have high expression of other melanocyte-lineage markers, notably Mlan and Mitf (Supplementary Fig. S2A). We then employed the variance-adjusted Mahalanobis (VAM) method, a scRNA-seq optimized approach to obtain accurate signaling pathway scores, to examine if YAP/TAZ-mediated gene expression was decreased in Braf\textsuperscript{V600E}-expressing mouse melanocytes relative to wild-type melanocytes utilizing previously published YAP/TAZ gene expression profiles\textsuperscript{35-37}. The most basic analysis, where all single cells were binned by genotype, demonstrated that YAP/TAZ gene expression targets were significantly reduced in Braf\textsuperscript{V600E}-positive melanocytes compared to wild-type (Fig. 2b).

We then performed unsupervised clustering, which generated five melanocyte subclusters, to clarify which unique populations of oncogenic Braf\textsuperscript{V600E}-expressing melanocytes exhibited the least YAP/TAZ activity (Fig. 2c). We theorized that clusters containing nevus melanocytes would be exclusively populated by cells isolated from Braf\textsuperscript{V600E} mice, and be the primary melanocytic subtype isolated from 50-day-old mice. Based on these criteria, we identified clusters 0 and 1 as Braf\textsuperscript{V600E}-expressing melanocytes isolated from nevi (Fig. 2c and Supplementary Fig. S2B, S2C). In support of this prediction, expression of Cdkn2a, which is upregulated in nevus melanocytes, was found to be the highest in clusters 0 and 1, although Cdkn2a read-depth was limited throughout all clusters (Supplementary Fig. S2D). Compared to all other melanocytes, nevus melanocytes (clusters 0 and 1) exhibited the lowest YAP/TAZ activity scores of any cluster, demonstrating that YAP/TAZ-mediated gene expression is reduced following oncogenic Braf expression in mouse nevus melanocytes (Fig. 2d–f). Importantly, expression of Hippo pathway components remained unchanged regardless of genotype or cluster, suggesting decreased YAP/TAZ signaling was due to Hippo pathway activation, not altered expression of YAP/TAZ regulators (Supplementary Fig. S2E, F).

Not all melanocytes captured from Braf\textsuperscript{V600E} mice exhibited low YAP/TAZ activity scores. Clusters 2 and 4, which contain an appreciable portion of melanocytes from both Braf\textsuperscript{V600E} and Braf\textsuperscript{V600E} mice, demonstrated much higher YAP/TAZ activity relative to nevus melanocytes (Supplementary Fig. S2G). However, within these clusters, YAP/TAZ activity was still decreased in Braf\textsuperscript{V600E} melanocytes compared to wild-type cells. This suggests that cell-intrinsic mechanisms following Braf\textsuperscript{V600E} expression are only partially leading to decreased YAP/TAZ activity and that other mechanisms, possibly cell-extrinsic cues, may play additional roles in vivo. We suspect cluster 3, the only cluster that did not exhibit this trend, may be comprised of proliferating, follicular melanocytes as this cluster mainly contains melanocytes isolated at P30 when most murine hair follicles are in anagen\textsuperscript{25}. Taken together, these data reveal Braf\textsuperscript{V600E}-expressing mouse melanocytes largely exhibit decreased YAP/TAZ activity, with the most significant decreases found within nevus melanocytes, strongly implying the Hippo pathway becomes activated in response to Braf\textsuperscript{V600E} expression and nevus formation in vivo. In support of these conclusions, immunofluorescence staining of three human benign nevi revealed YAP localization to be predominantly cytoplasmic and thus presumably inactivated in human nevus melanocytes, consistent with a previous study using a validated YAP antibody (Fig. 2g)\textsuperscript{38}.

Braf\textsuperscript{V600E}-induced Hippo activation restraints oncogenic melanocyte proliferation. We next investigated whether Hippo tumor-suppressor activation following Braf\textsuperscript{V600E} expression leads to reduced melanocyte proliferation in vitro. Population doubling assays demonstrated that expression of Braf\textsuperscript{V600E} reduced Mel-ST cell number ~30–40% relative to uninduced controls over a 4-day period, despite the fact these melanocytes were SV-40 immortalized (Fig. 3a). Live-cell imaging and proliferation assays revealed this was predominantly due to a proliferative arrest, rather than increased cell death (Supplementary Figs. S3A, B and 4A). To test whether the observed Hippo pathway activation induced by Braf\textsuperscript{V600E} expression was responsible for this proliferative defect, we used RNAi to knock down the LATS1/2 kinases in the context of Braf\textsuperscript{V600E} expression. We found that loss of LATS1/2 prevented YAP phosphorylation following induction of Braf\textsuperscript{V600E} and fully rescued cell growth and viability (Fig. 3b, c). We further found that inhibition of LATS1/2 with a potent small-molecule inhibitor also rescued cell growth (Fig. 3d)\textsuperscript{39}. We then validated these findings using soft-agelike growth assays. While parental Mel-ST cells and Mel-ST cells expressing Braf\textsuperscript{V600E} failed to efficiently grow under anchorage-independent conditions, Mel-ST cells expressing Braf\textsuperscript{V600E} together with a constitutively active YAP mutant (YAP-SSA) demonstrated significant colony formation (Fig. 3e and data not shown). These data reveal that fundamental inactivation of the Hippo pathway, through either LATS1/2 depletion/inhibition, or constitutive YAP activation, is sufficient to restore proliferation to Braf\textsuperscript{V600E}-expressing immortalized melanocytes in vitro.

We sought to determine to what extent human melanoma cells inactivate the Hippo pathway. We first interrogated the The Cancer Genome Atlas (TCGA), where we found that co-heterozygous loss of LATS1/2 is observed in ~15% of human melanomas (Fig. 3f and Supplementary Fig. S3C, D). We then stained a panel of human melanoma samples for YAP localization utilizing SOX10 as a marker for melanoma cells and, in agreement with previous studies, found that multiple melanoma tumors exhibited strong nuclear YAP localization, suggesting Hippo pathway inactivation (Fig. 3g and Supplementary Fig. S3E)\textsuperscript{31,38}. Collectively, these data reveal that a subset of oncogenic melanocytes during melanomagenesis will overcome or bypass Hippo pathway activation to regain proliferative capacity.

Braf\textsuperscript{V600E}-induced Hippo activation is ERK-dependent and partially mediated by changes in the actin cytoskeleton. We sought to understand the mechanisms through which Braf\textsuperscript{V600E} directly or indirectly activates the Hippo tumor-suppressor pathway. Oncogenic MAPK signaling has previously been shown to impair mitosis, and complete mitotic failure can lead to the generation of tetraploid cells that activate the Hippo pathway\textsuperscript{40-43}. We speculated that the expression of Braf\textsuperscript{V600E} may lead to Hippo pathway activation by disrupting the normal completion of mitosis. To test this possibility, we performed live-cell imaging of doxycycline-inducible Braf\textsuperscript{V600E} Mel-ST cells stably expressing the chromosome marker histone 2B-GFP (H2B-GFP). We observed that upon entering mitosis, cells expressing Braf\textsuperscript{V600E} often exhibited widely oscillating chromosomes and were unable to maintain a tightly aligned metaphase plate relative to uninduced controls (Fig. 4a). These chromosome alignment defects impaired the ability of many cells to satisfy the spindle assembly checkpoint, and consequently a portion of the Braf\textsuperscript{V600E}-expressing cells endured a significantly prolonged mitosis (Fig. 4a, b and Supplementary Fig. S4A, B). Cells that cannot satisfy the spindle assembly checkpoint either undergo mitotic cell death, or exit from mitosis without undergoing cell
Fig. 2 *Braf*<sup>V600E</sup>-expressing nevus melanocytes display decreased YAP/TAZ signaling. a UMAP of relative Dct expression of all single cells from nevus containing murine skin identifying a cluster of cells representing melanocytes from GSE154679. b YAP/TAZ VAM scores for melanocytes from indicated genotypes (*n* = 46 for *Braf<sup>+/+</sup>*, *n* = 543 for *Braf*<sup>V600E/+</sup>, two-tailed Mann–Whitney test). c Top, UMAP of melanocytes colored by subcluster; Bottom, UMAP of melanocytes colored by genotype and animal age (*n* = 589). d YAP/TAZ VAM score plotted by melanocyte subcluster. e YAP/TAZ VAM score comparing nevus (clusters 0, 1) and other melanocytes (clusters 2, 3, 4) (*nevus* *n* = 408, *other* *n* = 181, two-tailed Mann–Whitney test). f UMAP of melanocytes colored by gradient indicating YAP/TAZ VAM score. g Representative immunofluorescence staining of indicated proteins in two benign nevi cases with two different sets of antibodies, (r) = rabbit, (m) = mouse, DAPI (blue), YAP (green), SOX10 (magenta), scale bar = 50 µm. Source Data are provided as a Source Data file.
division, a phenomenon termed mitotic slippage\textsuperscript{44}. Cells that undergo mitotic slippage often generate multinucleated tetraploid cells, and multinucleated melanocytes have been observed in human nevi\textsuperscript{42,44,45}. We observed that upon induction of BRAF\textsuperscript{V600E} the number of mitoses producing tetraploid cells increased significantly (control: 2.47%, induced: 24.75%) and was mainly driven by an increase in mitotic slippage (control: ~1%, induced: ~20%) (Fig. 4b and Supplementary Fig. S4B). These data demonstrate that BRAF\textsuperscript{V600E} can impair mitosis leading to mitotic slippage and the formation of multinucleated tetraploid cells.
Fig. 4 Prolonged MAPK activation leads to cytoskeletal defects and Hippo activation. 

a Representative fluorescence and phase-contrast images from a live-cell video of dox-inducible BRAFV600E Mel-ST cells expressing the chromosome marker H2B-GFP (green) cultured ± dox (scale bar = 25 µm, hh:mm).

b Plot of mitotic duration and fate of individually tracked mitoses from (a) (n > 80 mitoses per condition from two independent experiments, graph shows mean ± SEM, dots represent individually tracked mitoses, black P value represent mitotic duration significance, two-tailed unpaired t test, blue P value represent significance for difference in frequency of whole-genome doubling events, two-sided Fisher’s exact test).

c Left, IB of indicated dox-inducible BRAFV600E Mel-ST cell lines cultured ± dox for 24 h along with indicated drugs at the following doses: ERKi (20 nM), MEKi-1 (10 µM), MEKi-2 (20 nM); right, intensity quantification of YAP phos-tag (n = 3 independent experiments, graph shows mean ± SEM, one-way ANOVA with multiple comparisons).

d Left, IB of dox-inducible BRAFV600E Mel-ST cell lines cultured ± dox for indicated time; right, intensity quantification of YAP phos-tag (n = 3 independent experiments, graph shows mean ± SEM, two-tailed unpaired t test).

e Left, representative IB of dox-inducible BRAFV600E Mel-ST cell lines treated ± dox for 24 h or with 1 mM hydroxyurea for 6 h; right, intensity quantification of YAP phos-tag (n = 3 independent experiments, graph shows mean ± SEM, two-tailed unpaired t test).

f Representative IB of RhoA-GTP pulldown in indicated dox-inducible BRAFV600E Mel-ST cell line ± dox; right, intensity quantification of RhoA-GTP to total RhoA (n = 3 independent experiments, graph shows mean ± SEM, two-tailed unpaired t test). Source Data are provided as a Source Data file.
melanocytes in vitro. However, two lines of evidence suggested that mitotic errors leading to tetraploidization were not the major underlying driver of Hippo pathway activation in \textit{BRAF}^{V600E}-expressing melanocytes. First, \textit{BRAF}^{V600E}-expressing Mel-ST cells arrested in G\textsubscript{1} (via thymidine) or G\textsubscript{2} (via RO-3306-mediated CDK1 inhibition) still experienced Hippo activation despite their inability to become tetraploid (Supplementary Fig. S4C). Second, immunofluorescence experiments revealed that mononucleated diploid cells also exhibited decreased nuclear YAP/TAZ, demonstrating tetraploidization was not necessary to observe Hippo pathway activation (Fig. 1d).

We next investigated whether \textit{BRAF}^{V600E} specifically, or rather hyperactivation of the MAPK pathway generally, is responsible for Hippo pathway activation. We found that dampening of MAPK signaling via inhibition of the downstream kinases MEK1/2 or ERK1/2 fully prevented Hippo pathway activation, as measured by YAP/TAZ phosphorylation status, in \textit{BRAF}^{V600E}-inducible cell lines (Fig. 4c and Supplementary Fig. S5A). These data demonstrated that Hippo pathway activation is entirely mediated by general hyperactivation of MAPK signaling and requires factors downstream of ERK. These data also discounted the possibility that oncocgenic \textit{BRAF}^{V600E} activates Hippo signaling via direct phosphorylation of key Hippo pathway components.

We noted that phosphorylation of YAP following \textit{BRAF}^{V600E} expression required sustained MAPK stimulation over a period of at least 12–16 h, as transient MAPK activation only minimally affected YAP phosphorylation (Fig. 4d and Supplementary Fig. S5B). We speculated that mounting oncogene-induced replication stress may be promoting Hippo pathway activation; however, induction of replication stress by hydroxyurea treatment alone was not sufficient to activate the Hippo pathway (Fig. 4e). Alternatively, it has been demonstrated that oncogenic activation of the MAPK pathway dramatically alters actomyosin cytoskeletal contractility and reduces RhoA activity in an ERK1/2-dependent manner\textsuperscript{46–48}. Reductions in active RhoA are well known to promote Hippo pathway activation and, furthermore, ERK1/2-dependent cytoskeletal changes have previously been shown to modulate YAP/TAZ activity in melanoma cell lines\textsuperscript{40,49,50}. We, therefore, posited that the reduction of RhoA activity may represent a mechanism by which \textit{BRAF}^{V600E}-expressing cells activate the Hippo pathway in vitro. Indeed, we observed that there was a significant reduction in the number of actin stress fibers in cells following activation of \textit{BRAF}^{V600E}, indicating reduced RhoA activity (Supplementary Fig. S5C, D). We then directly measured RhoA activity via pull-down assay, which revealed significantly decreased levels of active RhoA in Mel-ST cells expressing \textit{BRAF}^{V600E} relative to controls (Fig. 4f). These data suggest that \textit{BRAF}^{V600E}-induced Hippo pathway activation is at least partially mediated by prolonged MAPK hyperstimulation leading to ERK1/2-dependent cytoskeletal dysregulation. Supporting this view, endogenous \textit{Braf}^{V600E} expression in mouse embryonic fibroblasts has been shown to drastically reduce actin stress fibers, and a recent study has also demonstrated that expression of \textit{BRAF}^{V600E} in RPE-1 cells leads to decreased RhoA activity in vitro and cytokinesis failure in zebrafish (Darp et al. unpublished)\textsuperscript{31}.

\textit{Lats1}\textsuperscript{1/2}\textsuperscript{−/−} deletion promotes melanomagenesis. Our data suggested that functional inactivation of the Hippo tumor-suppressor pathway may enable \textit{BRAF}^{V600E}-expressing melanocytes to evade growth arrest and facilitate melanoma development. To test this, we generated mice carrying floxed alleles of both \textit{Lats1} and \textit{Lats2}\textsuperscript{24} with \textit{Tyr::CreER}\textsuperscript{22} to allow for inducible, melanocyte-specific inactivation of the Hippo pathway (Tyr::CreER\textsuperscript{T2}/\textit{Lats1}\textsuperscript{1/2}/\textit{Lats2}\textsuperscript{1/2}). Deletion of \textit{LATS1}/2 is well established to completely abrogate the Hippo pathway, and co-heterozygous loss of \textit{LATS1}/2 is observed in ~15% of human melanomas, making deletion of \textit{Lats1}/2 clinically relevant (Fig. 3f and Supplementary Fig. S3C, D)\textsuperscript{33,35}. We also crossed \textit{Tyr::CreER}\textsuperscript{T2}/\textit{Lats1}\textsuperscript{1/2}/\textit{Lats2}\textsuperscript{1/2} (\textit{Lats1}/2\textsuperscript{−/−}) mice with mice expressing the Cre-activatable oncogenic \textit{Braf} allele (\textit{Braf}\textsuperscript{K46E}), generating \textit{Tyr::CreER}\textsuperscript{T2}/\textit{Braf}\textsuperscript{K46E}/\textit{Lats1}\textsuperscript{1/2}/\textit{Lats2}\textsuperscript{1/2} (\textit{Braf}\textsuperscript{K46E}/\textit{Lats1}/2\textsuperscript{−/−}) mice (Fig. 5a and Supplementary Fig. S6A). We confirmed the melanocytic specificity of our \textit{Tyr::CreER}\textsuperscript{T2} expressing mice via incorporation of a fluorescent lineage trace (YFP\textsuperscript{46}), whose expression was only observed in cells that co-stained for melanocyte markers (Supplementary Fig. S6B).

We observed that \textit{Braf}\textsuperscript{K46E}/\textit{Lats1}/2\textsuperscript{−/−} mice were highly prone to developing spontaneous dermal tumors within weeks after birth, even without topical 4-hydroxytamoxifen (4-HT) administration. A similar melanoma mouse model, \textit{Tyr::CreER}\textsuperscript{T2}/\textit{Braf}\textsuperscript{V600E}/\textit{Pten}\textsuperscript{fl/fl} (\textit{Braf}\textsuperscript{V600E}/\textit{Pten}\textsuperscript{−/−}), has also been shown to be prone to spontaneous melanoma formation in the absence of topical 4-HT, due to leakage of the inducible Cre recombinase\textsuperscript{44,45}. This suggests that deletion of \textit{Lats1}/2 plays a major role in promoting melanoma development, as \textit{Braf}\textsuperscript{V600E} expression alone in murine melanocytes does not generate tumors\textsuperscript{44,45} (Supplementary Fig. S6A). In the few mice where spontaneous tumorigenesis was absent or delayed, 4-HT administration to \textit{Braf}\textsuperscript{V600E}/\textit{Lats1}/2\textsuperscript{−/−} flanks resulted in the potent form of tumors which appeared histologically similar to the spontaneously arising neoplasms (Fig. 5b, c and Supplementary Fig. S6C). These tumors exhibited strong nuclear YAP/TAZ staining, indicating Hippo inactivation, and positively stained for the melanocytic markers SOX10 and S100 (Fig. 5d). SOX10 staining was nuclear and homogenous whereas S100 staining was weakly heterogeneous. Subsequent histopathologic analysis by a dermatopathologist confirmed these infiltrative, spindle cell tumors to be mouse melanoma. Unlike other \textit{Braf}\textsuperscript{V600E}-driven mouse melanoma models (e.g., \textit{Braf}\textsuperscript{V600E}/\textit{Cdkn2a}\textsuperscript{−/−}, \textit{Braf}\textsuperscript{V600E}/\textit{Trp53}\textsuperscript{−/−}), which still mainly induce nevus formation, we were unable to appreciate any obvious neovgenesis in \textit{Braf}\textsuperscript{V600E}/\textit{Lats1}/2\textsuperscript{−/−} mice. These data imply oncogenic \textit{Braf}\textsuperscript{V600E}-positive melanocytes may be incapable of entering an enduring growth arrest without a functional Hippo tumor-suppressor pathway.

We also investigated the consequences of \textit{Lats1}\textsuperscript{1/2} loss in melanocytes in the absence of oncogenic \textit{Braf}. We found that following melanocyte-specific deletion of \textit{Lats1}/2, mice exhibited no obvious hyperpigmentation, yet still rapidly developed cutaneous tumors with 100% penetrance after 4–5 weeks (Fig. 5e–g and Supplementary Fig. S6C–E). Co-heterozygous deletion of \textit{Lats1}/2 also promoted cutaneous tumorigenesis, albeit at prolonged time scales (Supplementary Fig. S6F). Analysis of \textit{Lats1}/2\textsuperscript{−/−} tumor sections revealed non-pigmented neoplasms which were remarkably similar to invasive \textit{Braf}\textsuperscript{V600E}/\textit{Lats1}/2\textsuperscript{−/−} mouse tumors, exhibited a comparable staining profile, and were subsequently diagnosed as mouse melanoma (Fig. 5h). Previous studies have identified TEAD and AP-1 transcription factors as major regulators of the melanoma invasive state, which is marked by dedifferentiation and loss of pigmentation signatures\textsuperscript{56,57}. We hypothesized that YAP/TAZ-TEAD activation, driven by \textit{Lats1}/2 deletion, may enable melanocytes to directly access this invasive gene program explaining our observed lack of pigmentation. In support of this hypothesis, both \textit{Braf}\textsuperscript{V600E}/\textit{Lats1}/2\textsuperscript{−/−} and \textit{Lats1}/2\textsuperscript{−/−} mouse melanomas exhibited markedly low staining for mature, differentiated melanocyte markers (Supplementary Fig. S6G).

Given \textit{Lats1}/2\textsuperscript{−/−} tumors did not exhibit overt signs of pigmentation, we sought to generate additional data to validate that these neoplasms were melanocytic in origin. It has recently been demonstrated that initiation of the hair follicle cycle, via depilation, strongly promotes melanocyte transformation in
Braf^{V600E}/Pten^-/- mice. We leveraged this unique characteristic of mouse melanomagenesis to test if Lats1/2^-/- tumor formation was also promoted by depilation, suggesting a melanocytic origin. We induced loss of Lats1/2 on opposing mouse flanks and then depilated only one flank so as to compare the tumorigenic rate from depilated and non-depilated regions (Fig. 5i). We observed that skin regions depilated following 4-HT treatment demonstrated significantly faster tumorigenesis, with palpable tumors observed in 70% of depilated areas compared to 20% of non-depilated areas about one month following treatment.
(Fig. 5j). Not only did tumors appear faster in depilated areas, but these tumors also grew significantly larger (Fig. 5k). Collectively, our data demonstrate that melanocyte-specific loss of Lats1/2 alone, or in conjunction with oncogenic Braf expression, promotes mouse melanocyte transformation and the formation of mouse melanoma.

We then investigated whether Hippo pathway inactivation was also occurring in other mouse models of melanoma. We performed gene set enrichment analyses (GSEA) using gene expression data collected from benign and transformed melanocytes from BrafV600E/Cdkn2a+/− and BrafV600E/Cdkn2a−/−/Lkb1−/− mice. GSEA revealed that YAP/TAZ gene sets were significantly enriched in murine melanoma cells as compared to both arrested nevus melanocytes and proliferating non-tumorigenic melanocytes (Fig. 6a and Supplementary Fig. S7A–D). These data reveal that the Hippo tumor-suppressor pathway becomes attenuated as these murine melanocytes transform into melanoma.

Active YAP drives melanoma development. We aimed to further define how deletion of Lats1/2 promotes melanoma development in vivo. While it is well-described that Lats1/2 loss functionally inactivates the Hippo pathway and leads to the activation of YAP and TAZ, LATS1/2 can also impinge upon additional signaling pathways that promote tumor development. For example, recent research has revealed inactivation of the Hippo pathway can promote mTOR signaling via multiple routes including YAP-driven expression of a micro-RNA, miR-29, which targets PTEN mRNA for silencing60–62. However, we detected no observable changes in PTEN protein levels following either RNAi-mediated knockdown of LATS1/2 or expression of constitutively active YAP (YAP-SSA) or TAZ (TAZ-4SA) in Mel-SC cells (Fig. 6b, c). We also could not appreciate any significant change in phosphorylated S6 levels following LATS1/2 silencing (Fig. 6b). Furthermore, examination of Lats1/2−/− tumors revealed PTEN remained strongly expressed in vivo (Fig. 6d). These data reveal that loss of Lats1/2 is not driving melanomagenesis by activating mTOR via miRNA-mediated depletion of PTEN. It has also been demonstrated that active LATS2 can bind and inhibit MDM2 leading to increased p53 protein levels63, raising the possibility that deletion of Lats1/2 leads to decreases in p53, which may facilitate BrafV600E−driven murine melanomagenesis64. Discounting this, we found that p53 still accumulates in Lats1/2−/− tumors (Fig. 6d). Previous studies have also demonstrated that Lats1/2 knockout can induce cytokinesis failure and whole-genome doubling (WGD) in MEFs. Since WGD is well known to facilitate tumorigenesis, and a significant fraction of human melanomas are WGD, we speculated that Lats1/2 loss may drive tumorigenesis in vivo through an initial WGD event. To test this possibility, we performed copy-number analysis using ultra-low pass whole-genome sequencing (ULP-WGS) to examine if genomic alterations, such as WGD events, were enriched in BrafV600E/Lats1/2−/− tumors relative to BrafV600E/Pten−/− tumors. However, sequencing revealed that all tumors from these models were diploid, excluding the possibility that Lats1/2 deletion was primarily inducing melanoma through a WGD intermediate (Supplementary Fig. S8A).

We then assessed whether YAP activation alone was sufficient to promote melanoma development. To do so, we generated a transgenic zebrafish model that expresses constitutively active YAP (YAP-SSA) in zebrafish melanocytes utilizing the mini-CoopR system65. These Tg(mi3taa:YAP-SSA) zebrafish rapidly developed pigmented fish melanoma (Fig. 6e), demonstrating that constitutively active YAP is sufficient to induce melanoma development in a zebrafish model.

As activation of YAP/TAZ signaling was observed to promote melanomagenesis, we investigated whether depletion of YAP/TAZ could inhibit melanoma cell growth. RNAi-mediated knockdown of YAP/TAZ in the BrafV600E/Pten−/− mouse melanoma cell line (D4M.3A) resulted in significantly decreased viability as compared to immortalized melanocytes (Supplementary Fig. S7E). We further explored YAP/TAZ dependency in a panel of human melanoma cell lines utilizing the pan-TEAD inhibitor MGH-CP1 and found that a number of melanoma cell lines were strikingly sensitive to inhibition of YAP/TAZ activity (Fig. 6f and Supplementary Fig. S7F). While YAP is not commonly mutated in human melanoma, YAPI amplifications and mutations have been observed, and YAP staining in primary human melanoma has been shown to significantly correlate with reduced patient survival31,38,67. Together, these data implicate YAP as a cutaneous melanoma oncogene and novel therapeutic target to further explore in the treatment of human melanoma.

Discussion

Discerning the molecular pathways that govern the growth arrest of nevus melanocytes, and how melanocytes ultimately overcome these barriers, is critical to fully understanding the mechanisms of melanomagenesis. A considerable body of work supports a role for OIS in preventing tumorigenic growth of melanocytes in vitro and in vivo; however, expanding lines of evidence demonstrate melanocytes within nevi retain proliferative capacity, in conflict with the absolute growth arrest implied by OIS16. Indeed, up to 30% of melanomas are predicted to arise from pre-cursor nevi17. These observations highlight nevi as being the product of “stable clonal expansion” rather than senescence16. Several genetic alterations that have the capacity to overcome the stable growth arrest of melanocytes expressing oncogenic MAPK mutations have been identified, including CDKN2A, PTEN, and TP5333,3–7,64. While deletion of Cdkn2a and Trp53 with BrafV600E expression induces murine melanomagenesis, the vast majority of melanocytes still enter a stable growth arrest to form nevi7. Further, recent murine single-cell sequencing analyses reveal BrafV600E-positive nevus melanocytes do not exhibit expression of senescence signatures25. Together, these discoveries suggest additional unidentified tumor suppressive mechanisms exist to enforce the arrest of nevus melanocytes.

We discovered that expression of BrafV600E promotes activation of the Hippo tumor-suppressor pathway across multiple cell lines and that BrafV600E-positive murine nevus melanocytes display significantly decreased YAP/TAZ signaling in vivo. Our data demonstrate that oncogenic Braf expression induces Hippo pathway activation and cell cycle arrest in vitro by way of a cell-intrinsic mechanism, in which hyperactive MAPK signaling alters the cytoskeleton in part through decreased RhoA signaling and thus indirectly leads to Hippo pathway activation. However, our data also suggest that cell-extrinsic cues, perhaps secondary to the nevus microenvironment, may also be impinging upon YAP/TAZ signaling in vivo. Our single-cell sequencing analysis of mouse melanocytes indicated that while BrafV600E-expression leads to a general repression of YAP/TAZ signaling in most melanocytes, the effect is strongest in nevus melanocytes (Fig. 2d). A recent modeling study has proposed that the growth arrest of nevus melanocytes may be due to cells sensing and responding to their collective overgrowth, rather than cell-autonomous mechanisms25. This growth arrest model is highly analogous to the collective cell processes mediating contact inhibition and organ growth, which are governed by the Hippo pathway. It is therefore tempting to speculate that melanocyte overgrowth induced by oncogene expression significantly increases melanocyte density over multiple rounds of division, ultimately promoting mounting activation of the Hippo tumor-suppressor pathway, eventual growth arrest, and nevus formation (Fig. 6g). If
true, one would predict Hippo pathway activation would only engage after a nevus passes a critical size threshold and that nevus melanocyte growth should be restored by reducing local melanocyte density. Intriguingly, nevi that are partially resected have been observed to regain proliferative capacity and the majority of these recurrent nevi did not grow beyond the limits of the original surgical scar, suggesting these nevus melanocytes arrest once they attain a similar size. While growth arrest of oncogene-expressing melanocytes is conspicuous due the formation of pigmented nevi, it is also possible that oncogene-induced growth arrest of cells from other tissues is similarly mediated by activation of the Hippo pathway.
We also demonstrated that functional impairment of the Hippo pathway in melanocytes in vivo, either through deletion of Lats1/2 in mice or expression of constitutively active YAP1 in zebrafish, promotes cutaneous melanomagenesis. This finding has clinical relevance as co-heterozygous loss of any appreciation in oncogenic WGD disease. It is believed that activating MAPK mutations are critical for tumor-suppressor pathway promotes melanocyte growth arrest in vitro and tumor development in vivo in multiple model organisms of melanomagenesis. Collectively, our data implicate the Hippo pathway as an important melanoma tumor suppressor and highlight YAP/TAZ as promising therapeutic targets to investigate for the treatment of human melanoma.

**Methods**

**Cell culture.** Immortalized melanocyte (Mel-ST) cells were a gift from the lab of Dr. Robert Weinberg. Mel-ST cells, and all derivative cell lines generated in this study, were grown in DMEM media containing 5% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin. Human Embryonic Kidney 293 (HEK293A) cells, and all derivative cell lines, were grown in DMEM media containing 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. hTERT-BJ fibroblasts, and all derivative cell lines, were grown in DMEM:F12 media containing 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. BrafV600E/JPen−/− mouse tumor cells (DM3.A) were a generous gift of Dr. Constance Brinckerhoff. DM3.A tumor cells were grown in DMEM:F12 media containing 5% FBS. Primary adult epidermal melanocytes were purchased from ATCC (PCS-200-013) and maintained in Dermal Cell Basal Medium (ATCC PCS-200-030) supplemented with an Adult Melanocyte Growth Kit (ATCC PCS-200-042). All FBS used in these studies was confirmed to either be non-human or absent of tetracyclines or below 20 ng/mL by the manufacturer. All cells were maintained at 37 °C with 5% CO2 atmosphere and maintained at subconfluent levels for passaging and all experiments. Cultures were regularly checked for mycoplasma contamination utilizing a PCR detection kit (G238, ABM) or Hoechst staining. Bright-field images of tissue culture cells were captured on an Echo Revolve Hybrid Microscopy system at ×10 or ×20 (Echo Laboratories).

**Cell-line generation.** To generate the BrafV600E doxycycline-inducible system Mel-ST, HEK293A, or hTERT-BJ fibroblasts were first infected with lentivirus generated from pLenti CMV TetR Blast (Tet Repressor) and selected. Following selection, cells were infected with lentivirus generated from pLenti CMV/TO BrafV600E Neo or pLenti CMV/TO Braf Neo, and single-cell cloned to establish cell lines which demonstrated no basal expression at baseline and strong induction after doxycycline addition. The expression of BrafV600E was confirmed using two different mutant-specific antibodies (VE1 and RM8 clones). To stably express H2B-GFP lines, cells were infected with lentivirus generated from pLenti H2B-GFP Blast. Mel-ST cells stably expressing empty vector (pLVX Pur), YAP-TAZ (pBARE YAP-TAZ-AS4A), and TAZ-AS4A (pLVX Flag TAZ-AS4A) were generated via viral infection followed by selection. Tetraploid cells were generated by treating asynchronous cells with 4 µM DCB for 16 h, followed by gentle washing to remove drug (5 × 5 min); completion of cytokinesis was confirmed by phase-contrast imaging.

**Viral infections and transfections.** Mel-ST, HEK293A, or BJ Fibroblasts were infected for 12–16 h with virus-carrying genes of interest in the presence of 10 ng/mL polybrene, washed, and allowed to recover for 24 h before selection or single-cell cloning. Short-term viral infection of primary melanocytes was carried out...
similarly, but with 2 µg/mL of polybren. All RNAi transfections were performed using 25–50 nM siRNA with Lipofectamine RNAi MAX according to the manufacturer’s instructions. Cells were transfected with 12-well microplates for 60 min with a transfection agent by addition of reverse transcription mixture overnight for 18 h, which was then washed with PBS and replaced with fresh media, or forward transfection mixture for 4 h, which was then replaced with fresh media. Cells were then incubated for 48–72 h prior to lysis at sub-confluent levels.

**Plasmid generation.** Plasmids encoding the Tetracycline Repressor, pLenti CMV TetR Blast (176-1), was a gift from Eric Campeau & Paul Kaufman (Addgene Plasmid #17492). To create pLenti CMV/TO BRAF Neo and pLenti CMV/TO BRAFV600E Neo, we performed Gateway cloning using Gateway LR Clonase II (Invitrogen) according to manufacturer instructions to insert BRAF or BRAFV600E into pLenti CMV/TO Neo DEST (685-3) using pENTR BRAF or pENTR BRAFV600E and pENTR BRAF and pLenti CMV Neo DEST (685-3) was a gift from Eric Campeau & Paul Kaufman (Addgene plasmid #17292). To generate pLenti CMV/TO NRASG12R1K Neo we performed Gateway cloning to insert NRASG12R1K from the donor vector pDONR223 NRASG12R1K into the destination vector pLenti CMV/TO Neo Dest. pDONR223 NRASG12R1K was a gift from Jesse Boehen, William Hahn, and David Root (Addgene plasmid #81652).

**Immunofluorescence and confocal microscopy.** Cells were plated on glass coverslips, treated as indicated, washed in 1× phosphate-buffered saline (PBS) (Becton Bioproducts), and fixed in 4% paraformaldehyde for 10 min. Cells were then washed in PBS-0.01% Triton X-100, extracted in PBS-0.2% Triton X-100 for 10 min, rinsed in PBS to remove unincubated primary antibodies and then blocked for 1 h in 10% goat serum in TBS. Immunofluorescence images for analysis were collected on a Nikon Ti-E inverted microscope equipped with a Zyla 4.2 PLUS (Andor) and X-Cite 120 LED light source at the same exposure. Confocal immunofluorescence images were collected on a Nikon Ti-E inverted microscope equipped with a C2 + laser scanning confocal head with 405 nm, 488 nm, 561 nm, 640 nm laser lines. Z-stacks were acquired with a series of 0.5–1 µm optical slices which were then converted into a single, high-resolution image. Images were analyzed using NIS-Elements Advanced Research (AR) and ImageJ (Version 1.51). To assess YAP localization, two small square regions of interest were drawn at random in individual cells with one in the nucleus, and one in the cytoplasm. The background-corrected, mean fluorescence intensity of YAP was subsequently measured in these regions of interest and a nuclear to cytoplasmic ratio was determined. To assess stress fiber quantity, images were background-corrected, contrast normalized, and then fibers obvious to the naked eye were counted.

**Live-cell imaging.** Cells stably expressing H2B-GFP were grown on glass-bottom 12-well tissue culture-treated dishes (Cellvis) and treated with drugs of interest. Immediately post-treatment imaging was performed on a Nikon Ti-E inverted microscope equipped with the Nikon Perfect Focus system. The microscope stage was enclosed within a temperature and atmosphere-controlled environment at 37 °C. For live-cell imaging, cells were incubated with 250 nM trinitrophenylmaleimide, and then blocked for 1 h in 10% goat serum in TBS (Sigma-Aldrich). For IF, tissue sections were blocked for 1 h in 10% goat serum in TBS. Following serum block, if necessary, tissue was incubated with Rodent Block M (Biocare Medical) for 30 min to block endogenous mouse IgG prior to primary antibody addition. Primary antibodies were diluted in 10% goat serum in TBS and incubated overnight at 4 °C in a humidified chamber. Following primary addition for IHC, slides were washed with TBS-0.01% Tween-20, incubated with anti-rabbit or mouse SignalStain Boost IHC detection reagent (Cell Signaling Technology) for 30 min and then developed with SignalStain DAB substrate and DAB chromogen (Cell Signaling Technology) according to manufacturer’s instructions. Counterstaining was performed using hematoxylin, followed by dehydroxylation, clearing, and mounting with Cytoseal XYL (Thermo Fisher). Images were captured at randomly selected points using a Nikon Ti-E inverted microscope equipped with a DS-Ri2 (Nikon). For IF, slides were incubated with species-specific fluorescent secondary antibodies (Molecular Probes) and 2.5 µg/mL Hoechst. For 1 h at room temperature in a dark humidified chamber. Auto-fluorescence was quenched using Vector TrueVIEW according to the manufacturer’s instructions, and slides were mounted using Prolong Gold Antifade (Invitrogen). Images were captured at randomly selected points using a Nikon Ti-E inverted microscope equipped with a Zyla 4.2 PLUS (Andor) and X-Cite 120 LED light source. For all staining experiments, tissue-specific secondary controls were included in each staining experiment to ensure specificity and control for endogenous tissue pigment levels.

**Human nevi and melanoma samples.** Human skin tissues with melanocytic nevi and melanoma were retrieved from archived material in the pathology laboratory at UMass Medical Center in compliance with all relevant ethical regulations and were determined to be exempt by the Institutional Review Board at UMass Medical Center (IRB H00007200). The tissue blocks were deidentified before tissue sections (10 µm) were cut and stained by the author’s laboratory. These studies were reviewed in compliance with all relevant ethical regulations and were determined to be exempt by the Boston University School of Medicine Institutional Review Board (IRB H-37967).

**RNA isolation and qRT-PCR.** Total RNA from cultured cells was isolated using a Quick-RNA kit (Zymo Research). cDNA libraries were generated from RNA using the Superscript III kit and random hexamer primers (Invitrogen). Quantitative real-time PCR was performed using SYBR Green reagents in a StepOnePlus system (Applied Biosystems) according to manufacturer protocol. For each individual experiment, a technical triplicate was run which was then averaged to generate a single biological replicate. Primer sequences were as follows:

- **CYR61:** forward, AGGCCTCGCATCTTATACACC; reverse, TTCTTCTCACAAGGGCCAGCT
- **AMOTL2:** forward, TTGGAAATCTGCAAATCGCC; reverse, TGCTGTTCGTA
- **GCGCCCAGCT
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**Antibodies.** The antibodies used herein categorized by technique and company. *Immunochemistry (cells):* Santa Cruz Biotechnology: YAP 63.7, 1250 (detects both YAP/TAZ, sc-101199). *Immunochemistry (tissue):* Cell Signaling Technologies: YAP (1A12) #12395, 1:1000; YAP (DS811X) #14074, 1:100. Abcam: gp100 (ab137078), 1:250; SOX10 (Rabbit, ab180862), 1:250; SOX10 (Mouse, ab216020), 1:250.
Immunohistochemistry; Cell Signaling Technologies: YAP/TAZ (D24E4) #8418, T:300; YAP (DSH1) #14074, 1:000; GFP (D5.1, cross reacts with YFP) #2956, 1:100; phosphorylated ERK1/2 (Thr202/Tyr204) #9101, 1:100; Abcam: gp100 (ab137078), 1:250; SOX10 (Rabbit, ab180862), 1:250; SOX10 (Mouse, ab216200), 1:250; Melan-A (ab210546), 1:500. Dako: S100 (IS504), pre-diluted by the manufacturer.

Immunoblotting; Cell Signaling Technologies: B-Raf (D5T6S) sc-1000, 1:100; phosphorylated p44/42 ERK1/2 (Thr202/Tyr204) #9101, 1:1000; Ras-related C3 botulinum toxin substrate 1 (RhoA pull-down activation assay).

ImageJ (version 1.51). Microscopy on a Nikon Ti-E inverted microscope equipped with a Zyla 4.2 PLUS high-speed camera. Images were acquired using an Echo Revolve Hybrid Microscopy system at ×10 (Echo Laboratories), ×20 (Echo Laboratories) or ×40 (Echo Laboratories) magnification, with an exposure time of 0.015-0.1 second. Images were processed with Fast Blast with 0.015-0.1 second exposure.

Soft-agar assays. In six-well dishes, sterile 2% noble agar stock solution in water was dissolved by heating to 40-45 °C, mixed with warm media, plated at a final concentration of 0.6%, and allowed to cool and solidify at 4 °C. After solidification, gels were warmed to 37 °C. Next, cells were trypsinized, counted, 1 × 10^4 cells were plated in 0.3% noble agar and allowed to solidify at solidification temperature or briefly at 4 °C. Plates were maintained in a cell culture incubator for 2–4 weeks with feedings of 1.5 mL of 0.3% agarose solution weekly. All drugs were maintained at 2× concentration in underlays and independent experiments were done in technical triplicate. Total colonies per well were counted using phase-contrast imaging on an Echo Revolve Hybrid Microscopy system at ×10 (Echo Laboratories). For imaging, gels were stained for 20 min with 0.1% Crystal Violet, gently washed multiple times overnight, and imaged on a Chemi-Docs XRS+ system under Fast Blast with 0.015-0.01 sec exposure.

EdU assays. Mel-SC cells were seeded on coverslips a day before each collection at a density of 4.5 × 10^4 cells/well in a 12-well culture dish. Cells were treated with 10 µM EdU for 1 h, fixed with 4% PFA at different timepoints since the addition of doxycycline. Incorporation of EdU was visualized using the Click-iT EdU kit from Invitrogen according to the manufacturer’s protocol (C10337), imaged via fluorescence microscopy on a Nikon Ti-E inverted microscope equipped with a Zyla 4.2 PLUS (Andor) and X-Gite 120 LED light source at the same exposure, and analyzed in ImageJ (version 1.51).

RhoA pull-down activation assay. Active, GTP-bound RhoA was assayed using the RhoA activation assay, bead pull-down format, from Cytoskeleton, Inc (BK036) according to manufacturer instructions. Briefly, dox-inducible BRAFV600E expressing Mel-SC cells were plated at 3 × 10^5 cells in six well dishes and allowed to grow for 2–3 days. Prior to reaching 70% confluence, cells were serum-starved and treated with 1 mM GTP gamma S for 24 h, then stimulated with complete media for 6 h prior to ice-cold lysis. Following lysis, input was isolated from total lysate prior to freezing with liquid nitrogen and storage at −80 °C. Active RhoA pull-down was performed according to manufacturer instructions utilizing GST-tagged Rhotekin-RBD protein on agarose beads. Samples were then analyzed using immunoblot according to manufacturer protocols.

Drug treatments. The concentrations used for the MAPK pathway inhibitors (MEK1-1/2 and ERK1/2) were determined experimentally to be the doses at which phosphorylation of ERK1/2 and RSK1/2 returned to baseline despite the presence of oncogenic BRAF expression. The reagents used in these studies are as follows: MEKi-1: U0126 (Selleck Chemicals), 10 µM; MEKi-2: Trametinib (GSK1120212), 7 µM; MEKi-1/2 and ERKi were determined experimentally to be the doses at which expression was < 30% of that observed in control cells.

Gene expression analysis. Gene expression analysis was performed according to manufacturer instructions utilizing GST-tagged Rhotekin-RBD protein on agarose beads. Samples were then analyzed using immunoblot according to manufacturer protocols.

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constructs were injected with 20 mg/ml tamoxifen (Sigma, T5648) dissolved in corn oil to be injected daily with 100 µl tamoxifen for 5 consecutive days. Tumor development as well as on an approximately 2 cm length of the tail for 3 consecutive days. Two topically applied to each square for 3 consecutive days as above on the right flank, and a higher concentration of 4-HT was required; 4-HT was dissolved in dimethylsulfoxide (DMSO) to a concentration of 25 mg/ml, and topical administration was performed as above. For the depilation experiments using Lats1/2−/− mice, a 2 cm2 was shaved on both the left and right flank of each mouse, and 5 mg/ml 4-HT was topicaly applied to each flank daily for 3 consecutive days as above on the right flank, as well as on an approximately 2 cm length of the tail for 3 consecutive days. Two days after the final application, chemical depilation was performed by application of Nair hair remover onto the right flank for 15 s, followed immediately with wiping with a damp tissue to prevent irritation. For systemic knockout experiments, mice were injected with 20 mg/ml tamoxifen (Sigma, T5648) dissolved in corn oil to induce deletion in all Tyrinosine expressing cells. 8–12-week-old mice were injected daily with 100 µl tamoxifen for 5 consecutive days. Tumor development was measured weekly using calipers.

Zebrafish studies. Zebrafish were handled in accordance with protocols approved by the University of Massachusetts Medical School IACUC (Protocol A-2171). Constructs ProenfishEGFP-A and ProenfishYAP-SSA were used in the miniCoopR assay as previously described. Briefly, mitfa(−/−) mutant animals were bred, and single-cell stage embryos were injected with 25 pg of a single construct and 25 pg of Tol2 transposable mRNA. Successful Tol2-mediated integration of the construct into the genome rescued the mitfa(−/−) phenotype, enabling melanocyte development. Equal numbers of male and female zebrafish were used. Melanocyte rescue was scored at 4–5 days of development, and rescued animals were grown to adulthood and monitored weekly for the presence of melanosomes.

Statistical analysis. All quantitative data are presented as mean ± SEM, unless otherwise indicated. The number of samples (n) represents the number of biologic replicates or animals in study, unless otherwise indicated. Prism 9 was used for all statistical analyses and for the construction of most graphs.

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Acknowledgements

We would like to thank Jackie King and Ross King for their unwavering resolve to promote melanoma awareness and research, as well as Qi Sun, Shuyang Chen, and the entire Garab Lab for wisdom and advice. We would also like to thank April Deng, Karen Dresser, Constance Brinckerhoff, Robert Weinberg, and Arthur Lander for sharing cell lines, reagents, and/or assistance. The results published herein are partly based upon data generated by the TCGA Research Network (http://cancer.gov/tcga). We thank all the patients who donated specimens to both the TCGA and the MSKCC database and the Molecular Biology Core Facilities at the Dana-Farber Cancer Institute for their assistance with the copy-number analysis. M.A.V. is supported by a Ruth L. Kirchstein National Research Service Award (F30) from the NCI (1F30CA228388) and was previously supported by a training grant from the NIGMS (T32GM085451). E.K. and S.M. were both supported by an award from the Boston University Undergraduate Research Opportunities Program. X.V. is supported by the NHLBI (R01HL124392) and the American Cancer Society - Ellison New England Research Scholar Grant (RSG-17-138-01-CSM). N.M.K. was supported by the NHLBI (F31HL146163). C.J.C. was supported by the USA Department of Defense (W81XWH2010288). N.J.G. is a member of the Shannan and Ashraf Dohab Breast Cancer Research Laboratories and this work was supported in his lab by the NIGMS (GM117550), the Harry J. Lloyd Charitable Trust, the Jackie King Young Investigator Award from the Melanoma Research Alliance, and the Searle Scholars Program. This work was also supported by a pilot grant from the ACS and the Boston University Clinical andTranslational Science Institute Bioinformatics Group (1UL1TR001430).

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M.A.V. and N.J.G. conceptualized the study, designed the in vitro experiments, and wrote the manuscript. M.A.V., N.J.G., X.V., and N.J.G. designed all in vivo studies. M.A.V. performed most of the cell biological assays, tissue staining, and imaging analysis. N.K. performed in vivo experiments, with the assistance of M.A.V. and K.K., and prepared all figures. N.K., X.V., and N.J.G. designed all in vivo studies. M.A.V. and N.J.G. conceptualized the study, designed the in vitro experiments, and wrote the manuscript. M.A.V., N.K., X.V., and N.J.G. assisted in preparing all figures.

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and tissue assays. X.X. provided dermatopathology consult and tissue analysis. R.D. and C.C. performed zebrafish experiments. R.H. and J.D.C. completed the single-cell analysis and M.A.V. performed GSEA. L.H. and D.L. provided critical reagents.

**Competing interests**
The authors declare no competing interests.

**Additional information**

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-31399-w.

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**Peer review information** *Nature Communications* thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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