AP-1 and TGFβ cooperativity drives non-canonical Hedgehog signaling in resistant basal cell carcinoma

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Tumor heterogeneity and lack of knowledge about resistant cell states remain a barrier to targeted cancer therapies. Basal cell carcinomas (BCCs) depend on Hedgehog (Hh)/Gli signaling, but can develop mechanisms of Smoothened (SMO) inhibitor resistance. We previously identified a nuclear myocardin-related transcription factor (nMRTF) resistance pathway that amplifies noncanonical Gli1 activity, but characteristics and drivers of the nMRTF cell state remain unknown. Here, we use single cell RNA-sequencing of patient tumors to identify three prognostic surface markers (LYPD3, TACSTD2, and LY6D) which correlate with nMRTF and resistance to SMO inhibitors. The nMRTF cell state resembles transit-amplifying cells of the hair follicle matrix, with AP-1 and TGFβ cooperativity driving nMRTF activation. JNK/AP-1 signaling commissions chromatin accessibility and Smad3 DNA binding leading to a transcriptional program of RhoGEFs that facilitate nMRTF activity. Importantly, small molecule AP-1 inhibitors selectively target LYPD3+/TACSTD2+/LY6D+ nMRTF human BCCs ex vivo, opening an avenue for improving combinatorial therapies.
Results

nMRTF BCC chromatin accessibility resembles HF matrix. We have previously shown that active nuclear MRTF (nMRTF) in BCCs confers Hh target gene transcription and persistent growth in the presence of SMO inhibitors (SMOi) including vismodegib. However, the key driving factors that lead to active nMRTF remain unclear. To understand how MRTF interacts with the Hh pathway in the context of normal skin homeostasis, we examined MRTF cellular localization in mouse skin. Intriguingly, while BCCs are thought to derive from the bulge and outer root sheath (ORS)18, we found that nMRTF is restricted specifically to the matrix transit-amplifying cells (TACs) of the HF and dermal papilla (DP) (Fig. 1a, Supplementary Fig. 1a), with other subdomains of the skin showing predominantly cytoplasmic localization. While both the ORS and TACs respond to Shh signaling, TACs are committed HF progenitors that undergo Shh-dependent expansion during anagen. Notably, the HF matrix contains cells that express the highest levels of Hh signaling19, and they are known to be highly responsive to TGFβ and Wnt secreted from the DP20,21 (Fig. 1a, Supplementary Fig. 1a).

To further characterize the differentiation state of nMRTF cells, we interrogated MRTF cellular localization in clinical samples of benign skin tumors with known cellular origins. Strong and uniform nuclear MRTF was observed in HF matrix-derived pilomatricomas (PMX), while epithelial inclusion cysts (EIC) derived from interfollicular epidermis (IFE) as well as trichilemmal/pilar cysts (PC) derived from ORS displayed cytoplasmic MRTF localization22,23 (Fig. 1b). Furthermore, we found that out of 1201 MRTF target genes identified in resistant BCCs (Supplementary Data 1), 65% of those genes are upregulated in basal and/or suprabasal TACs24. These observations reinforce the relation of MRTF activity with the hair matrix lineage.

In parallel, we took a global approach to analyze the chromatin landscape relationships between BCC and normal skin by comparing the Assay for Transposase Accessible Chromatin (ATAC-seq) profiles of resistant and sensitive BCCs with those of various epithelial cell types (Fig. 1c, Supplementary Fig. 1b, Supplementary Table 1). We compared open chromatin of murine nMRTF BCC cell line ASZ001-25, previously selected to be highly resistant to SMOi-induced changes in cell growth (res-BCC)26, with other published ATAC-seq datasets from sensitive BCC generated from $K14$-creER; $Ptch1^{fl/fl};Tg53^{f/f}$ mice (sens-BCC)27, basal and suprabasal TACs (basal TAC and suprabasal TAC), bulge HF stem cells, hair germ (HG)24, and interfollicular epidermal stem cells (IFE)28. Principal component analysis (PCA) representation of these relationships reveals that the chromatin accessibility profiles of nMRTF resistant BCC cells cluster most closely with those of suprabasal and basal TAC, while sensitive BCC clusters with HG and bulge stem cells (Fig. 1c and Supplementary Fig. 1b).

We also compared nMRTF cells to residual BCC cells (resid-BCC), reversible Wnt-dependent, IFE-like tumor cells identified in a murine BCC model after vismodegib treatment. We see that multiple resistant nMRTF BCC cell lines (ASZ and BSZ)25 have quite disparate chromatin accessibility profiles from sensitive BCC as well as residual BCC (Supplementary Fig. 1d). Furthermore, the genes with increased chromatin accessibility in resistant nMRTF BCC comprise a uniquely pro-proliferative, pro-migratory program, suggesting they represent a distinct cell fate (Supplementary Fig. 1e). Significant phenotypic differences also exist between these BCC cell types, as nMRTF cells maintain high Hh signaling and proliferation rates when treated with vismodegib12 (see below), while residual tumor cells resume growth only when treatment is discontinued27. We conclude that SMOi-resistant nMRTF BCCs possess a distinct cellular state within naive tumors most closely resembling HF matrix TACs.

The observation that MRTF-SRF-Gli chromatin occupancy confers SMO-independent enhancement of Gli activity suggests the intriguing hypothesis that nMRTF functions in the hair to extend hair matrix proliferation at a distance from the Shh signal...
localized to the lateral disc\(^2\). Indeed, in mouse anagen HFs, proliferating matrix cells extend to the line of Auber on average 4–5 cell divisions and cease as nMRTF becomes cytoplasmic (Fig. 1a, d). We tested this hypothesis by treating mouse HF explants with CCG-1423, an MRTF-inhibitor (MRTF \(i\)), and found markedly reduced proliferation in the matrix (Fig. 1d, e) as measured by Ki67 staining, and a concomitant decreased total HF length (Fig. 1f, g). Altogether, we conclude that MRTF nuclear localization is linked to a distinct cell state of differentiation, allowing it to extend the cellular response to Shh signaling.
LYPD3/TACSTD2/LY6D mark the nMRTF population in patient BCCs. We have shown previously that naive human BCCs, such as those excised through standard Mohs surgical techniques, contain a heterogeneous number of cells displaying active nMRTF, which is predictive of the effectiveness of MRTF versus SMO1 for BCC treatments12. Therefore, we took advantage of the naturally occurring heterogeneity and conducted scRNA-seq on four naive human BCC tumors to identify and segregate the MRTF-active vs. inactive subpopulations for further study (Supplementary Fig. 2a, b). From the 45,656 total cells analyzed, the epithelial tumor cells were segregated from fibroblast, endothelial, and immune populations through Krt14 expression (Supplementary Fig. 2c–g). Samples were batch-corrected using canonical correlation analysis (CCA) before dimensional reduction and visualization of clusters via tSNE (Fig. 2a).

Cells with the highest MRTF activity were identified through an enrichment score based on the expression of a signature list of MRTF target genes (Fig. 2b). The list is derived from the intersection of SRF target genes identified by ChIP-seq, and MRTF–dependent genes identified by RNA-seq in resistant BCC cells treated with MRTF inhibitor CCG-142312 (Supplementary Fig. 2h, Supplementary Data 1). Clusters were then ranked based on their mean MRTF signature enrichment score, and the 12 clusters were separated into four groups: high MRTF, med-high, med-low, and low MRTF (Fig. 2c). Interestingly, the four tumors were not equally distributed among the clusters, as evidenced by the significant chi-squared statistic. The high MRTF clusters contained higher percentages of both of the four tumors (BCC8 and BCC3), consistent with our previous observation that at least 50% of naive clinical BCC samples contain nMRTF12 (Supplementary Fig. 2i).

We then determined the most highly upregulated genes in the high MRTF group vs. low MRTF group (Fig. 2d). To separate MRTF-active BCC cells by fluorescence-activated cell sorting (FACS), we selected target genes of MRTF whose protein products are expressed on the cell surface. The top three most highly enriched surface markers in the MRTF high group were LYPD3, TACSTD2 (also known as TROP-2), and LY6D (Fig. 2e, Supplementary Fig. 2j–l). To validate the specificity of these surface markers, we immunostained human and mouse BCC tumor cells along with MRTF. We found the protein levels of all three markers was significantly higher in tumor regions with nMRTF (Fig. 2f, g). Interestingly, the surface markers are also expressed at high levels in the HF matrix in normal mouse skin, correlating with nuclear MRTF (Fig. 2f). We also treated naive patient BCC explants with MRTF1 CCG-1423 and observed significantly reduced protein levels of all three surface markers, further confirming that expression of these markers is dependent on MRTF activity (Fig. 2h). To correlate the expression of the surface markers with functional resistance, we treated naive patient BCC explants with SMO, vismodegib, and found that protein expression of all three markers is significantly correlated with maintenance of Gli1 mRNA levels, indicating diminished response to SMO inhibition ex vivo (Fig. 2i). These data confirm that the three surface proteins LYPD3, TACSTD2, and LY6D are reliable indicators of nuclear MRTF activity as well as prognostic markers of SMO resistance in human BCC cells.

To further investigate the chromatin landscape and transcriptional differences specific to resistant nMRTF BCC, we sorted fresh naive clinical BCC samples first based on epithelial ITGA6 expression, then on those three surface markers (Fig. 2j, Supplementary Fig. 2m). Interestingly, we see significant co-expression of LYPD3 and TACSTD2, while LY6D shows much scarcer expression levels, consistent with our scRNA-seq results (Fig. 2e, Supplementary Fig. 2j–l). For this reason, we sorted ITGA6+/LYPD3+/TROP2+/LY6D+/− cells as surface marker positive (SM+) population and ITGA6+/LYPD3− TROP2− LY6D− cells as surface marker negative (SM−) and compared SM+ and SM− RNA-seq and ATAC-seq profiles (Supplementary Fig. 2p, q). Importantly, the expression of the MRTF signature gene list was significantly enriched by GSEA analysis in the SM+ population, further validating the three surface proteins as reliable markers of MRTF activity (Fig. 2k, l). As additional support of the specificity of these surface markers for resistant nMRTF BCC, we examined their expression in Gorlin syndrome patient BCCs, which are caused by inherited loss of PTCH1 and respond to vismodegib with almost no resistance20, as well as being consistently MRTF-inactive12. Indeed, Gorlin’s syndrome BCC tumor cells lack expression of the three surface markers (Supplementary Fig. 2n). Chromatin accessibility profiles of Gorlin BCCs are more similar to sorted SM− than SM+ naive patient BCCs by PCA (Supplementary Fig. 2o), providing further support that the SM+ surface phenotype can prospectively enrich for BCC cells possessing the distinct resistant nMRTF cell fate, allowing further characterization from heterogeneous BCC populations.

**BCC resistance requires coincident AP-1 and TGFβ signaling.** We used BETA analysis31 to integrate transcriptomic and chromatin accessibility data in order to identify differentially regulated genes and TF motifs between SM+ and SM− patient BCC cells (Supplementary Fig. 3a). Interestingly, the top Gene Ontology (GO) Biological Process terms32 enriched in the SM+ cells included terms associated with epidermal development and keratinocyte differentiation, underscoring the idea that resistant nMRTF BCC resemble a more differentiated cell state than sensitive BCC (Fig. 3a). To find driver TF pathways, we looked at enriched TF motifs as well as target gene expression from Chip-X Enrichment Analysis (ChEA)33 (Fig. 3b, c). Notably, AP-1, Smad2/3, and p63...
were among the top TFs acting in SM+ cells. As p63 is a known master regulator for epidermal development, we focused on AP-1 and Smad as potential activators of MRTF and non-canonical Hh signaling.

First, we assessed the necessity of TGFβ and AP-1 signaling in resistant BCC through genetic and pharmacologic perturbation. Treatment of BCC cells with various AP-1 small-molecule inhibitors (T522434 and SR1130235) resulted in a dose-dependent decrease in expression of Gli1 measured by mRNA and protein, and cell viability (Fig. 3d, e and Supplementary Fig. 3b, c). We also observed elevated levels of phosphorylated JNK in active nMRTF BCCs in comparison to inactive cMRTF BCC (Supplementary...
Fig. 2 LYPD3/TACSTD2/LY6D mark the nMRTF subpopulation in patient BCCs. a tSNE plot of unbiased clustering of tumor epithelia from 4 naive human BCC tumor scRNA-Seq datasets post multi-CCA alignment, filtered for positive Krt14 expression. b MRTF signature enrichment score (generated from genes in Supplementary Data 1) overlaid on tSNE clusters from (a). c Clusters ranked by mean MRTF signature enrichment score, then grouped as follows: clusters 1-3 = high MRTF, clusters 4-6 = med high, clusters 7-9 = med low, clusters 10-12 = low MRTF. d Heatmap of top 50 genes most enriched in high MRTF group (clusters 1-3) vs. low MRTF group (clusters 10-12). e Violin plots of gene expression levels per group for surface markers LYPD3, TACSTD2, and LY6D. f Representative IF images of surface marker expression in human and mouse MRTF-nuclear vs. MRTF-cytoplasmic BCC regions or HF matrix of normal mouse skin. Scale bar = 50 μm. g Quantification of LYPD3, TACSTD2, and LY6D fluorescence intensity normalized to DAPI. h Heatmap of all 7 genes ranked by mean normalized DAPI intensity, averaged over at least three tumors. i Dose-dependent decrease in phosphorylated Smad3 levels (Supplementary Fig. 3k). Consistent with this observation, siRNA knockdown of Gli1 expression or cell viability (Supplementary Fig. 3d). By contrast, inhibitors of p38 or ALK5 inhibitors on resistant BCC cells did not result in any drug therapeutic window. Notably, the combination of AP-1 and Smad3 inhibitors worked together in resistant BCC cells. These pathways may have a redundant or synergistic effect on cell viability, suggesting that these pathways may be redundant or acting upstream of a shared pathway (Supplementary Fig. 3f).

We next wanted to determine the specific AP-1 and TGFβ family members that operate in resistant BCCs. Although AP-1 consists of a family of Jun/Fos dimers all capable of binding to the TGA(C/G)TCA consensus sequence, different homo or heterodimer pairs can have drastically different transcriptional outputs39. c-Jun, JunB, and JunD are all expressed robustly in BCC cells, while FosL2 is the only Fos family member expressed at significant levels (Supplementary Fig. 3i). Consistent with this observation, siRNA knockdowns of c-Jun, JunB, JunD, or FosL2 in BCC cells resulted in significant decrease in Gli1 expression, with JunD knockdown having the biggest effect, while knockdown of Fos or FosB did not change Gli1 expression (Fig. 3f, Supplementary Fig. 3i). In parallel, siRNA knockdown of TGFβ family members TGBf1, TGBf3, Smad3, and Alk5 decreased Smad2/3 phosphorylation (Supplementary Fig. 3m) and reduced expression of Gli1 (Fig. 3f, Supplementary Fig. 3i, m).

nMRTF activity depends on RhoA activation13, so we next examined whether AP-1 and TGFβ cooperate to induce Rho signaling. Using a Rho G-LISA assay to specifically measure levels of active GTP-bound RhoA, we found that exogenous TGFβ3 ligand was sufficient to activate Rho signaling, which could be attenuated by the addition of ALK5 or AP-1 inhibitor (Fig. 3g). In addition, we found that inhibition of TGFβ or AP-1 signaling disrupted both actin polymerization and MRTF nuclear localization (Fig. 3h, i). Importantly, while TGFβ signaling regulates Rho activity in both Smad-dependent and independent pathways40, knockdown of Smad3 expression is sufficient to inhibit MRTF nuclear localization (Fig. 4g) and Gli1 expression (Fig. 3f). This suggests that in resistant BCCs, TGFβ acts in a Smad3-dependent manner to promote non-canonical Hh signaling.

If AP-1 and/or TGFβ promote a non-canonical resistance mechanism to drive Hh signaling, then inhibiting these TF pathways should increase the sensitivity of cells to canonical SMO inhibition. Indeed, we see that combining SMOi treatment with AP-1 inhibition or ALK5 inhibition in resistant BCC cells leads to increased levels of cell death than with SMOi alone (Fig. 3i). In contrast, neither AP-1 inhibition nor ALK5 inhibition have any additive effect on cell viability when combined with MRTFi CCG-1423 (Supplementary Fig. 3j), suggesting that these pathways work upstream of MRTF. These initial experiments connect JNK-mediated signaling via Jun/Fos and TGFβ signaling via Smad3 to maintain activation of Rho and MRTF, leading to non-canonical Hedgehog signaling in resistant BCC cells.

AP-1 and Smad3 induce transcription of Rho GEFs. To identify how AP-1 and Smad3 transcriptional targets activate Rho, we performed RNA-seq on resistant BCC cells treated with ALK5 or AP-1 inhibitors. Intriguingly, the top Molecular Function GO terms of genes dependent on both pathways included guanylate-nucleotide exchange factor (GEF) activity, proteins that directly activate RhoA. Knockdown of AP-1 and Smad3 induced transcription of GEFs, including RhoA GEFs that work together in mouse resistant BCCs to promote non-canonical Hh signaling. For example, the top GEFs that work together in mouse resistant BCCs to promote non-canonical Hh signaling.

These findings suggest that TGFβ and AP-1 signaling confers resistance by increasing transcription of Rho GEFs such as Arhgef17, which in turn activate RhoA and subsequent actin polymerization, leading to nuclear localization of MRTF and SRF, which as act non-canonical cofactors for Gli1 (Fig. 4i). In order to confirm this resistance pathway, we conducted a series of
Epistatic studies using small-molecule inhibitors or siRNA knockdowns at various stages of the pathway (Fig. 4i) simultaneously paired with overexpression constructs, measuring Gli1 expression as the final output (Fig. 4j, Supplementary Fig. 4d, e). Treatment with SMO inhibitor vismodegib leads to a decrease in Hh signaling, which can be rescued by concurrent administration of TGFβ3 ligand or transient overexpression of JunD, Arhgef17, RhoA or constitutively active MRTF (MRTF-N) (Fig. 4j). Importantly, the
effects of ALK5 inhibition cannot be rescued by overexpression of JunD or other AP-1 subunits and conversely, AP-1 inhibition cannot be rescued by TGFβ ligand administration (Fig. 4j, Supplementary Fig. 4e). These experiments together indicate that coincidental TGFβ and AP-1 signaling are required for Arhgef17, RhoA, and MRTF activation that leads to non-canonical Hh signaling and tumor resistance.

JunD/AP-1, but not TGFβ, is sufficient to drive nMRTF. To interrogate whether AP-1 or TGFβ are sufficient to drive MRTF-mediated resistance, we used our NIH-3T3 model where overexpression of constitutively active MRTF (MRTF-N) in tandem with subthreshold Smo agonist (SAG) enhances Gli signaling. 3T3 cells are a useful proxy for sensitive BCC cells in vitro because they respond to canonical Hh signaling but do not intrinsically express the non-canonical MRTF-driven resistance pathway. We confirmed that the expression of MRTF-N, Arhgef17, Arhgef18, or RhoA is sufficient to amplify Gli1 mRNA expression (Fig. 5a). Interestingly, we find that overexpression of JunD as well as other Jun family members is sufficient to amplify Gli1, whereas increased TGFβ signaling alone is not sufficient (Fig. 5a, Supplementary Fig. 4f). We find that this sufficiency is operating at the transcriptional level, as JunD but not TGFβ overactivation is sufficient to increase the transcription of selected GEFs (Fig. 5b). We confirmed that a basal level of TGFβ signaling exists and responds to ligand stimulation with significant elevation in phosphorylated Smad2/3 levels (Supplementary Fig. 4g) and expression of the canonical target gene Serpine1 (Fig. 5b).

Consistent with a primary role in driving nMRTF activity, the effects of JunD overexpression can be attenuated with inhibition of either ALK5 activity, Arhgef17 expression, or MRTF activity (Fig. 5c). Overexpression of Arhgef17 or JunD is sufficient to increase RhoA activation (Fig. 5d) and MRTF nuclear translocation in 3T3 cells (Fig. 5e, f, Supplementary Fig. 4i, j). Importantly, AP-1 activity appears to have little effect on canonical Hh signaling driven by SMO, as its inhibition does not affect Gli1 expression levels in Hh-responsive 3T3 or C2C12 cells treated with SAG (Supplementary Fig. 4h). Therefore, we conclude that JunD/AP-1 is sufficient to amplify non-canonical Gli activity that depends on TGFβ, RhoGEFs, RhoA, and MRTF signaling.

AP-1 establishes Smad3 DNA binding profile of resistant BCC. AP-1 has been shown to commission enhancers in conjunction with cell-type specific TFs. Due to JunD, but not Smad3 sufficiency in driving the AP-1/Smad/Rho/MRTF resistance pathway, we hypothesized that JunD/AP-1 regulates the unique chromatin accessibility profile that functions with TGFβ signaling in resistant nMRTF BCC. Indeed, AP-1 binding motifs are highly enriched in the differential open chromatin of sorted SM+ naive human BCC (Fig. 3c), while few differences arose in canonical Smad3 binding motifs. This is consistent with previous findings that Smad proteins bind to their canonical DNA binding element (SBE) with 100-fold lower affinity than their interacting TF partners, and therefore need to cooperate with other TFs to influence transcription. To test this hypothesis, we conducted ATAC-seq and phospho-Smad3 chromatin immunoprecipitation sequencing (ChIP-seq) in resistant nMRTF BCC cells treated with AP-1 inhibitor. We found that AP-1 increases chromatin accessibility at Smad3 binding sites and modifies Smad3 DNA binding on a genome-wide level (Fig. 6a, Supplementary Fig. 5a–c, e). Interestingly, the intersection of genes dependent on AP-1 for maximal mRNA expression, chromatin accessibility, and Smad3 binding are responsible for interaction with the cytoskeleton and RhoGEF activity (Fig. 6b, c, Supplementary Data 3). These findings suggest that in resistant BCC, AP-1 shapes the chromatin accessibility landscape to open alternative Smad3 binding sites, allowing AP-1 and Smad3 to cooperatively induce expression of Rho regulators including GEFs.

To illustrate the AP-1/Smad3 chromatin cooperativity, we focused on accessibility changes in the regulatory regions of the Arhgef17 locus, although we observed similar patterns in the regulatory regions of other GEF loci (Fig. 6d, e, Supplementary Fig. 5d). We identified an AP-1 dependent ATAC peak in resistant BCC cells within the first intron, which is closed in SMO-sensitve BCC, and showed that Smad3 binding within the ATAC peak disappears with AP-1 inhibition (Fig. 6d). To interrogate the function of this AP-1 dependent open chromatin region, we used the CRISPR-Cas9 system in resistant nMRTF BCC cells to delete the region of the ATAC peak (Supplementary Fig. 5f). Care was taken to ensure the neighboring exon was excluded from the targeted region (Fig. 6d) to avoid disrupting protein function. Arhgef17 ATAC-peak deletion (Arhgef17ΔΔ) cells displayed diminished expression of Arhgef17 (Fig. 6f), and significant reduction of Gli1 levels, which could be rescued by overexpression of full-length Arhgef17, RhoA, or MRTF-N, but not TGFβ3 ligand stimulation (Fig. 6g, h). Arhgef17ΔΔ cells revealed diminished actin polymerization and decreased levels of nuclear MRTF (Fig. 6j, k), similarly to Arhgef17 siRNA knockdown (Fig. 4g, h). Furthermore, the Arhgef17ΔΔ cells also display enhanced sensitivity to inhibition of Gli1 expression by SMOΔ or MRTFΔ (Fig. 6i). These genetic studies show that resistance-specific, AP-1-dependent accessible chromatin in regulatory regions of Arhgef17 and likely other Rho activator genes are crucial for optimal Smad3 binding and transcription.
Since AP-1 signaling drives SMO
resistance through MRTF activation, we predicted that in treatment of patient tumors, AP-1 inhibitors, like MRTF inhibitors\(^1\), would only display efficacy in BCC populations with nMRTF. Indeed, we see that in naive human BCC explants, tumors containing nuclear MRTF respond significantly to AP-1 inhibition with a decrease in Gli1 expression, while tumors with only cytoplasmic MRTF are less responsive. Furthermore, there is a significant correlation between the expression of surface markers LYPD3, TACSTD2, and LY6D and the responsiveness to AP-1 inhibition (Fig. 6i). We also see

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This text is a continuation of the discussion on the role of AP-1 signaling in SMO resistance through MRTF activation. The text mentions that AP-1 inhibitors, like MRTF inhibitors, would only display efficacy in BCC populations with nMRTF. Tumors containing nuclear MRTF respond significantly to AP-1 inhibition with a decrease in Gli1 expression, while tumors with only cytoplasmic MRTF are less responsive. The text also highlights a significant correlation between the expression of surface markers (LYPD3, TACSTD2, LY6D) and the responsiveness to AP-1 inhibition. Further, the text supports this with Fig. 6i.
that patient BCC explant treatment with AP-1 inhibitors results in significantly reduced levels of nuclear MRTF (Supplementary Fig. 5g, h). We conclude that AP-1 drives the chromatin accessibility profile conducive to AP-1/Smad3-dependent nMRTF BCC resistance, and the identified surface proteins can act as prognostic markers for response rate to AP-1 inhibitors.

To further evaluate the clinical potential of these pathway inhibitors, we treated naive patient BCC explants with combinations of ALK5, AP-1, and/or SMO inhibitors. Similar to our findings in the mouse BCC cell line, SMO plus AP-1 inhibitors have an additive effect in reducing Gli1 expression, while AP-1 plus ALK5 inhibitors do not (Supplementary Fig. 5i). These findings support the potential efficacy of combination therapies targeting the canonical and non-canonical pathways simultaneously.

Discussion

In this study, we have identified three surface markers in BCC—LYPD3, TACSTD2, and LY6D—that reliably define the nMRTF BCC cell state and can be used as prognostic markers for resistance to SMO and responsiveness to AP-1 inhibitors. We show that this resistant cell state closely resembles the differentiation state of suprabasal TACs of the hair matrix, and that AP-1 mediated chromatin accessibility facilitates a Smad3-dependent transcriptional program of RhoGEFs that drive nuclear MRTF activity and resistance. Human BCC sensitivity to AP-1 inhibitors ex vivo correlates with nMRTF status and surface marker expression, providing pre-clinical support for AP-1 inhibition as a viable target for combination therapy along with canonical SMO inhibition for resistant BCCs.

Our discovery that MRTF is active in the matrix TACs of normal skin uncovers a novel interactor with the Hh pathway, given its ability to amplify Gli signaling. Hh ligands control developmental patterning through concentration-dependent signaling in nearly every developing metazoan body tissue. In the skin, Shh is only expressed in the lateral disc, the group of cells in the matrix directly adjacent to the DP that eventually form the skin, Shh is only expressed in the lateral disc, the group of cells in the matrix directly adjacent to the DP that eventually form the skin, Shh is only expressed in the lateral disc, the group of cells in the matrix directly adjacent to the DP that eventually form the skin, Shh is only expressed in the lateral disc, the group of cells in the matrix directly adjacent to the DP that eventually form the skin, Shh is only expressed in the lateral disc, the group of cells in the matrix directly adjacent to the DP that eventually form the skin. Given its ability to amplify Gli signaling, Hh ligands control the developmental role in tissue patterning.

Interaction between AP-1 and Smad TFs is also complex, having either antagonistic or cooperative functions depending on the regulatory region. Previous studies have shown that at promoters with consensus Smad binding elements (SBEs), Jun TFs inhibit Smad-dependent transcription, while at promoters with canonical AP-1 binding sites, or TPA response elements (TRES), Smad binding acts synergistically with Jun to enhance transcription. This suggests that when AP-1 and Smad are simultaneously active, AP-1 shifts the Smad binding profile away from canonical SBEs and instead favors TRED activation. This is consistent with our Smad3 ChIP-seq data in resistant BCC cells, where we observe that when AP-1 is active, Smad3 binding sites are more enriched for AP-1 motifs rather than Smad motifs.
(Supplementary Fig. 5e). Whether Jun and Smad proteins are actually forming heterocomplexes on AP-1 binding sites, or binding distinct nearby cis elements is a question that is still under debate and merits further study.\(^56\)–\(^58\).

Finally and importantly, we have shown that nMRTF BCC cells exist as a distinct cell state, which can be effectively targeted with small-molecule MRTF and AP-1 inhibitors, using LYPD3, TACSTD2, and LY6D as prognostic markers. We have also shown that in heterogenous tumors, combining AP-1 and SMO inhibitors can be a useful way to increase efficacy and widen the therapeutic window. Further pre-clinical in vivo studies are necessary to gauge its potential as a therapeutic for BCC. By discovering the surface markers as a predictor of the appropriateness of combination therapies, we have advanced the possibility to target both the canonical and non-canonical drivers of resistance at once, and therefore maintain tumor therapeutic response.

**Methods**

**Human samples.** Written informed consent was obtained for all human subject samples and was reviewed by the Stanford University Institutional Review Board, protocol #18325 (Stanford, CA).
**Mice.** C57BL/6 mice (Stock number: 000664) were obtained from the Jackson Laboratory. Tumor blocks for Ptch1+/−;K14-creER;p53fl/fl mice came from previous work. All mouse work was approved by the Institutional Animal Care and Use Committee (IACUC) at Stanford University.

**Mouse skin explants.** C57BL/6J mice were sacrificed at p26, backs shaved, skin removed, and then 6-mm punch (Integra; 33-36) was used to generate circular punches, which were subsequently attached to the bottom of 6-well TC plates (Corning; 3516) with 6μL of Matrigel (Corning; 356237). Explants were cultured in 154CF media (Gibco; M15CF500) supplemented with 15% chelated FBS (GE HyClone; SH30396.03), 1% Penicillin Streptomycin (Gibco; 15140-122), 0.05 mM CaCl2 (Gibco; 50-9702), 0.4% Amphotericin B (Gibco; 15290-026), 0.01% Plasmocin (Invitrogen; NC9880956), and 1% HKGS (Gibco; S-0001-5). Paired explants from same mouse were either treated with 150μm CCG-1423 (Tocris; 5277) or...
Fig. 6 AP-1 establishes the Smad3 DNA binding profile of resistant BCC. a Heatmap and line graphs of ATAC-seq signal in resistant ASZ001 cells treated with and without 20 μM AP-1 inhibitor T5224 across pSmad3 ChIP binding sites. b Overlap of genes displaying loss of chromatin accessibility (ATAC-seq), Smad3 binding (ChIP-seq), and/or expression (RNA-seq) in response to AP-1 inhibitor treatment in resistant BCC cells, defined as log2 FC = −1 and p < 0.05. Genes listed in Supplementary Data 3. c GO molecular function terms enriched in genes showing AP-1 dependence of chromatin accessibility, Smad3 binding, and mRNA expression levels. P-values calculated by Fisher exact test. d Visualization of ATAC and ChIP peaks at Arhgef17 regulatory locus. ATAC peak of interest has been highlighted, and scissors represent target sites for CRISPR guide RNAs. e List of RhoGEFs dependent on AP-1 by chromatin accessibility, Smad3 binding, and mRNA expression levels in resistant BCC cells. f Arhgef17 qRT-PCR in WT ASZ and Arhgef17 ATAC peak deletion (Arhgef17ΔD) cell line. **p < 0.001. g GilT qRT-PCR in WT and Arhgef17ΔD ASZ cell lines, treated with 5 ng/ml of TGFβ3 ligand for 24 h or 1 μg/ml of Rho activator II for 6 h. *p < 0.0344, **p < 0.0003. h GilT qRT-PCR in WT and Arhgef17ΔD ASZ cell lines transiently transfected with overexpression constructs. **p = 0.0002. Open circles on all bar graphs represent independent biological replicates. All error bars represent mean ± SD. P-values calculated using unpaired, two-tailed Student’s t-test. i GilT qRT-PCR analysis in WT and Arhgef17ΔD ASZ cell lines, treated with increasing dosages of vismodegib or CCG1423. Calculated IC50 of ASZ WT shown in blue, ARHGEF17ΔD in red. **p < 0.01, ***p < 0.001. j If images of MRTF protein localization and actin polymerization in WT ASZ and Arhgef17ΔD cell lines. k Quantification of MRTF fluorescence intensity across cell radius in (j), measured as μm distance from cell center. Nuclear boundaries represented as DAPI intensity. Mean intensities of >50 cells shown as solid lines, with SEM as dotted lines. l Correlation of relative reduction in GilT mRNA expression of human tumor explants treated with 40 μM AP-1 inhibitor T-5224 for 24 h, with their relative intensity of surface marker immunostaining. Tumors were categorized as nuclear MRTF if immunofluorescent staining of MRTF colocalizing with DAPI was present in at least one of four separate 200 μm × 200 μm microscopy fields, otherwise they were categorized as cytoplasmic MRTF.

**DMSO control. Explanats were cultured for 72 h, then fixed in 4% PFA for 24 h and imbedded in paraffin blocks for H&E and immunofluorescence staining.**

**Immunofluorescence staining and imaging.** Human and mouse BCC tumors, benign skin tumors, or normal mouse skin were fixed with 4% paraformaldehyde and embedded in paraffin blocks. 5 μm sections were mounted on slides and stained with H&E or were immunolabeled using the antibodies listed below. Antigen retrieval was performed in pH 6 citrate buffer (Vector Laboratories H-3300). Cell lines were plated in 8-well chamber slides (Millipore) were fixed using 3.7% formaldehyde diluted in PBS for 10 min. Sections or chamber slides were immunostained using Cell Signaling Technologies IF General Protocol using the following antibodies and dilutions at 4 °C overnight: anti-KrT14 (1:500, BioLegend SIG-3476-100), -MKLI-MRTF (1:200, Novus NBP1-88496), -HHIP (1:100, Santa Cruz Technology sc-293265), -Krt15 (1:500, Origene BP577), -phospho-Smad2/3 (1:200, Abcam ab22903), -Ki-67 (1:500, Invitrogen MA5-14550), -Lysyl oxidase (LYP) (1:200, R&D Systems AF5350), -TACSTD2/Trop2 (1:200, Abcam ab224488), -LY6D (1:200, Proteintech 17361-1-A1), -Arhgef17 (1:200, Lifespan Biosciences LS-C83529), -phospho-JNK (1:200, Invitrogen PAI-9594), and -HA tag (1:200, abcam ab103275). The fluorescent-labeled secondary antibodies were used as follows: anti-goat Alexa Fluor 488 (1:500, Life Technologies, A-11055), -anti-mouse Alexa Fluor 488 (1:500, Life Technologies, A-21202), -rabbit-anti Alexa Fluor 555 (1:500, Life Technologies, A-31572), -anti-mouse Alexa Fluor 594 (1:500, Life Technologies, A-21203), -anti-guinea pig Alexa Fluor 594 (1:500, Life Technologies A-11076), and -anti-chicken Alexa Fluor 647 (1:500, Jackson Immuno Research, 703-606-155). Nuclear visualization was performed using Hoechst 33342 (1:1000).

Confocal imaging was carried out using a Leica SP8 microscope equipped with an adjustable white light laser and hybrid detectors, and when making comparisons between images, laser intensities and imaging settings were held constant. To quantify protein expression in BCCs, mean pixel intensity was measured using ImageJ in regions that stained positive for keratin 14, then normalized against mean pixel intensity of DAPI in the same region. For each condition, at least three 100 × 100 μm microscopy fields were counted in at least four tumors. Subcellular localization of MRTF was quantified through multiposition intensity profiles using the ImageJ multi-plots plug-in. Line averaging was calculated on at least 50 unique nuclei per condition with the line starting in the nucleoplasm, ending in the cytoplasm, and centering on the edge of the DAPI signal. DAPI staining was used as a guide to determine nuclear localization. Measurements were then normalized to the maximum pixel intensity in each line dataset. Actin filament staining was carried out using phalloidin 488 and 647 (Life Technologies).

**Human BCC single-cell RNA-sequencing processing and analysis.** The FASTQ files for four naive human BCCs (BCC1, BCC2, BCC3, BCC8, BCC10) were aligned using the 10X Genomics Cell Ranger 2.1.1. Each library was aligned to an indexed GRCh38 genome using the Cell Ranger Count function. The Cell Ranger Aggr function was utilized to normalize the number of mapped reads across all of the sample libraries, resulting in 45,656 cells total, with median of 2,473 genes per cell and 7,935 UMI counts per cell. The clustering of cells was performed using the Seurat v3.3.4 R package18. In brief, initial bulk samples containing single cell data matrices were column-normalized and log-transformed. Quality control parameters were used to filter cells with 200–5000 genes with a mitochondrial percentage under 10%. Effects of cell cycle phase were calculated and regressed out using the Seurat QC workflow function. The four samples were merged and aligned using the MultiIICF function, using the top 1000 genes that were highly variable in at least two datasets. To identify cell clusters, the top 15 aligned CCs with a resolution = 0.6 were used to obtain 22 clusters total. To present high dimensional data in two-dimensional space, we performed t-SNE analysis using the same 15 aligned CCs. For subclustering of epithelial cells, the datasets were subset for clustering with average KrT14 expression >0.5. The multi-CCA alignment and clustering was repeated as above, resulting in 12 clusters.

We assigned an MRTF enrichment score using the AddModuleScore function based on a list of MRTF target genes (Supplementary Data 1). This list was generated from the intersection of genes mapped by GREAT to SRF-Chip-seq peaks12, and genes that are differentially expressed (log2 fold change >1 or <−1, p < 0.05) in resistant ASZ001 cells with MRTF inhibitor treatment12. Then clusters were ranked and re-ordered from 1–12 by their mean MRTF enrichment score. The 12 clusters were separated into four groups: high MRTF, med-high, med-low, and low MRTF, and differentially expressed genes were identified between the high MRTF and low MRTF groups using the FindMarkers function with default parameters.

**FACS analysis of naive human BCC tumors.** Tumors were minced, then dissociated in 0.5% collagenase in HBSS for up to 1 h, and 0.05% trypsin for up to 15 min. Cells were strained through a 70 μm filter, then washed twice using FACS buffer (2% BSA /PBS), then stained with the following antibodies for 30 min at 4 °C in 1:1 dilution: anti-LYPD3 (Sino Biological 11836-R213-P), -anti-Trop2/TACSTD2 (R&D systems FAB650-V), -ly-ly6D (E480 cytoky, of Dr. Ruud Braekenhoff), and -anti-CD49f/ItgA6 (Millipore MAB1378). Propidium iodide was used at a concentration of 1:1000 as a dead cell marker. During FACS, live epithelial cells were first isolated by positive ItgA6 expression >10. Then MRTF enrichment and clustering were separated based on surface marker expression. Because LY6D was rarely expressed, both LY6D-positive and -negative cells were included in the surface marker positive (SM+) population, resulting in final expression profiles SM+ (ItgA6+ LYPD3+ TACSTD2+ LY6D+/−) vs. SM− (ItgA6+ LYPD3+ TACSTD2+ LY6D−). Sorted cells were then used for RNA-seq and ATAC-seq, n = 4 biological duplicates from two tumors. All samples were stained and FACS analyzed with the same parameters.

**RNA-seq library preparation and data processing.** RNA extraction was performed using RNeasy Plus (QiAGEN) from the samples reported in this research, per manufacturer’s instructions. RNA-seq from human BCC tumors used four biological samples per condition from two tumors. RNA-seq from ASZ001 cells used two or three biological replicates per condition. Library preparation, sequencing, and mapping were carried out with minor modifications. The RNA-seq libraries were constructed by TruSeq Stranded mRNA Library Prep kit (Illumina) and sequenced on an Illumina Hiseq2000 sequencer. The alignment was performed using TopHat20 with mm9 as a reference genome. Raw counts and RPMK values were called using analyzeRepeats.pl in Homer21. Differential gene expression analysis was performed using the DESeq2 R package22 with cutoffs at log fold change >1 or <−1 and p value <0.05. GO and ChEA enrichment analysis were performed using the clusterProfiler R package23. Gene set enrichment analysis (GSEA)24 was performed to determine whether a priori defined set of genes shows statistically significant difference between biological samples. The MRTF signature gene list (Supplementary Data 1) was uploaded as a custom gene set, and gene expression values from the RNA-seq analysis of the human BCC samples SM+ and SM− were used to perform the enrichment. The four datasets were merged and aligned following the developer’s protocol. The normalized enrichment score and nominal p value are presented to determine the significance of enrichment level.
Cell culture. ASZ001 and BS22 BCC cells were cultured in 154CF medium (Life Technologies) supplemented with 2% chelated FeBS and 0.05 mM CaCl2. Experiments were carried out using low serum conditions with 154CF medium containing 0.2% chelated FeBS and 0.05 mM CaCl2, NIH-3T3, HaCat, and C2C12 cells (all from ATCC) were cultured in DMEM supplemented with 10% FBS. Hh induction experiments were carried out using low-serum DMEM containing 0.5% FBS supplemented with SAG (Sigma). Transient transfection of mammalian expression vectors was performed with Lipofectamine 2000 (Invitrogen) or calcium phosphate precipitation. 

Cell viability assays. Cells were plated subconfluent in 96-well plates, then serum starved and treated with the following inhibitors: vismodegib (GDC-0449; SelleckChem), CCG-1423 (Cayman Chemicals), T-5224 (SelleckChem), Flag-JunWT-Myc (Addgene 47443), PC2Flag-JunB (Addgene 29697), pCMV JunD (Origene MR205123), PCDH-MRTF-N12, pEGFP-RhoCA32, and pCDH-GFP51.

Quantitative reverse transcription PCR. RNA isolated using QIAGEN RNeasy Mini Kit according to manufacturer protocol. Brilliant II SYBR Green QRT-PCR 1-Step Master Mix (Agilent) was used for reverse transcription and quantitative PCR. Primer sequences are listed in Supplementary Table 2. Each reaction was performed in triplicate and values were normalized to beta-tubulin.

Small interfering RNA (siRNA)-mediated gene knockdown. ASZ001 cells were transfected with 30 pmol of siRNAs (Sigma-Aldrich) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) with standard protocol. The siRNA oligo sequences were designed, synthesized and fluorescent labeled by Sigma-Aldrich MISSION siRNA (Supplementary Table 3). To achieve higher knockdown efficiency and avoid off-target effect, we initially screened three individual siRNAs targeting different regions and chose the top two for the subsequent study. The transfection experiment was performed in triplicate over three times, and the knockdown efficiency was measured by qRT-PCR. Only the oligos that achieved successful target gene knockdown >80% were used for additional gene expression analysis. Gene expression was normalized to cells transfected with Cy-3 tagged universal negative control siRNA (Sigma-Aldrich SIC003).

Rho activity assays. Rho activity in ASZ001 or NIH-3T3 cells was quantified using the RhoA-GFP (Invitrogen) as a positive control. Cells were transiently transfected with vectors expressing GFP, Arhgef17, JunD, or RhoA (SelleckChem), and/or 20 µM AP-1 inhibitor T-5224 (SelleckChem). NIH-3T3 cells were transiently transfected with vectors expressing GFP, Arhgef18 (Origene MC206181), PCMV Fosl2 (Origene MR204779), Flag-JunWT-Myc (Addgene 47443), PC2Flag-JunB (Addgene 29697), pCMV JunD (Origene MR205123), PCDH-MRTF-N12, pEGFP-RhoCA32, and pCDH-GFP51.

Chromatin immunoprecipitation. Protein-DNA complexes were isolated from ASZ001 cells, and these were used to map chromatin occupancy of phosphorylated Smad2/3. ASZ001 cells were grown to 80% confluence, then serum starved for 24 h with DMSO vehicle control or 20 µM AP-1 inhibitor TIS24. Cells were stimulated with 5 ng/ml TGFβ3 for 2 h prior to fixation. Fifty million cells per replicate were cross-linked with 1% formaldehyde for 10 min, followed by quenching with 0.25 M glycine for 5 min at room temperature (RT). Cells were lysed in modified RIPA buffer (50 mM Tris, 150 mM NaCl2, 1% Triton X-100, 0.75% SDS, 0.5% sodium deoxycholate), which was supplemented with protease and phosphatase inhibitor cocktail (Roche). Cellular extracts were sonicated using a Covaris tissue ultrasonicator for 30 seconds at 50% power and 80% duty cycle to achieve 200–1000 bp chromatin fragments in length. The cleared extract was incubated with 10 µg of antibody against phosphorylated Smad3/3 (abcam ab52903) or nonspecific rabbit IgG control antibody (Cell Signaling) overnight and precipitated using magnetic Protein A Dynabeads (Invitrogen). Beads were washed with ChIP wash buffer (100 mM Tris pH 8.0, 500 mM LiCl, 1% Igepal) and then deoxyscholic acid wash (1% deoxyscholic acid, 0.1% SDS). The protein–DNA complexes were eluted with IP elution buffer (1% SDS, 50 mM NaCOH). Cross-links were reversed by incubation with 0.2 M NaCl at 67 °C overnight while shaking at 1000 r.p.m. on a thermostorer. RNA was digested with 0.2 µg/ml RNase A at 37 °C for 30 min, and protein was digested using proteinase K for 1 h at 37 °C. DNA was isolated using Qiagen MinElute columns following the manufacturer’s instructions, and used for ChIP-seq library prep.

ChIP-sequencing library preparation and data processing. ChIP–seq libraries were generated using the standard protocol for the NEBNext Ultra II DNA Library Prep Kit for Illumina (Nextera DNA Choice) and sequenced on the Illumina MiSeq. Raw reads were mapped to the human genome (hg19) and quantified using the NGSpeak tool. Enrichment peaks were determined using HOMER software (http://homer.grch37.hg19). Enrichment peaks were annotated for gene associations using the GREAT tool (http://greatgene.org). A peak list was generated from ATAC-seq data of ASZ001 cells, and these were used to map chromatin occupancy of phosphorylated Smad2/3.

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Motif analysis of peaks from ATAC-seq and ChIP-seq. Motif analysis on peak regions was performed using HOMER function findMotifsGenome with default parameters to calculate the occurrence of a TF motif in peak regions compared to that in background regions. We used -log10 (p-value) to rank the enrichment level of TF motifs.

ATAC-seq signal intensity around TF bound regions. A window from −5 kb upstream to +5 kb downstream of peaks was taken and divided into 50 bp bins. The number of uniquely mapped and properly paired ATAC-seq tags overlapping each bin was counted, normalized by library size and log-transformed using an in-house script. Therefore, we generated a matrix with each row representing a peak region, and each column containing the normalized tag counts from a 50 bp bin in a consecutive manner within the 10 kb window. To visualize the signal intensity across all the TF bound regions, the data matrix was presented as a heatmap by Java TreeView. To get an average intensity, we took the mean of fragment count in each bin of all the binding regions and plotted the result as a line graph.

CRISPR/Cas9-mediated targeted gene deletion/replacement. We used CRISPR/Cas9-mediated genome editing to generate our Arhgef17 ATAC peak deletion (Arhgef17Δ5p) ASZ001 line. We designed two gRNAs to target independent loci surrounding the 3290 bp genomic region that we wished to delete, from chr7: 108073710–108077000. The sequences of the gRNAs are as follows: TCCCAAGTCCCGCGCAGG and GAACGTAGTGATCTGAGG. A donor sequence was synthesized as 5′-phosphorylated gene blocks (IDT), and the gRNAs were incorporated into a DNA fragment with all components necessary for gRNA expression. Constructs were transfected into ASZ001 cells alongside an hCas9 expression plasmid, using Lipofectamine LTX Reagent with PLUS Reagent (Thermo Fisher Scientific, 15331000) and the recommended manufacturer’s protocol. After 48 h, we added puromycin to the cells to select out positive cells for gRNA expression. Multiple rounds of transfection and puromycin selection were conducted to achieve a pure population. The deleted region was verified by genomic PCR.

Primary patient BCC explant culture and drug treatment. Freshly resected tumors were obtained from patients with advanced BCC receiving Mohs surgery. Informed consent was obtained in writing for all patient samples and was reviewed by the Stanford University Institutional Review Board #18325. The tumor subtype was verified through the immediate histological examination of resected BCCs. Patient specimens were minced and cultured in EpiLife medium (Life Technologies) supplemented with 0.05 mM CaCl2 with or without 1 µM of SMOi vismodegib, 40 µM AP-1 inhibitor T-5224, 10 µM AP-I inhibitor SR13102, 40 µM ALK5 inhibitor, and/or 10 µM MRTF inhibitor CCG-1423 for 24 h. Drug-treated tissues were suspended in RLT buffer (Qiagen) and homogenized using 2 ml tissue lysing matrix E tubes (MP Biomedicals). RNA was isolated from tumors using the RNeasy standard protocol with RNA extracts were used to carry out TaqMan probes for human Gli1 and G phápdi (Thermo Fisher). MRTF localization and surface marker expression was assessed in explant specimens through freezing samples in O.C.T. reagent and sectioning blocks for immunofluorescence analysis.

Quantification and statistical analysis. Statistical tests used in each experiment as well as information on replicates is indicated in Figure Legends. In general, data represent similar results from three or more independent, biological samples and cell cultures, unless otherwise described. Single-cell RNA sequencing is analyzed from four individual tumor datasets. Sorted human BCC cells for RNA-seq and ATAC-seq analysis include four biological replicates per condition from two tumors. Deep sequencing data of ASZ001 cells are from two or three biological samples per condition. For IF analyses of human BCC tumors, n = 9 tumors with nuclear MRTF and n = 11 tumors with cytoplasmic MRTF. For IF analyses of cultured ASZ001 and NIH-3T3 cells, n > 50 for each quantification. Scale bars, radial distribution, and quantification of pixel intensity was measured using FIJI software23. Bar and line graph results are presented as the mean ± SD. Significance values were generated using GraphPad Prism 8 and denoted by asterisks as indicated in figure legends. Unpaired student’s t-test was used to determine the significance of differences with the annotations: *p < 0.05, **p < 0.01, and ***p < 0.001. Details of statistical methods for specific analysis are described in the corresponding methods sections. A normal distribution was observed for all data.

Data availability. Sequencing data generated for this manuscript are available in the Gene Expression Omnibus using SuperSeries with GEO accession numbers GSE156855 and GSE141526. Previously published sequencing datasets used for analysis for this manuscript are available in the Gene Expression Omnibus using GEO accession numbers GSE100876, GSE116966, and GSE89928. Source data are provided with this paper.

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Additional information

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