An oncogenic MYB feedback loop drives alternate cell fates in adenoid cystic carcinoma

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Translocation events are frequent in cancer and may create chimeric fusions or ‘regulatory rearrangements’ that drive oncogene overexpression. Here we identify super-enhancer translocations that drive overexpression of the oncogenic transcription factor MYB as a recurrent theme in adenoid cystic carcinoma (ACC). Whole-genome sequencing data and chromatin maps highlight distinct chromosomal rearrangements that juxtapose super-enhancers to the MYB locus. Chromosome conformation capture confirms that the translocated enhancers interact with the MYB promoter. Remarkably, MYB protein binds to the translocated enhancers, creating a positive feedback loop that sustains its expression. MYB also binds enhancers that drive different regulatory programs in alternate cell lineages in ACC, cooperating with TP63 in myoepithelial cells and a Notch program in luminal epithelial cells. Bromodomain inhibitors slow tumor growth in ACC primagraft models in vivo. Thus, our study identifies super-enhancer translocations that drive MYB expression and provides insight into downstream MYB functions in alternate ACC lineages.

Chromosomal rearrangements that create a chimeric fusion gene or drive oncogene overexpression are common in cancer. The discovery of the Philadelphia chromosome translocation in chronic myelogenous leukemia, which creates the BCR-ABL1 fusion gene, ushered in an era of targeted therapy with kinase inhibitors. Oncogenic rearrangements that juxtapose a strong enhancer near an oncogene, triggering overexpression of this gene, are also frequent in leukemia and lymphoma1–3. Recently, a similar enhancer-hijacking mechanism was described in medulloblastoma4, wherein chromosomal translocations involving enhancers cause overexpression of GFI1 or GFI1B, which function as transcriptional repressors of tumor-suppressor genes. In other cases, translocation events drive the expression of an oncogene by replacing its promoter with a highly active promoter, as is the case for TMPRSS2-ERG fusions in prostate cancer5.

ACC is a malignant neoplasm that arises within the secretory glands, most commonly in the salivary glands of the head and neck. Although typically slow growing, these tumors are locally aggressive, with a tendency to spread along nerves. Perhaps most challenging clinically, ACC can recur locoregionally or with distant metastases decades after primary tumor resection, requiring careful long-term surveillance of all patients. Because of the resistance of these tumors to chemotherapy and radiation therapy, non-resectable cases are usually fatal.

The MYB-NFIB translocation is a molecular hallmark that is present in a majority of ACCs7. The MYB locus encodes a master transcription factor involved in cellular differentiation and proliferation. It functions as an oncogene in a variety of cancers, including breast cancer, pancreatic cancer and leukemia8. The MYB-NFIB translocation reportedly disrupts the MYB 3′ UTR, which contains a microRNA regulatory site that downregulates MYB expression9. However, MYB translocations that retain the 3′ UTR are still associated with high MYB expression, indicating the existence of additional mechanisms for MYB overexpression in ACC.

Here we identify the juxtaposition of super-enhancer regions to the MYB locus as the unifying feature of ACC translocations. Detailed genomic and epigenomic analyses of ACCs identify alternate rearrangements that translocate super-enhancers in the NFIB and TGFBR3 loci either upstream or downstream of the MYB gene. MYB protein binds these super-enhancers, which loop to the MYB promoter, thereby establishing a positive feedback loop that

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sustains expression of this master regulator. MYB also binds a larger repertoire of enhancers across the genome, which appear to underlie alternate ACC expression signatures in the myoepithelial and luminal epithelial compartments of ACC. BET bromodomain inhibitors, which disrupt enhancer functions, slow tumor growth in ACC primagraft models in vivo. However, these inhibitors appear to be ineffective against high-grade ACCs that harbor activating mutations in the Notch pathway. Thus, we identify a new mechanism of transformation in which rearrangement of a regulatory element creates a positive feedback loop between an oncogenic transcription factor protein and its gene locus, with implications for diagnosis and therapeutic strategies in ACC.

RESULTS
New MYB translocations in ACC
A diagnostic feature of ACC is a (t(6;9)) rearrangement that translocates MYB to the NFIB locus and results in high MYB expression. This translocation creates a fusion gene whose coding sequence is almost identical to that of MYB but has an altered 3′ UTR that lacks negative regulatory elements and leads to increased transcript stability. Yet, although nearly all ACCs overexpress MYB, only about 30% carry an actual fusion transcript.

We therefore examined whole-genome sequencing data for 18 ACCs, including 12 published primary ACCs and six primary patient-derived xenografts (ACC primagrafts). Consistent with previous reports, we identified MYB translocations as the main recurrent event (13 of 18 ACCs) in these tumors (Fig. 1a). We confirmed the presence of MYB rearrangements in four of these primagrafts by PCR. MYB rearrangements in the primagrafts were previously verified by FISH. We also confirmed by PCR representative rearrangements involving other loci that were detected in the sequencing data (Supplementary Fig. 1 and Supplementary Table 1). These results support the validity of the rearrangements detected from whole-genome sequencing data for these 18 ACCs. Finally, we identified MYB rearrangements in two additional tumors by targeted paired-end sequencing. This yielded a total of 15 (of 20) ACCs with MYB rearrangements.

We identified canonical NFIB-MYB fusions with loss of the MYB 3′ UTR in six of the 20 tumors (30%; Fig. 1b and Table 1).

![Figure 1](https://example.com/fig1.png)

**Figure 1** MYB translocations involve alternate partners and frequently retain the MYB 3′ UTR. (a) Circos plots of interchromosomal translocations in six ACC primagrafts. Only MYB translocations (purple) occur in more than two tumors. (b) For a cohort of 20 tumors, the pie chart depicts the fraction of MYB translocations that involve the NFIB locus, with or without loss of the MYB 3′ UTR, or that rearrange to other loci (TGFBR3 or RAD51B). The rearrangements to alternative partners retain the MYB 3′ UTR. (c) Log plot showing MYB mRNA expression in ACC primagrafts (color-coded as in b) relative to normal salivary gland. Error bars, s.e.m. (n = 3 technical replicates per sample). P < 1 x 10^-5 as compared to normal salivary gland in all cases. These data suggest that neither 3′ UTR loss nor NFIB fusion is sufficient to explain the robust MYB overexpression in ACC.

| Sample Origin | MYB translocation | MYB 3′ UTR Source | MYB 3′ UTR | Notes |
|---------------|------------------|-------------------|------------|-------|
| PD3185 Primary | Not detected     |                   |            | 1     |
| PD3186 Primary | Not detected     |                   |            | 1     |
| 2012 Primary   | Not detected     |                   |            | 2     |
| 2128 Primary   | Not detected     |                   |            | 2     |
| 6536 Primary   | Not detected     |                   |            | 2     |
| PD3176a Primary | MYB-NFIB fusion  | Lost              | 1          | Inversion |
| PD3208a Primary | MYB-NFIB fusion  | Lost              | 1          | Inversion |
| 505 Primary    | MYB-NFIB fusion  | Lost              | 2          | Inversion |
| 131169 Primary | To NFIB locus Retained |            | 2          |       |
| PD3226a Primary | To NFIB locus Retained |            | 1          | Complex |
| PD5912a Primary | To NFIB locus Retained |            | 1          | Complex |
| PD3177a Primary | To TGFBR3 locus Retained |            | 1          |       |
| ACCX5M1 Primagraft To MYB-NFIB fusion Lost 4 Inversion | |
| ACCX11 Primagraft To MYB-NFIB fusion Lost 4 Inversion | |
| ACCX16 Primagraft To MYB-NFIB fusion Lost 3 Inversion | |
| ACCX9 Primagraft To NFIB locus Retained 3 | |
| ACCX19 Primagraft To NFIB locus Retained 3 | |
| ACCX22 Primagraft To NFIB locus Retained 3 | |
| ACCX2 Primagraft To TGFBR3 locus Retained 3 | |
| ACCX12 Primagraft To RAD51B locus Retained 3 | |

MYB translocations as detected in 12 primary ACCs and eight primagrafts. The sources of the samples are denoted as (1) whole-genome sequencing data from EGA, data set EGAD00001000062, (2) whole-genome sequencing data from Ho et al., (3) data from whole-genome sequencing conducted in this study and (4) paired-end ChIP-seq data for H3K27ac and input control generated in this study.

An additional six tumors (30%) harbored an NFIB-MYB rearrangement but retained the MYB 3′ UTR. We also identified new translocations involving the MYB locus: two tumors harbored rearrangements involving other loci. These data suggest that neither 3′ UTR loss nor NFIB fusion is sufficient to explain the robust MYB overexpression in ACC.
Translocations occur within the technical replicates. (a) H3K27ac (enhancer) profiles are shown for alternate ACC rearrangements: MYB-NFIB translocation with loss of the MYB 3′ UTR (ACCX16); MYB-NFIB translocation with the MYB 3′ UTR retained (ACCX19); and MYB-TGFBR3 translocation with the MYB 3′ UTR retained (ACCX6). Arrows between the chromosomes indicate the rearrangements. H3K27ac signal is scaled in fragments per million (fpm). (b) Candidate enhancers ranked by H3K27ac signal in the ACC primagrafts (positive samples, blue) and TGFBR3 (negative samples, red) loci satisfy super-enhancer criteria. These enhancers score similarly in other tumors (Supplementary Fig. 3). (c) H3K27ac (enhancer) profiles for the NFIB locus (negative strand shown) in five ACCs and six MYB-NFIB-positive primagrafts. Enhancers are numbered as indicated (En1–En8). Translocations occur close to the 5′ UTR of NFIB near the En1 enhancer (black triangles). The bars below the peaks mark the positions of super-enhancers. (d) 3C demonstrates looping of translocated enhancers to the MYB promoter in ACCX19. The plot depicts, for each enhancer (En1–En8) or control site, the relative interaction frequency (RIF) to the MYB promoter. Significant interactions (P < 0.05) are marked by an asterisk; error bars, s.e.m. (n = 5 technical replicates). (e) H3K27ac profiles for the TGFBR3 locus (negative strand) in five ACCs and two MYB-TGFBR3-positive primagrafts. Translocations occur within TGFBR3, near the E11 enhancer (black triangles). (f) 3C demonstrates looping of translocated enhancers to the MYB promoter in ACCX6 (MYB-TGFBR3 rearrangement), as in d. Error bars, s.e.m. (n = 5 technical replicates). These data suggest that alternate ACC rearrangements juxtapose super-enhancers to the MYB locus that physically interact with the MYB promoter and activate its expression.

between MYB and the TGFBR3 locus, and one tumor harbored a rearrangement between MYB and the RAD51B locus. We used quantitative RT-PCR to confirm that all of these rearrangements were associated with high levels of MYB expression (Fig. 1c). Notably, several rearrangements occurred at the 5′ end of the MYB gene, which is inconsistent with the production of any fusion protein (Fig. 2a). These findings indicate that neither a fusion gene product or 3′ UTR loss is a unifying feature of ACC rearrangements and raise the alternate possibility that these translocations increase MYB expression through regulatory alterations.

Enhancer rearrangements act as drivers of MYB activation

We postulated that ACC translocations might reposition distal regulatory elements in proximity to MYB, thereby triggering its overexpression. We therefore mapped the chromatin landscapes of 13 ACCs, including five primary specimens and eight primagrafts. We mapped histone H3 lysine 4 trimethylation (H3K4me3), a promoter-associated mark, and histone H3 lysine 27 acetylation (H3K27ac), a marker of active enhancers and promoters. In a subset of samples, we also mapped the enhancer-associated BET bromodomain protein BRD4. The overall H3K27ac patterns were similar across the primary ACCs and primagraft models but distinct from that of an ACC cell line derived by viral transformation14. The ACC landscapes were distinct from those of other tumor types and non-malignant tissues (Supplementary Fig. 2). The conserved epigenomic landscapes for primary tumors and primagrafts, together with the conserved histology12, support the fidelity of the in vivo primagraft models.

We next examined the genomic loci that were translocated to MYB in the various tumors—specifically, the regions downstream of NFIB, TGFBR3 and RAD51B. We found that all three regions contained large clusters of enhancers that are active in ACCs (Fig. 2c,e). Indeed, when we collated super-enhancers in ACCs on the basis of the expanse and signal intensity of H3K27ac15 and BRD4 occupancy16, we identified several super-enhancers in the rearranged portions of NFIB and TGFBR3 (Fig. 2b,c,e and Supplementary Fig. 3). We also identified smaller enhancers downstream of RAD51B, which was rearranged...
in one ACC (Supplementary Fig. 4). Interestingly, NFIB, TGFBR3 and RAD51B are all highly expressed in normal salivary gland, suggesting that these regions are indeed active before transformation (Supplementary Fig. 5). These findings suggest that the various rearrangements in ACC may act by repositioning potent regulatory elements close to MYB.

To test whether specific enhancers within the translocated super-enhancers might activate the MYB promoter, we examined their physical proximity to the promoter using chromosome conformation capture (3C). First, we examined an ACC with a translocation involving MYB and the NFIB locus. We examined eight acetylated elements located between 13 and 750 kb from the MYB promoter. We identified four elements that demonstrated a significant interaction (P < 0.05) with the MYB promoter (Fig. 2d). We also examined a second ACC with a MYB-TGFBR3 translocation. In this case, seven of the nine H3K27ac peaks tested interacted with the MYB promoter (Fig. 2f). These data suggest that the translocations reposition super-enhancers that subsequently loop to the MYB promoter and sustain high-level MYB expression.

Figure 3 MYB protein binds translocated super-enhancers and other active enhancers. (a) MYB binding and H3K27ac profiles are shown for the NFIB locus in ACCX16 and the TGFBR3 locus in ACCX2 (negative strands shown). MYB-bound enhancers looping to the MYB promoter are labeled as in Figure 2c-f. (b) Box plots depicting the distribution of MYB signal over enhancers in ACCs. Each box shows quartiles (q1, q2, q3), and the whiskers extend to q3 + 1.5 × (q3 – q1). Super-enhancers in the NFIB locus are top ranked MYB targets in tumors with MYB-NFIB translocation (red points; number 5 in ACCX5M1 and number 17 in ACCX16). Super-enhancers in the TGFBR3 locus are top ranked MYB targets in tumors with MYB-TGFBR3 translocation (number 77 in ACCX2). (c) Schematic depicting the positive feedback loop, engaged by chromosomal rearrangements, that sustains MYB overexpression in ACC. (d) High-confidence MYB peaks in three grade 2 primagraffs (Online Methods) were annotated as ‘promoter’ (±2 kb from a transcription start site; top) or ‘enhancer’ (bottom). Heat maps show MYB and H3K4me3 signals over 2,776 promoters (rows; 5-kb regions centered on MYB peaks, ranked by MYB signal) or MYB and H3K27ac signals over 10,502 enhancers (rows; 5-kb regions centered on MYB peaks, ranked by MYB signal). (e) Expression of MYB target genes (purple), as compared to control genes (orange), in ACC primagraffs (left) and normal salivary gland (right). High expression of genes near MYB-binding sites supports a role for MYB as a transcriptional activator in ACC. (f) MYB target genes ranked by cumulative MYB signal over the promoter and nearby enhancers (Notch pathway genes are shown in red). (g) Heat map showing enhancers with preferential H3K27ac in grade 2 (top) or grade 3 (bottom) primagraffs. Transcription factor motifs enriched in the respective enhancer groups are indicated.

Positive feedback MYB circuit
To examine potential downstream targets of MYB overexpression, we mapped MYB protein binding across the genome in three ACC primagraffs using chromatin immunoprecipitation and sequencing (ChIP-seq) (Fig. 3). The MYB binding profiles were similar across the three ACC primagraffs and were strongly enriched for the MYB motif in all samples (CAGTT; P < 1 × 10−759). The MYB binding patterns differed from published data sets for other human and mouse tissues17,18 yet showed a statistically significant overlap, including 62% overlap with MYB-bound promoters in MCF-7 human breast cancer cells (P < 1 × 10−6) and 60% overlap with MYB targets in mouse myeloid progenitors (P < 1 × 10−51).

Notably, MYB was bound to the enhancers in the NFIB and TGFBR3 loci that were translocated to the MYB locus in ACC (Fig. 3a). When we ranked MYB-bound enhancers per gene by binding signal, the translocated enhancers were near the top ranked genes (NFIB, number 5 in ACCX5M1 and number 17 in ACCX16; TGFBR3, number 77 in ACCX2; Fig. 3b and Online Methods). Moreover, in the respective rearranged tumors, these MYB-bound enhancers...
MYB binding to translocated enhancer clusters may augment its own expression by activating transcription of the MYB gene (Fig. 3c). To test whether the translocated enhancers can drive transcription in a MYB-dependent manner, we cloned five 250-bp intervals from the NFIB and TGFB3 enhancers into a minimal promoter reporter vector. We tested these reporter constructs in Jurkat human T cells, which express high levels of MYB protein at baseline. We found that four of the five elements strongly induced reporter activity. Moreover, we found that the activity of two of these elements was diminished when we mutated their MYB motifs (Supplementary Fig. 6). These data support the enhancer identity and MYB responsiveness of the sequence elements juxtaposed to the MYB locus by rearrangements. They are most consistent with a model in which positive feedback sustains MYB expression in this disease.

MYB-related regulatory programs in ACC

To infer potential downstream effects of MYB overexpression, we called 13,278 high-confidence MYB-binding sites (Online Methods and Supplementary Table 2). A majority of these sites coincided with distal regulatory elements (75%), whereas a minority coincided with promoters (Fig. 3d). MYB shows a strong preference for active elements as marked by H3K4me3 (promoters) or H3K27ac (enhancers). We predicted MYB target genes by assigning MYB-bound enhancers to nearby genes that are expressed in ACC (Online Methods). These genes were expressed at relatively higher levels in the primagrafts, as compared to all expressed genes, but were weakly expressed in normal salivary gland (Fig. 3e). We refer to these genes as putative MYB targets, as they are probabilistic predictions based on binding profiles and expression patterns, whose further validation will require the development of faithful in vitro models for ACC.

Putative MYB targets in ACC were enriched for genes related to development, migration, cell signaling, cell cycle, transcription regulation and angiogenesis (Reactome, Gene Ontology and MSigDB; false discovery rate (FDR) < 1%; Supplementary Table 3). Specific examples include MYC, BCL2, AURKA, CCND1, MET, FGFR2, IGF1R, MALAT1, CASC4 and NENF. We compared the expression patterns for these genes to those in normal salivary gland (Supplementary Fig. 7). Of 4,853 highly expressed MYB-bound genes in ACC, 50% were also highly expressed in normal salivary gland, 38% showed low levels of expression and 12% were not expressed. Functional annotation of putative MYB targets that were also expressed in the normal counterpart demonstrated enrichment for genes involved in neurodevelopmental processes. In contrast, putative MYB targets that were uniquely expressed in ACC were enriched for cell cycle regulators, including CDK6 and GMNN (Supplementary Table 2). Thus, MYB may engage two distinct regulatory circuits in ACC, one that reinforces a preexisting neurodevelopmental program in salivary epithelial cells and another that drives proliferation.

We next sought to identify other transcription factors or pathways that mediate or cooperate with MYB-driven regulatory programs in ACC. We scanned the high-confidence MYB peaks collated above for enriched transcription factor motifs. As expected, the top ranked motif corresponded to the MYB consensus motif. The motif ranked second was the TP53/TP63/TP73 consensus motif (P < 1 × 10−348). TP63 was also identified as a putative MYB target (Supplementary Fig. 7). To directly test whether TP63 co-binds with MYB, we mapped binding of this transcription factor by ChIP-seq. Remarkably, we found that 81% of TP63-binding sites in ACC were co-bound by MYB (Supplementary Fig. 8). We next collated putative target genes near the top ranked MYB-binding sites, focusing on transcription factors and transcriptional regulators (Fig. 3f and Supplementary Table 4). This identified activators such as EN1, recently established as a biomarker for high-grade ACC, the ARID1A chromatin remodeler, which is mutated in ACC, and NOTCH1. In addition to NOTCH1, the Notch activators JAG1 and JAG2 and the Notch transcriptional repressor SPEN were identified among these highly ranked putative MYB targets. These data suggest that MYB, TP63 and Notch signaling may coordinately orchestrate the diverse expression programs in ACC.

Inter- and intratumoral epigenetic heterogeneity in ACC

ACC is notable for its biphenotypic differentiation, with myoepithelial and (luminal) epithelial cells arranged in a ‘cribriform’ pattern. This histology is seen in low-grade (grade 1 and 2) tumors, which constitute the majority of ACC cases. However, a smaller fraction of tumors have a ‘solid’ histology dominated by luminal epithelial cells and are more aggressive (grade 3). Grade 3 tumors can originate...
Table 2 MYB, Notch and TP63 immunohistochemistry

| Tumor  | MYB                  | ICN1                  | TP63                                             | Grade | Tumor site   |
|--------|----------------------|-----------------------|--------------------------------------------------|-------|--------------|
| ACCD1  | Diffuse positive     | Diffuse positive      | Minor subset positive, periphery (<5%)           | 3     | Trachea      |
| ACCD2  | Diffuse positive     | Diffuse positive      | Minor subset positive, periphery (<5%)           | 3     | Parotid      |
| ACCD3  | Diffuse positive     | Diffuse positive      | Minor subset positive, periphery (<5%)           | 3     | Trachea      |
| ACCD4  | Diffuse positive     | Diffuse positive      | Negative                                          | 3     | Maxillary sinus |
| ACCD5  | Diffuse positive     | Diffuse positive      | Negative                                          | 3     | Trachea      |
| ACCS1  | 70%                  | 60%                   | 40%                                              | 2     | Parotid      |
| ACCS2  | Staining failed      | 60%                   | 30%                                              | 2     | Trachea      |
| ACCS3  | 60%                  | 10%                   | 90%                                              | 2     | Parotid      |
| ACCS4  | 70%                  | 30%                   | 70%                                              | 2     | Auditory canal |
| ACCS5  | 90%                  | 50%                   | 20%                                              | 2     | Parotid      |
| ACCS6  | 80%                  | 40%                   | 60%                                              | 2     | Parotid      |
| ACCX2  | NA                   | 20%                   | 50%                                              | 2     | Parotid      |
| ACCX5M1| NA                   | 30%                   | 80%                                              | 2     | Metastatic tumor to lungs |
| ACCX6  | NA                   | 30%                   | 80%                                              | 2     | Metastatic tumor to lungs |
| ACCX9  | NA                   | 100%                  | 0%                                               | 3     | Parotid      |
| ACCX11 | NA                   | 100%                  | 0%                                               | 3     | Sinonasal cavity |
| ACCX12 | NA                   | <10%                  | 20%                                              | 2     | Trachea      |
| ACCX14 | NA                   | <10%                  | 90%                                              | 1     | Trachea      |
| ACCX15 | NA                   | 100%                  | 0%                                               | Unknown | Oral cavity |
| ACCX16 | NA                   | 30%                   | 80%                                              | 2     | Bronchus     |
| ACCX19 | NA                   | 30%                   | 60%                                              | 2     | Oral cavity  |
| ACCX20M1| NA                 | 30%                   | 50%                                              | 1     | Metastatic tumor to liver |
| ACCX2002| NA                  | 30%                   | 50%                                              | 2     | Parotid      |
| ACCX21 | NA                   | 30%                   | 70%                                              | 1     | Parotid      |
| ACCX22 | NA                   | <10%                  | 30%                                              | 1     | Parotid      |
| ACCX24 | NA                   | <10%                  | 0%                                               | 2     |              |
| ACCX29 | NA                   | 40%                   | 80%                                              | 2     |              |

Tumors of all grades express MYB in all cells. Grade 3 tumors have strong intercellular NOTCH1 (ICN1) staining but no TP63 expression, whereas grade 1 and 2 tumors express TP63 in some cells and ICN1 in others. NA, not available.

from grade 2 tumors but more commonly present independently. We therefore considered how MYB might promote these alternate cell fates in ACC.

We focused in particular on regulatory programs related to TP63 and Notch, which were both highlighted by our epigenomic analysis. We first examined the expression of these regulators in 19 grade 2 ACCs (Fig. 4a, Table 2 and Supplementary Fig. 9). Immunohistochemistry confirmed strong staining for TP63—a marker of myoepithelial cells—specifically in myoepithelial compartments. TP63 was conspicuously absent from luminal epithelial tumor cells, which stained positive for KIT. We also stained these tumors for ICN1, the active intracellular form of NOTCH1. ICN1 was expressed only in luminal epithelial cells, and its expression was mutually exclusive with that of TP63 (Fig. 4b). This mutual exclusivity is consistent with established antagonism between TP63 and NOTCH1 during development.

Thus, MYB appears to coordinate seemingly opposing regulatory programs in the distinct cellular compartments of ACC.

We next examined TP63 and NOTCH1 expression in eight grade 3 ACCs. These more aggressive specimens lacked TP63 staining, consistent with loss of the myoepithelial component. Remarkably, they all showed strong diffuse staining for ICN1. ACCs can harbor activating mutations in NOTCH1 or loss-of-function mutations in the RBPJ repressor SPEN. These mutations were present in seven of the nine grade 3 tumors but in none of the lower-grade tumors examined (Supplementary Table 5).

To gain further insight into the circuits that drive these respective regulatory programs, we compared enhancer patterning between low- and high-grade ACCs. First, we performed unsupervised clustering of putative active enhancers on the basis of their H3K27ac patterns across 13 primagrafts and primary tumors (Supplementary Fig. 10). This analysis distinguished sets of enhancers preferential to either grade 2 or grade 3 tumors, which we then scanned for over-represented transcription factor motifs (Fig. 3g). The TP63 motif was highly enriched in grade 2–specific enhancers, whereas the RBPJ/Notch motif was enriched in grade 3–specific enhancers. TP63 exists in two main isoforms, TAp63, a transcriptional activator, and ∆Np63, which lacks the transactivation domain and exerts stem-like and oncogenic functions. Only the oncogenic isofrom, ∆Np63, was transcribed in our ACC cohort (Supplementary Fig. 7), as is frequently the case in salivary tumors.

Thus, TP63 appears to be a mediator of the MYB regulatory program in the myoepithelial component of low-grade ACCs. Conversely, Notch signaling is active in luminal epithelial components of low-grade ACC. Its further activation by somatic Notch pathway gain-of-function mutations likely underlies the switch to solid histology and the aggressive clinical course of grade 3 tumors.

**BET and Notch inhibitors target alternate ACC phenotypes**

Our findings suggest that chromosomal rearrangements in ACC engage a positive feedback loop, in which MYB protein activates juxtaposed super-enhancers, which loop to the MYB gene and sustain its expression. BET bromodomain inhibitors have been shown to suppress MYB function in acute myeloid leukemia and, more generally, may suppress super-enhancers with strong BRD4 occupancy. These findings suggest that MYB target loci in ACC, which also have high BRD4...
occurrence (Supplementary Fig. 11), might be sensitive to BET bromodomain inhibition. We specifically hypothesized that grade 2 tumors would be particularly sensitive to bromodomain inhibitors given their prominent MYB regulatory circuits. In contrast, somatic Notch activation might render grade 3 tumors relatively less sensitive to bromodomain inhibition, as recently observed in T-ALL with activating NOTCH1 mutations. We therefore examined the in vivo efficacy of BET inhibitors in ACC primagrafts. To this end, we engrafted nude mice with four different ACCs, two grade 2 and two grade 3 tumors. We confirmed that both grade 3 primagrafts harbored genetic events leading to Notch activation (Supplementary Table 5) and stained strongly positive for ICN1 and the proliferation marker Ki-67. Randomized groups of five mice each were treated with vehicle or the BET bromodomain inhibitor JQ1, and tumor growth was measured over time (Fig. 5a). BET bromodomain inhibition significantly slowed tumor growth in the grade 2 primagrafts (Fig. 5b). We also detected a modest decrease in MYB levels and MYB target gene expression (Fig. 5c). In contrast, the grade 3 tumors did not respond to BET bromodomain inhibition, potentially reflecting a relatively stronger dependency on Notch signaling. Of note, we recently showed that Notch-mutant ACCs are sensitive to Notch inhibitors. Our results suggest that BET inhibitors may be sufficient to disrupt core MYB circuitry in low-grade ACC but are ineffective against high-grade tumors, which may instead be sensitive to Notch inhibitors.

**DISCUSSION**

ACC is an incurable disease with slow but chronic tumor progression that is refractory to conventional chemotherapy or radiation therapy. We have shown that most cases of ACC harbor translocations that juxtapose a super-enhancer to the MYB locus. A convergence of genetic, epigenetic and therapeutic data indicates that these rearrangements establish a positive feedback loop in which MYB protein binds the translocated enhancers, which in turn physically interact with the MYB promoter and drive its expression. Thus, although MYB is known to autoregulate itself in wild-type cells, enhancer-hijacking events perturb this physiological control in ACC, yielding a high degree of overexpression.

**Figure 5** BET bromodomain inhibition slows tumor growth in grade 2 ACC primagrafts. (a) Experimental design for ACC xenotransplantation trials with the BET bromodomain inhibitor JQ1. ACC cells from four different human tumors were transplanted into the flanks of nude mice. Once tumor size reached 200–300 cc, mice were randomized into two treatment groups (vehicle or JQ1). Mice were treated daily and were monitored for disease burden. The trial was stopped when mice became moribund. (b) Average tumor size from 3–9 mice per group is depicted during the period of the xenotransplantation trial (grade 2 tumors, ACCX6 and ACCX5M1; grade 3 tumors, ACCX9 and ACCX11). Error bars, s.e.m.; n = 3 technical replicates. BET bromodomain inhibition slows growth and leads to downregulation of MYB and MYB target gene expression in grade 2 tumors. MYB coordinates with distinct regulatory programs in alternate cell lineages in cribriform grade 2 tumors, cooperating with a TP63 program in myoepithelial cells or a Notch program in luminal epithelial cells. In grade 3 tumors, however, additional genomic events frequently lead to constitutive Notch activation and tip the balance toward the luminal epithelial fate and a solid histology. Grade 2 tumors appear dependent on bromodomain proteins to maintain MYB-driven enhancer programs, as indicated by their sensitivity to corresponding inhibitors. In contrast, grade 3 tumors with constitutive Notch activation are insensitive to bromodomain inhibitors, consistent with results in other Notch-driven tumors. In grade 2 tumors, immunohistochemical staining for MYB tends to be stronger in myoepithelial cells than in luminal cells, in line with previous reports, whereas grade 3 tumors exhibit diffuse MYB staining with variable intensity across tumors (Supplementary Fig. 9). Although these differences may in part reflect technical issues, they raise the possibility that lower MYB protein levels in grade 3 tumors influence tumor response to BET inhibitors. In conclusion, our study advances understanding of ACC biology and underscores how interplay between genetic and epigenetic alterations can affect malignant transformation, disease progression and therapeutic sensitivities.
METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. European Genome-phenome Archive (EGA), accession EGAS00001001457 and Gene Expression Omnibus (GEO) accession GSE76465.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

B.K. and Y.D. designed and performed experiments and analyzed the data. B.K. and R.E.B. designed the experimental strategy and supervised the study and analysis. Y.D. carried out computational analyses. Y.D., B.K. and R.E.B. wrote the manuscript. J.C.A., M.I.C., K.E.W., S.M.G., C.D.C., S.I.R., L.M.S. and M.I.W. contributed to experiments and data analysis. A.H.A., R.J.H.R., M.J.K., W.C.F., L.Q., J.Q., I.E.B., C.A.M., A.K.E.-N. and J.E.B. provided reagents, contributed to analysis and gave conceptual advice. All authors discussed the results and implications and reviewed the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests; details are available in the online version of the paper.

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ONLINE METHODS

Primary tumors. Primary ACCs were collected at the MD Anderson Cancer Center, University of Virginia and Massachusetts General Hospital with approval by the respective institutional review boards. Discarded specimens were processed under a waiver of informed consent from the institutional review board.

Primagraft experiments. To generate ACC primagrafts, viable ACC cells were injected into the flank of 7- to 8-week-old female nude (Fasxnu) mice. Tumors that grew were passaged through at least three rounds of nude mouse transplantation before being used in vivo drug testing. Studies were performed under the auspices of protocols approved by the University of Virginia Institutional Animal Care and Use Committee (IACUC). For in vivo drug testing, 1 × 10^6 viable ACC cells were injected into the flank of nude mice. Once tumors were visible, the mice were randomized to receive vehicle or JQ1 (50 mg/kg daily) diluted in 10:90 DMSO:10% hydroxypropyl-β-cyclodextrin orally until reaching a minimal tumor volume of 1,000 mm^3 (4–9 mice per group: ACCX5M1 and ACCX6, four treated versus nine vehicle-treated controls; ACCX9, four treated versus five vehicle-treated controls; ACCX11, five treated versus eight vehicle-treated controls). Tumor growth was monitored, and mice were weighed daily and euthanized when moribund. In these experiments, no statistical methods were employed to determine the sample size, and no blinding of investigators to mouse identity was performed. All mouse procedures used in this study were approved by the IACUC at START (San Antonio, Texas).

Cell lines. The human papillomavirus (HPV)-transformed ACC cell line ACC112 was derived from discarded specimens and cultured in RPMI supplemented with 10% FBS, epidermal growth factor, hydrocortisone and insulin (all from R&D) as previously described. Jurkat cells were obtained from the American Type Culture Collection, and Oci-Ly3 cells were obtained from the Broad-Novartis Cancer Cell Line Encyclopedia. Both cell lines were cultured in RPMI supplemented with 10% FBS and propagated at a density of 1–2 million cells/ml. Cells were not tested for mycoplasma.

Calling MYB translocations. MYB translocations were called from paired-end whole-genome sequencing data using the following data sets. Data for 12 primary tumors were obtained from EGA under study EGA5000010000030. Of the 12 tumors, five were not considered for analysis because of low coverage or unreliable paired-end alignment. Data for five additional tumors were recently published and included in the analysis. We performed 100-bp paired-end whole-genome sequencing for six additional patient-derived primagrafts with the Illumina HiSeq platform. MYB translocation in the ACC primagrafts ACCX5M1 and ACCX11 was detected by paired-end sequencing of H3K27ac ChIP-seq and input control. All fastq files were aligned to the reference genome (hg19) using Burrows-Wheeler Aligner (BWA) ALN. Reads from primagrafts that aligned to the mouse genome (mm10) with a maximal editing distance of 3 bp (on the basis of BWA alignment) were filtered out. Rearrangements were called with Dranger and BreakPointer. Because of the lack of matching normal controls, we could not use the default germline filtering. Instead, we filtered against a panel of 100 non-matched normal samples, defining the rearrangement score as 10 × q × t/n, where q is the quality (as defined by dRanger), t is the number of supporting reads in the normal sequence, and n is the average number of supporting reads in the normal sequences. Only rearrangements with a score ≥2 were kept. Known germline variants from the Database of Genomic Variants were filtered out. Intrachromosomal rearrangements that spanned less than 1 Mb were filtered out, as they were suspected to be germline. Rearrangements from ChIP-seq paired-end data were called as previously described. All MYB translocations were manually reviewed in the Integrative Genomics Viewer (IGV). Only MYB and NFIB were found to have recurrent rearrangements in more than two primagrafts (even when considering all rearrangements with a score ≥3). CDHB1, EYS, and TAF13 were rearranged in two primagrafts but not in the other 12 primary tumors. All new data have been deposited in EGA, which is hosted by the European Bioinformatics Institute (EBI), under accession EGAS000001001457.

Chromatin immunoprecipitation. We performed ChIP assays in primary tumors and primagrafts as described with the following modifications. Frozen tissue was chopped using a scalpel before fixation and further dissociated after fixation by shearing with an 18-gauge needle. Chromatin from formaldehyde-fixed cells (1–5 × 10^6 cells per histone mark, 1 × 10^6 cells for MYB binding) was fragmented to a size range of 200–700 bases with a Branson 250 Sonifier. Solubilized chromatin was immunoprecipitated with antibody against H3K4me3 (2.5 µl; Millipore, 07–473CA), H3K27ac (2.5 µl; Abcam, ab4729), BRD4 (5 µl; Abcam, A301–985A 100), MYB (10 µl; Abcam, A301–985A 100), and TP63 (5 µl; ActiveMotif, 39739). Each of these antibodies was validated by protein blot or dot blot as described. Antibody-chromatin complexes were pulled down with protein G magnetic beads (Dynabeads, 10003D), washed and then eluted. After cross-linking reversal and proteasome K treatment, the immunoprecipitated DNA was treated with RNase and purified with Agencourt AMPure XP (Beckman Coulter, A68380). Libraries were prepared according to Illumina’s instructions. ChiP DNA and input controls were sequenced with the Illumina HiSeq 2500 or the NextSeq 500 instrument. Reads were aligned to the reference genome (hg19) using BWA. Maps reading to more than two genomic loci were ignored. Reads aligned to the same position and strand were only counted once. Raw data were deposited at EGA under accession EGAS000001001457. Processed data were deposited at GEO under accession GSE76465.

Statistical analysis. Data for bone marrow–derived mesenchymal stem cells and gastric and fetal liver muscle tissues are publicly available through the Roadmap Project (GSM112792, GSM1013128 and GSM1058767); HMEC, Panc1 and MCF-7 cell line data were downloaded from the Encyclopedia of DNA Elements (ENCODE) (GSM733660, GSM818826 and GSM945854); data for MG028 and Ewing sarcoma were recently published; and MOLT3 data were taken from ref. 19. Peaks and motifs were called using HOMER. H3K27ac peaks were centered on nucleosome-free regions, set to be 400 bp in length with a minimal distance of 600 bp, and required to be fourfold higher than the signal for matching input. MYB peaks were called with default parameters. To call putative super-enhancers, BRD4 or H3K27ac peaks up to 12.5 kb apart were stitched together, and enhancers with a slope greater than 1 were considered to be super-enhancers, as described in ref. 15. H3K27ac heat maps were calculated after merging all H3K27ac peaks across samples. Signal was normalized by total signal per sample. Only peaks with normalized signal >10 fpm in at least one sample were considered. Between-sample correlations were calculated by Spearman’s ρ. Motifs were called with HOMER in a 300-bp region around the peak center. To identify top H3K27ac motifs, known motifs were sorted on the basis of median P value across all samples. To identify motifs at peaks that differed between tumor grades, we merged the H3K27ac peaks for all grade 2 and all grade 3 primagrafts and then identified peaks with more than fourfold higher signal, averaging across 2-kb regions, for one set over the other. High-confidence MYB peaks were called by merging the peaks from three grade 2 primagrafts, summing the MYB signal over each peak in each sample, normalizing each sample by the average signal of that sample and selecting peaks where the average signal over all samples was at least 0.75. To determine which genes were expressed, we used published microarray data, averaging over the three samples (log2 space). Any gene with an average expression level >5 was considered to be expressed. Peaks were assigned to genes using GREAT, limited to a 100-kb maximal distance. We calculated GOBP, MSigDB and Reactome annotation enrichments for all MYB-bound and expressed genes versus all expressed genes using Fisher’s exact test (FDR < 1%). To identify MYB-driven transcriptional regulators, we focused on targets annotated as ‘positive regulation of transcription, DNA-templated’ by GOBP (GO:0045893) that were found to be significantly enriched (P < 3.8 × 10^-7) in the analysis above. We then ranked all these genes by total normalized MYB signal for all MYB peaks assigned to each respective gene. Normal salivary gland RNA sequencing data were obtained from the Human Protein Atlas (HPA) as fragments per kilobase of transcript per million mapped reads (FPKM) for every gene. Genes were divided into ‘not detected’, ‘low’ or ‘high’ categories on the basis of HPA definitions, where high included both ‘medium’ and ‘high’ genes, according to HPA definitions. Annotation enrichment of expressed MYB targets in a given set was compared to all genes in that set that are expressed in ACC. To test the expression of MYB targets and non-MYB targets, we compared the average expression of the expressed MYB targets in ACC, as described above, to the average expression of other expressed (log2 ≥5) genes. To control for MYB-independent expression differences between those genes, we compared the expression of the same sets of genes in normal salivary
gland. To compare MYB binding profiles to previously published promoters bound by MYB in MCF-7 cells\(^1\), we compared the latter to the subset of our high-confidence MYB peaks, described above, at most 2 kb from a transcription start site. To compare these MYB binding profiles to previously published MYB profiles for mouse myeloid progenitors\(^1\), we compared bound genes in mouse (as listed in Supplementary Tables 5 and 6 of ref. 18) to homologous human genes (by NCBI HomoloGene) with an assigned high-confidence MYB peak in ACC. To estimate BRD4 overlap with MYB peaks, we called MYB and BRD4 signal on all MYB peaks detected in ACCX5M1 and ACCX9 and counted peaks with normalized binding >30 fpm. To estimate TP63 overlap with MYB peaks, we called MYB and TP63 signal on all TP63 peaks detected in ACCX5M1 and counted peaks with normalized binding >30 fpm.

To quantify MYB signal over enhancers per target gene, MYB peaks more than 2 kb away from the transcription start site were assigned to genes using GREAT, limiting the maximal distance to 1 Mb to allow for fair comparison of the wide range of translocated NFIB enhancers. We then compared the total MYB signal over the translocated enhancers to the total signal over all enhancers of any MYB target.

The significance of 3C analysis was called on the basis of a 95% confidence interval not intersecting zero interaction. The significance of JQ1 treatment in primagraft experiments was called by one-tailed Student’s t test at the last time point for which tumor measurements were obtained. The significance of expression differences as determined by quantitative PCR after JQ1 treatment was called by one-tailed Student’s t test in comparing JQ1- and vehicle-treated samples.

Quantitative RT-PCR analyses. Frozen tumor tissue was mechanically homogenized, and total RNA was extracted with TRizol (Life Technologies) followed by the RNeasy Mini kit (Qiagen) with on-the-column DNase treatment. Total RNA was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis system for RT-PCR. Quantitative PCR was performed with FastStart Universal SYBR Green Master Roche) on an ABI 7500 instrument (primer sequences are listed in Supplementary Table 6). Gene expression was measured by determining the log2 (C\(_t\)) value of the desired transcript as compared to GAPDH transcript. A one-tailed P value <0.05 was considered to be statistically significant.

Genomic breakpoint PCR analyses. For validation of genomic rearrangements, genomic DNA was extracted from ACC primagrafts using the QIAamp DNA mini kit (Qiagen). PCR reactions were performed with 2-min extension times to allow for adequate amplification of longer fragments. PCR products were visualized on a 1.5% agarose gel. MYB rearrangements and representative highest-confidence and lowest-confidence rearrangements were selected for validation (Supplementary Table 1). Rearrangements in four primagrafts were validated. The primer sequences are listed in Supplementary Table 6.

Reporter assays. Five translocated enhancer sequences and five controls with scrambled MYB consensus motifs (replacing CNGTGT with GAAA; Supplementary Table 6) were synthesized and cloned into the pGL4.23[luc2/ minP] vector (Promega) by BlueHeron. Enhancer activity was measured in six replicates as the relative luminescence of the pGL4.23[luc2/minP] vector as compared to the pGL4.73[hR luc/SV40] vector with the Dual-Glo Luciferase system (Promega) after 36-h co-nucleofection into Jurkat cells, following the manufacturer’s instructions (Amaxa Cell Line Nucleofector Kit V, Lonza).

Chromosomal conformation capture. 3C analysis was performed as described\(^24\). In brief, frozen tumor tissue was chopped using a scalpel before fixation and further dissociated after fixation by shearing with an 18-gauge needle. Cross-linked chromatin was digested with 500 U of HindIII (Roche) fixation and further dissociated after fixation by shearing with an 18-gauge needle. Cross-linked chromatin was digested with 500 U of HindIII (Roche) overnight at 37 °C followed by ligation. 3C products were phenol-chloroform extracted, ethanol precipitated and dissolved in Tris-EDTA buffer. Each PCR was performed under the following conditions: 95 °C for 10 min; 65 cycles at 95 °C for 15 s, 60 °C for 1 min and 72 °C for 1 min; and a final extension at 72 °C for 10 min. PCR products were analyzed by agarose gel electrophoresis. Primer and TaqMan probe sequences are listed in Supplementary Table 6. Any undetected quantitative PCR call or C\(_t\) >50 was considered as C\(_t\) = 50. 95% confidence intervals were used to call statistical significance.

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were cut at 4 microns and placed on Superfrost plus glass slides, which were baked for 60 min at 60 °C. Staining was conducted on a Leica Bond III automated immunohistochemical staining workstation. To stain for MYB, antigen retrieval was performed using Bond Epitope Retrieval 1 solution for 30 min. Staining was carried out by incubation with a MYB-specific rabbit monoclonal primary antibody (EP769Y, Abcam, ab45150) at 1:400 dilution for 30 min at room temperature, followed by incubation with a rabbit-specific secondary antibody linked to horseradish peroxidase (Bond Polymer Refine Detection kit). Staining was developed by incubation with diaminobenzidine (Leica Detection kit), and slides were then dehydrated and coverslipped. Dual staining for activated NOTCH1 (ICN1) and TP63 was carried out by first performing antigen retrieval using Bond Epitope Retrieval 2 solution for 40 min. Slides were then incubated with antibody to ICN1 (D3B8, Cell Signaling Technology, 4147) at 1:100 dilution for 60 min at room temperature, followed by incubation with a rabbit-specific secondary antibody linked to horseradish peroxidase (Bond Polymer Refine Detection kit). ICN1 staining was then developed by incubation with diaminobenzidine (Leica Detection kit). Slides were incubated in a secondary primary antibody, a mouse monoclonal antibody specific for p63 (AA4, Biocare, CM163A) at 1:250 dilution for 30 min. The second antibody was detected using the mouse-specific Bond Polymer Refine Red Detection kit, which detects staining using Fast Red, part of the detection kit. Slides were then dehydrated and coverslipped. Dual staining for ICN1 and KIT was performed as above, using a rabbit monoclonal antibody specific for ICN1 (D3B8, Cell Signaling Technology, 4147) at 1:100 dilution for 60 min at room temperature. A second primary antibody, a mouse monoclonal antibody specific for KIT (Dako, A4502), at a 1:250 dilution for 30 min.

To generate spectral libraries, single-stained tissue sections were imaged using the Mantra multispectral imaging platform (PerkinElmer). The spectrally resolved individual profiles between 420–720 nm of 3,3′-diaminobenzidine (DAB; ICN1), Fast Red (KIT or TP63) and hematoxylin counterstain were used to deconvolute staining patterns in triple-stained tissue sections. Three representative areas of each stained tissue section were imaged at 20× magnification and deconvoluted using the Inform 2.1 software package (PerkinElmer). Each image was manually divided into tumor and stromal tissue, and individual tumor cells were segmented using Inform 2.1 algorithms that score positive staining of nuclei and cell membranes for each color.

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