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BPTF Maintains Chromatin Accessibility and the Self-Renewal Capacity of Mammary Gland Stem Cells

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SUMMARY

Chromatin remodeling is a key requirement for transcriptional control of cellular differentiation. However, the factors that alter chromatin architecture in mammary stem cells (MaSCs) are poorly understood. Here, we show that BPTF, the largest subunit of the NURF chromatin remodeling complex, is essential for MaSC self-renewal and differentiation of mammary epithelial cells (MECs). BPTF depletion arrests cells at a previously undefined stage of epithelial differentiation that is associated with an incapacity to achieve the luminal cell fate. Moreover, genome-wide analysis of DNA accessibility following genetic or chemical inhibition, suggests a role for BPTF in maintaining the open chromatin landscape at enhancers regions in MECs. Collectively, our study implicates BPTF in maintaining the unique epigenetic state of MaSCs.

INTRODUCTION

Lineage commitment and cell differentiation are processes driven by the reorganization of chromatin accessibility, which allows fate-specific transcription factors (TFs) to control gene-regulatory networks. This mechanism is modulated by factors that change the "active" or "repressive" state of genes via deposition or removal of post-translational modifications of histone tails, or via ATP-dependent remodeling of histone-DNA interactions. In the case of MECs, chromatin maintenance and remodeling regulate the progression of MaSCs into committed luminal and myoepithelial cell fates. Several prior studies have described epigenetic regulators that play a role in MaSC self-renewal and pregnancy-induced tissue proliferation (Hesling et al., 2011; Li et al., 2009; Zou et al., 2014; Pal et al., 2013). However, it remains unclear how alterations of chromatin accessibility alter these important cell-fate transitions.

In a search for epigenetic factors regulating chromatin remodeling and MaSC maintenance, we found BPTF, which is known to regulate the accessibility of DNA and stabilize the recruitment of the NURF complex to promoter regions (Li et al., 2006; Ruthenburg et al., 2011). BPTF is essential for mouse embryonic development (Goller et al., 2008), thymocyte maturation (Landry et al., 2011), and melanocyte stem cell differentiation (Koludrovic et al., 2015). Here, we demonstrate that BPTF is required at multiple stages of mammary gland development and for MaSC activity.

We found that BPTF depletion results in the accumulation of an uncharacterized MEC type. This cell population has a unique transcriptional profile, and its accumulation may reflect a differentiation arrest associated with upregulation of apoptotic pathways. Further investigation into chromatin accessibility suggests that BPTF depletion leads to a considerable increase in the accessibility of regulatory regions located near genes with functions related to apoptosis and cell-cycle arrest. Importantly, these chromatin changes are associated with global changes in gene expression. Collectively, our data support a role for BPTF in regulating the differentiation and survival of MECs.

RESULTS

BPTF Is Expressed in MaSCs and Is Required for the Development and Maintenance of the Mammary Epithelium

In order to identify regulators of MEC lineage commitment and differentiation, we re-analyzed our previously published RNA-seq libraries from purified mammary gland cell types (dos Santos et al., 2013; Figure 1A). This analysis identified the bromodomain-containing protein *Bptf* as being highly expressed in MaSCs relative to the other cell types, a result we validated using RT-qPCR (Figure S1A). Immunofluorescence staining of mammary glands revealed BPTF protein in the majority of MECs, with strong nuclear staining in cytokeratin positive ($KRT5^+$) cells, which surround ductal structures and are enriched for mammary reconstitution units (dos Santos et al., 2013; Van Keymeulen et al., 2011). Interestingly, we found that BPTF staining poorly overlapped with DAPI nuclear staining in cytokeratin 8/18 positive ($KRT8/18^+$) cells, suggesting a more cytoplasmic localization in this cell type

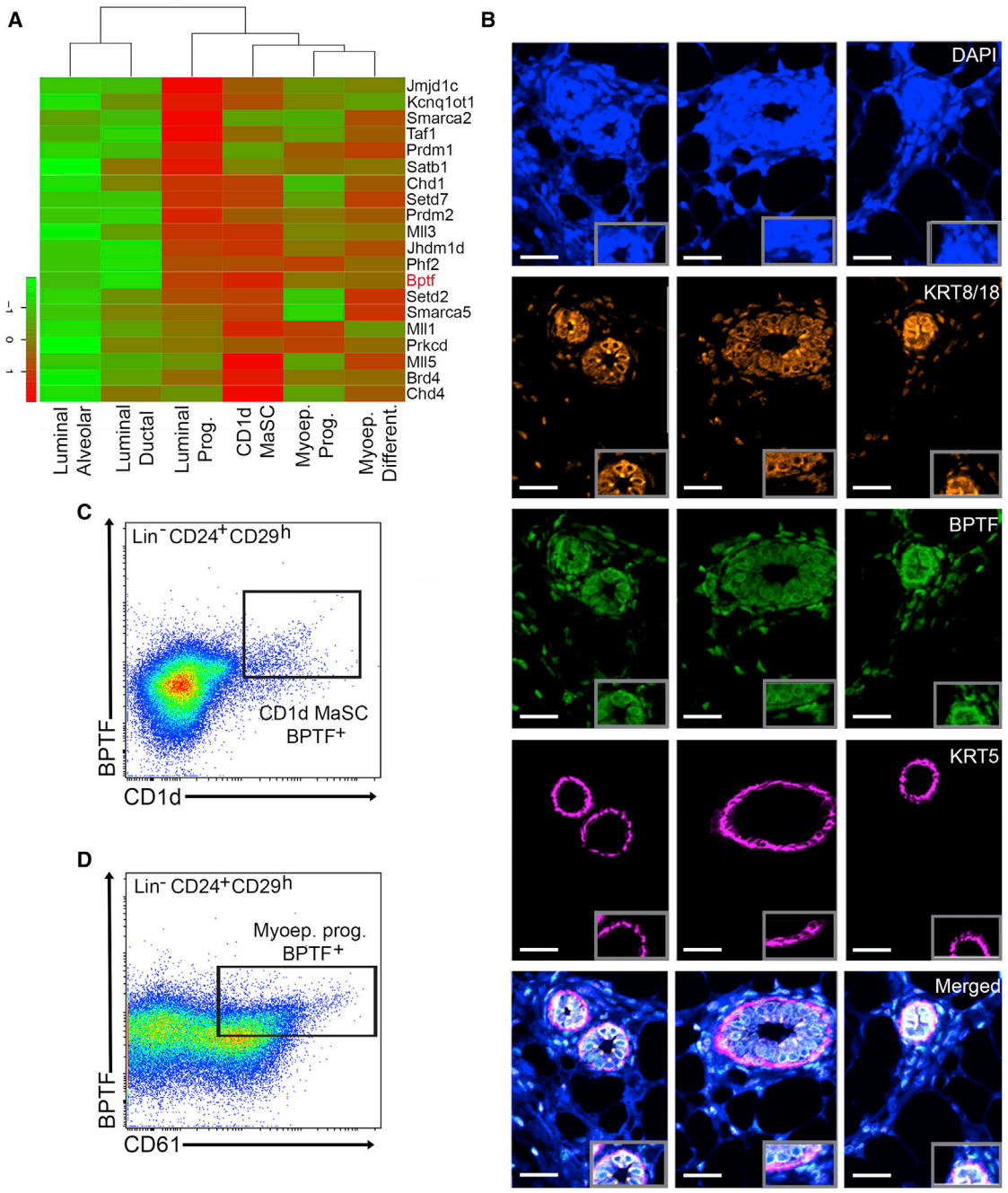

Figure 1. BPTF Is a Chromatin Remodeling Factor Expressed in MaSCs

(A) Transcriptional analysis of epigenetic factors. RNA-seq analysis of epigenetic factor on major MECs (reads per kilobase of transcript per million mapped reads cutoff of 10).

(B) BPTF protein levels in MECs. Representative IF images of mammary gland sections stained with DAPI (blue), anti-KRT8/18 (orange), anti-BPTF (green), and anti-KRT5 (purple). Scale bars, 100 μm.

(C and D) BPTF levels in less differentiated MECs. Representative FACS staining demonstrating BPTF levels in CD1d MaSCs (C) and CD61^{+} myoepithelial progenitors (D).

(Figures 1B and S1B). In addition, intracellular flow cytometry (FACS) analysis showed that the majority of CD1d MaSCs and a fraction of myoepithelial progenitors express high levels of BPTF protein, supporting the idea that its abundance may vary among different MEC types (Figures 1C, 1D, and S1C). Since BPTF has not been

previously implicated in mammary development, our findings prompted us to investigate the role of BPTF in MaSCs.

BPTF Depletion Affects Mammary Gland Development

To evaluate the consequences of BPTF depletion in MECs, we crossed *Bptf*-floxed mice (Landry et al., 2008) with a mouse expressing Cre-Ert2 under the control of the Krt5 promoter ($Krt5^{CRE-Ert2}$) (Indra et al., 1999). In doing so, we generated mice carrying the conditional $Bpft^{+/fl}$ allele (referred to here as WT), and mice carrying both the $Krt5^{CRE-Ert2}$ $Bptf^{fl/fl}$ alleles, referred to herein as KO (Figure S2A). To confirm deletion of *Bptf* exon 2, we performed RT-PCR using primers flanking *Bptf* exons 1, 2, and 3 (Figure S2A). We found that tamoxifen (TAM) treatment of KO MaSCs resulted in a PCR fragment corresponding to the truncated, exon-2 depleted, *Bptf* mRNA isoform (Figure S2B). In addition, analysis of MECs soon after TAM treatment showed decreased BPTF protein levels (Figure S2C) and mRNA levels (Figure S2D) in MECs from KO mice, indicating successful BPTF targeting.

To investigate the BPTF requirement during mammary gland development, TAM-treated WT and KO mammary glands were examined at pubescence, mid-pregnancy, or during involution (Figure S2E). Loss of BPTF was found to affect the pubescent development of the mammary gland, resulting in a decline of ductal structures. This notable effect was also present when BPTF was depleted in early pregnancy or during involution, suggesting a role for BPTF at all three developmental stages (Figures 2A and S2F). Furthermore, loss of BPTF during late involution (I14) resulted in an increased number of cleaved CASPASE-3^+ cells (Figures 2B and S2G) and a decreased number of $Ki67^+$ cells (Figure S2H). In addition, histological and FACS analysis of untreated and TAM-treated KO MECs confirmed that BPTF depletion caused a decline in ductal structures and increased the fraction of cells undergoing apoptosis (Figures S2I and S2J). Taken together, these results suggest a role for BPTF in the survival and proliferation of MECs during several stages of mammary gland development.

Next, we investigated the effects of BPTF depletion on the abundance of specific MECs using FACS analysis. We found that all major MECs were detected in KO mammary glands (Figures 2C–2G) with a slight decrease in the percentage of total luminal cells (WT ~11.7% and KO 7.3%) and myoepithelial progenitor cells (WT ~11.8% and KO 7.8%) in KO mammary glands, suggesting that targeting BPTF in MaSCs may affect the abundance of luminal and myoepithelial cell types (Table S1). The most striking observation, however, was the emergence of a unique MEC population in the KO mammary glands, which was not apparent in WT mice (Figure 2C). This population was distinct from the classic luminal ($CD24^{high}CD29^+$) and myoepithelial ($CD24^{low}CD29^{high}$) cell populations and accounted for approximately 5% of total KO MECs (Table S1). Hereafter, we refer to this cell population as BPTF-sensitive epithelial cells (Bsecs).

Phenotypic characterization of Bsecs demonstrated a unique pattern of expression for the common MEC surface markers (Table S2), displaying enrichment of a marker that identifies progenitor-like cells ($CD61^+$), while displaying lower enrichment for the luminal ductal cell marker ($CD133^+$), which suggests a progenitor-like identity of Bsecs (Table S2). Interestingly, we found a slight enrichment of $CD1d^+$ cells, suggesting that Bsecs may represent a cellular state that has just exited the MaSC fate toward a progenitor-like state. Taken together, these results suggest that BPTF may control the transition of MaSCs into a lineage-committed state, and place BPTF as a putative regulator of MaSC maintenance and differentiation.

Loss of BPTF Alters Gene Expression and MaSC Mammary Reconstitution Activity

Next, we performed transcriptome profiling (RNA-seq) of FACS-sorted BPTF WT and KO MECs to identify regulatory networks downstream of BPTF function (Figure 3A). Gene expression and ontology analysis highlighted several pathways upregulated in Bsecs compared with WT myoepithelial cells (Figure 3B). Several of these genes were previously shown to be required during expansion and maintenance of luminal progenitors (*Id2*, *Basp1*, *Sema3*, *Msx2*, *Hes1*, *Areg*, *Foxa1*) and during apoptosis (*Bbc3*, *Pmaip1*, *Ggct*), suggesting that Bsecs may represent a cell population committed to a luminal fate but with shortened life span due to BPTF depletion. In agreement with this, luminal and myoepithelial cells from KO mammary glands upregulated gene sets involved in apoptosis and extracellular matrix remodeling, a microenvironment change directly involved in MEC proliferation and differentiation (Schedin and Keely, 2011; Figure 3C). Together, these results suggest that BPTF depletion alters transcription of various genes that regulate MECs differentiation and apoptosis.

Since BPTF is expressed in MaSCs, we asked whether BPTF depletion influences MaSC self-renewal in transplantation assays. While we did not observe significant changes in the abundance of the MaSCs in KO versus WT mammary glands (Table S2 and Figure 2G), we observed a marked effect on the ability of KO MaSCs to incorporate bromodeoxyuridine in short-term pulse experiments, suggesting a role for BPTF in controlling the proliferation potential of MaSCs (Figure S3A). In addition, we found that BPTF depletion impaired the self-renewal abilities of MaSCs in transplantation assays. Injection of WT MaSCs into the cleared fat pad of recipient female mice yielded many ductal structures, yet the transplantation of KO MaSCs resulted in a 5-fold decrease in the number of mammary ducts (Figure 3D

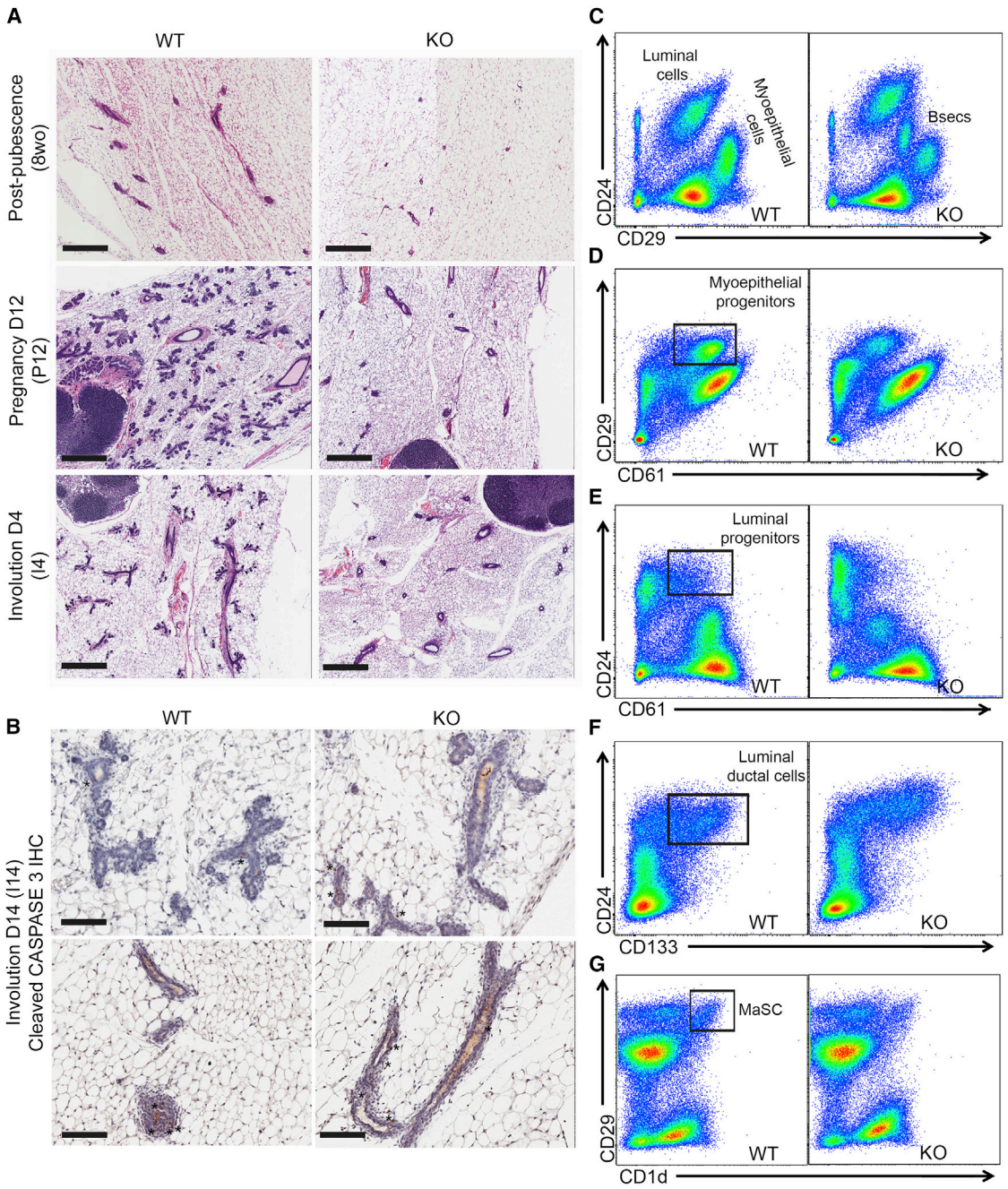

Figure 2. BPTF Is Required for the Active Stages of Mammary Gland Development

(A) BPTF depletion affects mammary gland development. Representative images from WT and KO glands at post-pubescence (8wo), mid-pregnancy (P12), and involution (I4). Scale bars, 400 μm.

(B) Cleavage CASPASE-3 IHC staining of WT and KO mammary glands at involution (I14). * highlights clusters of positive cells. Scale bars, 400 μm.

(C–G) Representative FACS plots of WT and KO MECs demonstrating the distribution of (C) Bsecs cells, (D) $CD61^+$ myoepithelial progenitor cells, (E) $CD61^+$ luminal progenitor cells, (F) $CD133^+$ luminal ductal cells, and (G) CD1d MaSCs.

and Table S3). We also tested whether pregnancy signals could improve the repopulation activity of KO MaSCs, and found a slight increase in the number of ductal structures in glands transplanted with KO MaSCs; however, this improvement was reduced when compared with WT transplants (Figure S3B and Table S3). Altogether, these

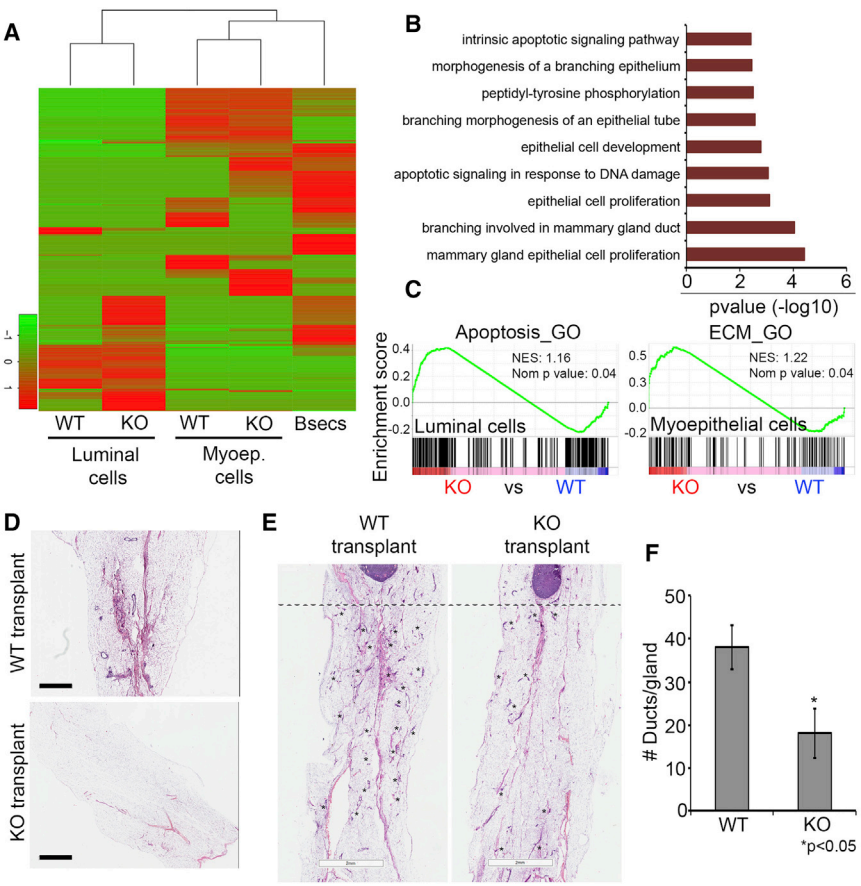

Figure 3. Loss of BPTF Alters Transcription Regulation and Impairs MaSC Self-Renewal

(A) Transcriptional profile of BPTF KO MECs. FACS-sorted WT and KO MECs were used for the preparation of RNA-seq libraries. Heatmap showing hierarchical clustering of Bsecs with WT and KO luminal and myoepithelial cells.

(B) Gene ontology analysis of genes upregulated in KO Bsecs compared with WT myoepithelial cells.

(C) Statistically significant gene set enrichment analysis predictions for genes upregulated in KO luminal cells (left panel) and in myoepithelial cells (right panel).

(D and E) Loss of BPTF affects MaSCs self-renewal. (D) Representative images of mammary glands transplanted with FACS-sorted, TAM-treated, WT and KO MaSCs. (E) Representative images of mammary glands transplanted with FACS-sorted, WT and KO MaSCs, following TAM treatment (2-weeks post-transplant). Scale bars, 2 mm. * highlights clusters of ductal structures.

(F) Duct quantification of mammary glands injected with WT and KO MaSCs. n = 3 independent experiments. t test. *p < 0.05.

results suggest that a self-renewing state is not fully maintained in the absence of BPTF expression.

A crucial step for successful cellular transplantation and expansion is tissue engraftment. Therefore, we transplanted WT and KO MaSCs into the cleared fat pad of recipient mice, which were then treated with TAM to achieve BPTF depletion (Figure S3C). Using this strategy, we found that transplantation of WT MaSCs yielded an average of 40 ductal structures per recipient fat pad, whereas injection of KO MaSCs exhibited approximately 18 mammary ducts per gland (Figures 3E and 3F), supporting the previous hypothesis that BPTF depletion affects the ability of MaSCs to self-renew, even after tissue engraftment. These results support a role for BPTF in maintaining the function of MaSCs.

BPTF Depletion Remodels Chromatin Accessibility in Mammary Myoepithelial Cells

Owing to its association with the NURF complex, we reasoned that BPTF might sustain the accessibility of select regulatory elements that regulate genes involved in self-renewal and lineage commitment. To investigate this possibility, we evaluated global DNA accessibility (ATAC-seq) in FACS-sorted BPTF WT and KO myoepithelial cells (Figure S4A). The genomic distribution of total ATAC-seq peaks revealed a decrease in the number of accessible regions located near transcription start sites of genes in KO myoepithelial cells compared with WT cells. We also observed a reciprocal increase in the number of accessible sites at genic and intergenic regions in KO cells, suggesting that BPTF may be controlling the open/close chromatin state of proximal and distal regulatory regions in mammary myoepithelial cells (Figure 4A). In fact, we found that the majority of ATAC-seq peaks overlapped with the center of distal (>2 kb away from promoter regions) H3K27ac-enriched chromatin, a histone modification that marks active regulatory regions (Figure S4B). Several of these ATAC-seq peaks were associated with a subset of differentially expressed genes (Figure S4C), suggesting a direct correlation between chromatin accessibility changes and gene expression. We found that distinct KO-specific accessible regions residing near genes upregulated in KO MECs were involved in the regulation of cell death (Figure 4B left panel and Figure 4C upper panel), which agrees with our finding that BPTF depletion increases the apoptotic rates of MECs. Interestingly, a subset of KO downregulated genes were

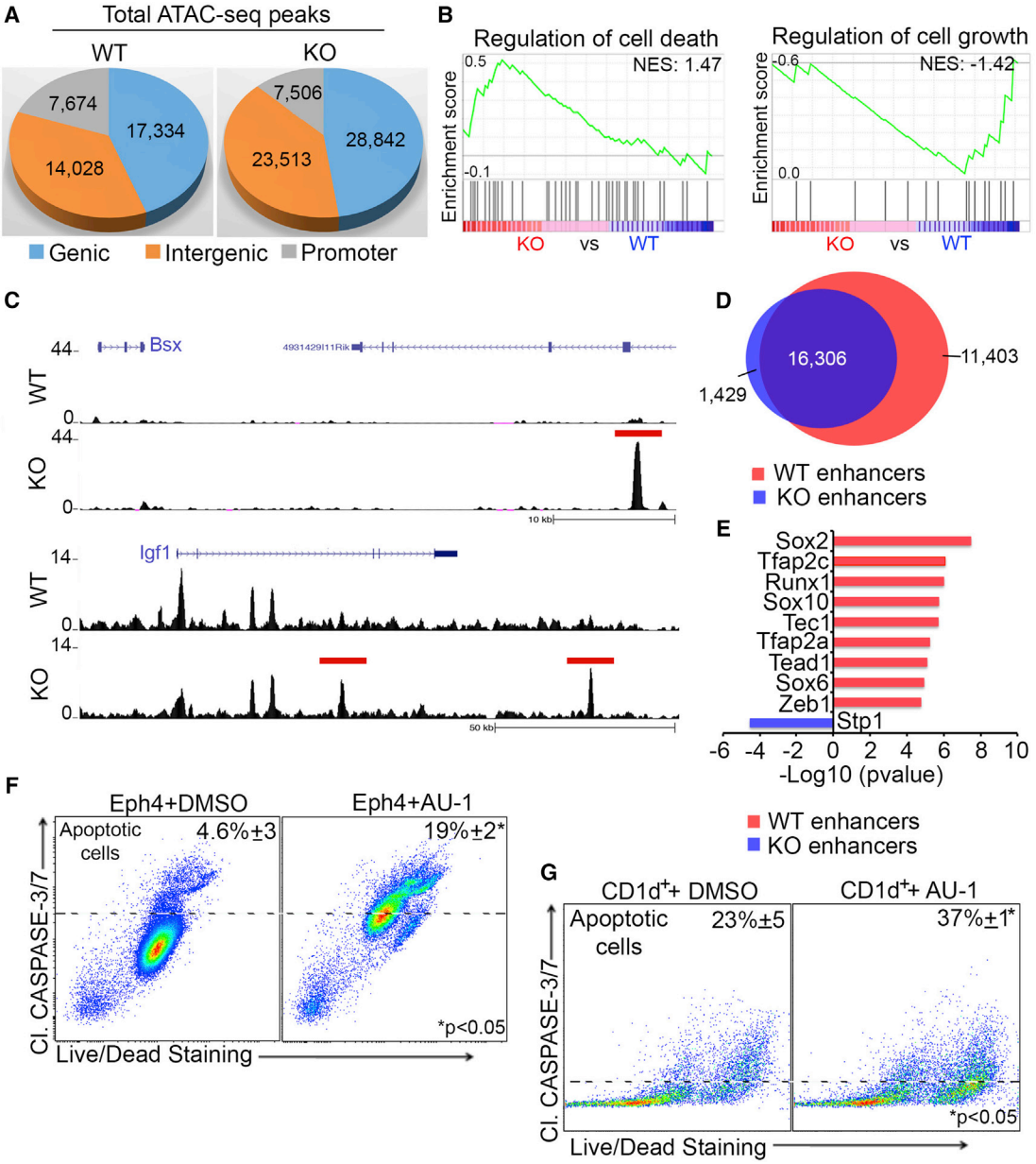

Figure 4. BPTF Depletion Alters the Chromatin Accessibility of MECs

(A) Chromatin accessibility analysis (ATAC-seq) of WT and KO MECs at distinct genomic regions.

(B) Statistically significant gene set enrichment analysis predictions for genes differentially expressed in KO MECs and associated with KO-specific ATAC-seq peaks.

(C) Genome browser images of KO-specific ATAC-seq peaks flanking genes upregulated (Bsx) or downregulated (Igf1) in KO MECs.

(D) Venn diagram showing chromatin accessibility of WT and KO MECs at putative enhancer regions.

(E) Top ten TF motifs enriched at WT enhancer (red) and KO enhancer (blue) regions.

(F and G) Effects of chemical BPTF inhibition on cell survival. (F) Eph4 cells and (G) MaSC short-term cultures were treated with either DMSO control or AU-1, followed by CASPASE-3 staining. n = 3 independent experiments. t test. *p < 0.05.

associated with cell growth and flanked by KO-specific ATAC-seq peaks (Figure 4B right panel and Figure 4C lower panel), and therefore downregulation of such genes is in agreement with the decreased proliferation rate of MECs after BPTF depletion. Altogether, the combination of phenotypic and molecular analyses supports a role for BPTF in regulating chromatin accessibility and transcriptional output of MECs.

Next, we investigated the effect of BPTF depletion on the overall enhancer landscape of mammary myoepithelial cells. We found a total of 27,709 enhancer regions in WT myoepithelial cells and only 12,832 enhancers in KO myoepithelial cells, suggesting that BPTF depletion influences the number of distal regions with open chromatin (Figure 4D). A substantial fraction of elements (11,403 regions) displayed reduced accessibility after BPTF inactivation, while 1,429 regions displayed elevated accessibility after BPTF depletion, suggesting that the predominant function of BPTF in MECs is to sustain chromatin opening. A motif analysis of WT-specific accessible regions revealed enrichment of DNA motifs recognized by several TFs, including Sox10, Runx1, and Tead1, which are known regulators of MaSCs differentiation (Dravis et al., 2015; van Bragt et al., 2014; Shi et al., 2015; Figure 4E). In addition, KO-specific open chromatin regions showed enrichment of a single TF, Stp1, a DNA-associated protein known to control cellular differentiation (Zhao et al., 2004; Figure 4E). Collectively, these analyses suggest that BPTF controls the chromatin state of MECs at regions that may support a stem-like phenotype, and its depletion changes the accessibility of regions associated with shortened life spans.

Chemical Inhibition of BPTF Bromodomain with AU-1 Modulates Chromatin Accessibility

Next, we sought to investigate whether chemical inhibition of BPTF would influence chromatin accessibility and cell survival. In these experiments, we utilized the small molecule AU-1, which was previously co-characterized by our group to suppress BPTF function in cells (Urick et al., 2015). We found that AU-1 treatment of Eph4 cells, a luminal-biased murine cell line model that can recapitulate stem cell activity (Pinkas and Leder, 2002), led to a decrease in c-MYC DNA occupancy, a TF previously described to rely on BPTF for its association with regulatory regions (Richart et al., 2016; Figure S4D). Furthermore, we found that AU-1 treatment decreased the proliferative capacity of Eph4 cells, with a G1 arrest of the cell cycle (Figure S4F), in addition to increasing the percentage of CASPASE-3^{+} cells (Figure 4F). We also tested the effect of AU-1 on CD1d MaSC short-term cultures, which resulted in diminished growth and increased apoptosis (Figures S4G and 4G), further supporting our observations in KO MaSCs. ATAC-seq analysis of AU-1-treated Eph4 cells revealed changes to chromatin accessibility (Figure S4E), in agreement with our ATAC-seq analysis of BPTF deficiency in primary MECs. Altogether, these chemical and genetic approaches implicate BPTF as a critical epigenetic regulator of MECs proliferation, self-renewal, and cell survival.

DISCUSSION

Our expression analysis prompted our investigation of BPTF as a regulator of MaSC self-renewal. We have found that BPTF sustains the cellular identity of MECs, and blocking its function results in chromatin alterations that influence cell differentiation. MECs are distinctly recruited at many of the post-birth mammary gland developmental stages; while MaSCs and early progenitors are recruited for mammary epithelium expansion during pre-pubescent and post-involution, lineage-committed progenitors and differentiated MECs are more responsive to hormonal influx, supporting tissue expansion during pregnancy (Rios et al., 2014; Rodilla et al., 2015). Here, we provide evidence that these developmental transitions require BPTF for their proper execution.

During the course of our study, we found that *Bptf* mRNA levels and protein localization differ in MaSC and luminal-committed cells, suggesting multiple layers that may control BPTF function during MEC differentiation. In this context, decreased BPTF levels, via transcriptional regulation and/or protein subcellular localization, would grant access to genomic regions associated with shortened life span and MEC differentiation. In addition, we identified an uncharacterized cell population (Bsecs), which accumulate in an aberrant fashion in BPTF-depleted glands. Accumulation of Bsecs, coupled with the simultaneous decrease of myoepithelial progenitors and total luminal cells, further support a role for BPTF in guiding the transition of stem/progenitor cells into a luminal-biased cell fate. Therefore, lack of BPTF would arrest cells in a stage that prevents their progression into the luminal fate, resulting in decreased ductal structures at different stages of mammary development.

Many of the factors known to control MEC lineage commitment and cellular differentiation operate at the level of transcriptional control. While the TFs Gata3, Elf5, and Runx1 regulate the commitment of MaSCs into luminal progenitors (Asselin-Labat et al., 2007; Chakrabarti et al., 2012; van Bragt et al., 2014), Stat5a/b regulate the proliferation of cells during pregnancy and lactation (Yamaji et al., 2009). Here, we showed that BPTF depletion perturbs the accessibility of regions recognized by master regulators of MaSC self-renewal and differentiation, illustrating how chromatin remodeling may change cellular state and influence the development of the mammary gland.

We also found that BPTF inhibition interferes with a transcriptional program supported by c-MYC activity, suggesting a role for targeting BPTF in MYC-dependent human cancers. It is worth mentioning that while normal c-MYC is important during mammary gland development (Hynes and Stoelzle, 2009), its overexpression is present in approximately 50% of basal-like breast cancers (Chen and

Olopade, 2008). Our observation that BPTF targets self-renewal networks and controls cellular proliferation and survival, extends beyond its known role in the control of c-MYC activity, and therefore suggests a potential role of BPTF in targeting additional breast cancer dependencies.

EXPERIMENTAL PROCEDURES

Mouse Lines

$K5^{Cre-ERT2}$ (FVB.Cg-Tg(KRT5-cre/ERT2)21pc/JeldJ) (Indra et al., 1999) and $Bptf^{fl/fl}$ (B6.129S1-Bptf<tm1.1Cwu>/J) (Landry et al., 2008) mice were purchased from The Jackson Laboratory. $K5^{CRE-Ert2}Bptf^{fl/fl}$ mice were backcrossed into FVB background. NOD.CB17-Prkdc<scid>/J female pups (15 days old) were utilized as recipients of transplantation assays. All experiments were performed in agreement with approved CSHL Institutional Animal Care and Use Committee.

Immunostaining

Immunofluorescence staining (IF) and Immunohistochemistry staining (IHC) were performed as described in Supplemental Experimental Procedures.

Mammary Gland Isolation

Mammary glands were harvested and processed as previously reported (dos Santos et al., 2013). MaSC short-term cultures, transplantation assay, FACS sorting, and analysis were performed as described in Supplemental Experimental Procedures.

Illumina Library Preparation and NextGen Sequencing

FACS-sorted and Eph4 cells were utilized for the preparation of NextGen Illumina libraries, as described in Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the raw and processed sequencing data reported in this paper is GEO: GSE98004.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.04.031.

AUTHOR CONTRIBUTIONS

C.O.D.S. designed the research and wrote the manuscript; C.O.D.S. and G.J.H. supervised the research; W.D.F., A.C., P.F.S., A.M.O., and C.O.D.S. performed experiments and analyzed results; S.E.K. and W.C.K.P. provided critical reagents.

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