MYB is essential for mammary tumorigenesis

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Abstract

MYB oncogene up-regulation is associated with estrogen receptor-positive (ER+) breast cancer (BRCA), but disease requirements for MYB function in vivo have not been explored. In this study we provide evidence of a critical requirement for MYB functions in models of human and murine breast cancer. In human BRCA, we found that MYB expression was critical for tumor cell growth both in vitro and in vivo in xenograft settings. In transgenic knockout mice, tissue-specific deletion of the murine Myb gene caused a transient defect in mammary gland development that was reflected in delayed ductal branching and defective apical bud formation. In MMTV-neu mice where tumors are initiated by activation of HER2, Myb deletion was sufficient to abolish tumor formation. In the more aggressive MMTV-PyMT model system, Myb deletion delayed tumorigenesis significantly. Together the findings in these transgenic knockout models implied that Myb was critical during an early window in mammary development when it was essential for tumor initiation, even though Myb loss did not exert a lasting impact upon normal mammary function. Two important MYB target genes that promote cell survival, BCL2 and GRP78/BIP, were each elevated compared to non-transformed mammary epithelial cells, thereby promoting survival as confirmed in colony formation assays in vitro. Taken together, our findings establish a role for MYB at the hub of ER- and HER2-dependent pathways in mammary carcinogenesis.

PRÉCIS: The MYB oncogene has been widely studied in blood cancers, but the importance of its function in solid tumors including breast cancers where MYB is often elevated has not been known.

Introduction

Breast cancer (BRCA) is heterogeneous in its genetic makeup and multifaceted in etiology (*1-2*). In addition to ERα, high expression of the receptor tyrosine kinase HER2 and the status of progesterone receptors are prognostic and therapeutic guides in BRCA management. Although the majority of BRCAs express one or more of these receptors, some are "triple-negative" (*3*); this subtype is often more aggressive and does not respond to conventional adjuvant therapies that target the above-mentioned receptors (*4-5*). With such phenotypic diversity, the prospect of identifying molecular points of convergence that drive BRCA may seem small although nonetheless enticing.

The appeal of considering transcription factors in the context of BRCA is that they are often downstream of multiple signaling pathways and their target genes can fall into pro-malignancy categories that cooperate in tumorigenesis. We have focused on the transcription factor, MYB (*6*) for which target genes in myeloid cells (*7*) and BRCA (*8*) have been reported. A central observation that underpins our attention on *MYB* is that it is frequently over-expressed in BRCA (*9*). We reported the direct regulation of *MYB* transcriptional elongation by ERα (*6*), that *MYB* is essential for proliferation of BRCA cell lines (*6*), and that it suppresses differentiation and apoptosis (*10*). As ~70% of BRCAs are ERα+ve, this work addressed a significant BRCA category. Nevertheless, we report here that MYB is also evident in some HER+ve BRCA in the absence of ERα. At the other end of the spectrum are *BRCA1*-mutant BRCA (11), representing only a few percent of all cases. Intriguingly however, 29% of *BRCA1*-mutant BRCA have amplified *MYB* (12) further suggesting that *MYB* has broad relevance to BRCA.

To specifically address the role of *MYB* in mammary cancer, we have now employed two transgenic mammary-specific tumor models. Both depend upon the mouse mammary tumor virus (*MMTV*) promoter to drive either the wild type (wt) rat *NEU* gene (13) or the Polyoma Middle T Antigen viral gene (*PyMT*) (14). These models have provided many important insights into mammary cancer progression. We report here that with the specific ablation of *MYB* expression in the mammary gland, neither transgenic model progresses normally to form tumors. *In vitro* studies demonstrate that MYB protects mammary epithelial cells from apoptosis, allows colony formation in soft agar and drives cancer-associated gene expression. Collectively, these data establish a central and pervasive role for MYB in mammary carcinogenesis.

Materials and Methods

Xenografts

One x 10^6 ZR-75-1 cells (stably expressing either inducible *MYB* shRNA or scrabbled (*SCR)* shRNA as well as constitutive eGFP under the control of *EF-1α* promoter (6) in 50 µL complete medium were mixed with 50 µL Matrigel (BD Biosciences) and injected into the vicinity of the mammary gland of 6-8 week old female NOD/SCID mice. Estradiol pellets (0.72mg/pellet, 60 day release; Innovative Research of America) were implanted three days before cell injection. Mice were imaged 2 weeks later with an Imaging Station (*In Vivo* FX; Eastman Kodak Company) for eGFP expression. After confirmation of the presence of tumors, mice were divided into two groups with equal sized tumors. To induce shRNA, mice were given chow supplemented with 600mg/Kg Doxycycline (Specialty Feeds). Tumor growth was measured by eGFP intensity at fortnightly intervals.

Mice

Mice with *loxP* sites introduced between *MYB* introns 2 and 6 were kindly provided by Jon Frampton (Birmingham, England) (15). These mice were then crossed with *MMTV-Cre* (16) (C57Bl/6) and/or *MMTV-NEU (wt rat Neu)* (FI – FVB/N: C57Bl/6 - mixed background littermates; six generations on C57Bl/6). F1 backcross litters were generated with matched genotypes (*MMTV-Cre*; *MMTV-NEU*; $MYB^{f/f}$; or *MMTV-PyMT* $MYB^{f/+}$; $MYB^{f/f}$*; MMTV-Cre* (C57Bl/6). All *MMTV-NEU* females experienced 2 pregnancies. Animals were bred and maintained in a pathogen-free environment and with approval by the PMCC Animal Ethics Committee.

Tumor Microarrays and Immunohistochemistry

Tumors (sixty-nine) from women without a family history of BRCA were collected with consent from a single site State Pathology Centre. These were processed for immunohistochemistry for HER2 (4B5; rabbit monoclonal Ventana), ERα (SP1; rabbit monoclonal Ventana) and pan-MYB (Mab1.1; Upstate Biotech) as described (17), detected by horseradish-peroxidase conjugated anti-mouse or anti-rabbit secondary antibodies (DAKO), visualised with 3,3'-diaminobenzidine tetrachloride (DAKO), scanned (Aperio^{R} slide scanner) and scored blinded by two observers. Mouse tissues were fixed in Methacan. Proliferating cell nuclear antigen (PCNA) was detected with mouse antibody PC-10 (Santa Cruz).

Cell culture and expression

Murine cell lines 67NR, 66cl4, 4T1, 4T1.2 were derived from a spontaneous mammary tumor (18) (verified by array CGH analysis, all 3 lines are essentially identical to the original 4T1 cell line, *unpublished data*). ZR-75-1 cells (purchased from ATCC) were reconfirmed by STR analysis in the last month (19). TA93 and PyMT cell lines were established from *MMTV-NEU and MMTV-PyMT* tumors respectively by us (*unpublished*). NMuMG cells were originally purchased from ATCC. Primary tumors were collected from *MMTV-NEU; PyMT or Wnt-1 mice*. Retroviral vectors pRufNeo-*MYB* (murine *MYB*, full length) and pRufNeo-Control were used to generate retroviral supernatants for stable transductions (20). Western analysis of was conducted as previously described (21-22).

Anchorage independent growth

NMuMG *MYB*-transduced and control cells were plated in 0.35% agarose in 6 well plates (15,000 cells/well). Medium was added every 4-5 days and colonies photographed at day 21.

Mammary gland analysis

Mammary glands whole mounts were spread on a glass slide before fixing in Carnoy fixative. Glands were stained in 0.2% carmine and 0.5% aluminum sulfate overnight. Subsequent analysis was carried out using Metamorph^{R} image software.

PCR

PCR was used to amplify genomic DNA for the detection of *MYB*^{f}, *Cre*, and *NEU* genes (primers - **Supplementary Table 1**). For RT-qRT-PCR analyses, primers were designed using Primer Express software (**Supplementary Table 2**) and transcripts were amplified using a Prism 7000 Sequence Detection system (ABI).

FACS

Fluorescence activated cell sorting was used to detect apoptotic cells by Annexin V antibody binding (BD Pharmagen) and 7-amino-actinomycin D (7AAD) staining. Data were analyzed using the FCS express program (De Novo software).

Statistical Analysis

Data were subject to analysis using Graph-Pad Prism5 (CA, USA).

Results

MYB Knock-down in vivo

Having established that MYB is required for proliferation of human ER+ve BRCA cells *in vitro* (*6*) we asked here if persistent expression was required for established tumors. ERα+ve ZR-75-1 cells with inducible shRNA directed against *MYB* (*6*) were generated with an eGFP expression module to allow *in vivo* xenograft imaging. Tumors were established orthotopically for two weeks before shRNA expression was induced with doxycycline (**Figure 1A**); subsequent monitoring (**Figure 1B-C**) showed that *MYB* expression is required for on-going tumor growth. Similar data were obtained with MCF-7 cells (***data not shown***). These data indicate that not only is *MYB* expression a feature of human ER+ve BRCA cells *in vitro* but that sustained *MYB* expression is required for tumor growth.

MYB in HER2+ve/ERα-ve Breast Tumors

MYB expression is a consistent feature of ERα+ve BRCA; reviewed in (9). We have shown above that it is required for growth *in vivo.* By evaluating BRCA from 69 women without a family history of BRCA in a TMA format we found most expressed MYB (64/69). Furthermore coincident expression of ERα+ve and MYB (56/64) (**Supplementary Figure 1A-C**) is consistent with previous reports based mostly on cell lines and mRNA analyses (6, 10, 23). Importantly Mab1.1 also detects other MYB family members and to confirm that the signal observed with the TMAs was MYB we employed another antibody Mab5.1 (22) that we determined to be MYB-specific (***data not shown***). Both Mab 1.1 and 5.1 gave comparable IHC (***data not shown***).

Our IHC observations were consistent with the mRNA profiling of BRCA where *MYB* tracks with ERα expression (reviewed in (9)), however the status of MYB in HER+ve tumors has been less clear. In TMAs we identified 13 tumors with robust HER2 expression; 12 of which were also MYB+ve. Of these we focused on the 4 that where MYB+ve and HER2+ve but ERα -ve which are shown in **Figure 2A** as serial sections stained for the three antigens. In one tumor (**Figure 2A-iv)** MYB expression was identified associated with tumor cells in the HER2+ve (ERα-ve) field as well as in an apparently normal ductal region where cells were ERα+ve, MYB+ve but HER2-ve (**Figure 2B-C)**. From these examples it is apparent that MYB expression can occur without ERα driving *MYB* transcription and pathways that are HER2-dependent may also be associated with *MYB* expression.

MYB expression in mouse mammary tumors and NEU-induced tumorigenesis

Here we extended previous studies with human BRCA cell lines to examine *MYB* in mouse mammary tumors. A range of spontaneously arising clones of different metastatic capacity (4T1.2, 66CL4 and 67NR) (24-25), cell lines from *MMTV-NEU* (TA93) and *MMTV-PyMT* (PyMT) tumors and primary tumors were compared to an immortalized mammary epithelial cell line (NMuMG) (26) or to normal mammary gland. *MYB* mRNA and protein was found to be higher in tumor cell lines and tumors (*MMTV-Her2/Neu; -PyMT; -Wnt-1)* compared to adult mammary gland (**Supplementary Figures 2A-C**). By IHC, we also demonstrated MYB nuclear staining in *MMTV-NEU* tumors (**Figure 3A**) and consistently elevated *MYB* mRNA in tumors resected from these mice (**Figure 3B**).

MMTV-NEU mice were then crossed with $MYB^{fl/fl}$ x *MMTV-Cre* mice. $MYB^{fl/fl}$ mice have been described previously (15) and have been used by us to show that MYB is required for

colon epithelial homeostasis (27). However they have not been employed to examine tumorigenesis in epithelial tissues. To ensure both efficient tumorigenesis as well as Cre-mediated recombination all females were subjected to two rounds of pregnancy and lactation. All *MMTV-NEU;MYB*$^{f/f}$ females developed at least one mammary tumor between 300 - 500 days consistent with other reports (13). By contrast, at 500 days no *MMTV-NEU;MMTV-Cre;MYB*$^{f/f}$ females had palpable tumors. Later, three mice in this cohort of 14 mice developed tumors (**Figure 3C**) while the remaining mice died of old age, tumor-free. These late onset tumors from KO mice retained *MYB* expression albeit at lower levels (but higher than normal mammary glands) (**Figure 3D**) indicating that *MYB* was not completely deleted in these cases. To ensure that the expression of the oncogenic *MMTV-NEU* transgene was not under the transcriptional influence of MYB in normal mammary tissue RNA (DNAse-treated) was prepared from adult female KO and wt mammary glands and subjected to qRT-PCR whereby rat *NEU* cDNA expression was found to be relatively robust and unaffected by the loss of *MYB* (**Supplementary Figure 3A**).

MYB expression is required for timely MMTV-PyMT tumorigenesis

Given that very few *MMTV-NEU*;*MMTV-Cre;MYB*$^{f/f}$ mice developed mammary tumors and that the incidence of tumor formation in the *MMTV-NEU;MYB*$^{f/f}$ control mice was 100% (28/28), *MYB* expression is clearly shown to be a limiting factor in this model. The *MMTV-NEU* model takes almost one year before mice begin to develop mammary tumors. By contrast, the viral oncogene *Polyoma Middle-T* (*PyMT*) leads to a rapid onset of multifocal mammary tumors beginning at 10 weeks (14). Initially we established that primary tumors from *MMTV-PyMT* females consistently expressed MYB (**Supplementary Figure 2B; Figures 4A-B**). We then introduced the *MYB*^{f} locus and the *MMTV-Cre* transgene. The deletion of one *MYB* allele led to

a significant delay in time to cull of tumor bearing *MMTV-PyMT;MMTV-Cre*;*MYB*$^{f/+}$ females while deletion of both alleles (*MMTV-PyMT;MMTV-Cre*;*MYB*$^{f/f}$) markedly impeded tumor development and doubling life expectancy (**Figure 4C**).

Approximately one quarter of this cohort of mice was still disease-free at 700 days which is remarkable given that the *PyMT* transgene typically leads to highly aggressive and multifocal mammary carcinogenesis. By contrast, in *MMTV-NEU* mice where tumors were rarely detected, at least not until 500 day in KO mice *MMTV-PyMT* females did develop tumors. To confirm that the basis for tumor delay was not due to an effect of KO on *PyMT* expression, *PyMT* mRNA levels were evaluated in tumors from wt and KO mice. Although levels of *MYB* varied, perhaps indicative of tumor heterogeneity and KO, it was very clear that *PyMT* transgene expression was consistently high regardless of *MYB* level (**Supplementary Figure 3B**). Interestingly, tumors were isolated that had either very low or robust *MYB* mRNA expression on a background KO, indicating that the cells that gave rise to some of these tumors had not been subject to *MYB* deletion as might have been expected in the presence of MMTV-Cre. Such mice were reconfirmed to be of the designated genotypes (***data not shown***). This suggests that PyMT can transform both *MYB*-ve and +ve mammary target cells more rapidly than in cells expressing the *NEU* transgene.

(c-)MYB and A-MYB in developing mammary glands

In view of the inhibition or delay of mammary tumorigenesis in mice on a KO background, perhaps *MYB* loss might lead to the ablation of cells that are targeted for tumorigenesis or perhaps even a general loss of mammary epithelial cells. Mammary gland development has been characterized extensively (28) but a role for MYB in this tissue has not been examined as global *MYB* KO mice die at E14.5 (29). By contrast *A-MYB* KO mice are

viable but have defects in mammary gland function (30). We compared the expression patterns of *MYB* and *A-MYB* during mammary gland development compared to house keeping gene *GAPDH* (**Figure 5A)** (and *Cytokeratin-18*; **Supplementary Figure 4**) expression. *MYB* expression was initiated earlier and at higher levels relative to *A-MYB* in virgin glands before it declined. Notably, luminal MYB is most evident in virgin glands (**Figure 5B-D**) and proportionally more cells were MYB+ve than those actively engaged in the cell cycle (PCNA+ve) or those that were ERα+ve (**Supplementary Figure 5**).

To test whether *MYB* loss might be of functional consequence, we examined the mammary glands of $MYB^{fl/fl}$ mice crossed with *MMTV-Cre* mice. **Figures 5E-G** reveal delayed ductal branching and terminal end bud (TEB) formation in KO glands. Importantly, female mice carrying the mammary-specific *MYB* deletion became pregnant, give birth to litters of normal size and suckle their pups to normal body weights (***data not shown***), indicating that the *MMTV-Cre* line employed by us was not causing lactation defects (31). The presence and ultimate resolution of mammary gland developmental defects in the KO mice was in marked contrast to other KO mice such as *CyclinD1*, *SRC* and *A-MYB* which showed a lasting negative effect on mammary gland development (32-33). It is therefore reasonable to speculate that the delayed mammary development may be in part responsible for impeding a key event in the initiation of mammary carcinogenesis that is MYB-dependent

MYB induces Anchorage-independent Growth and Enhances Cell Survival

We next sought to establish how MYB might contribute to mammary cell transformation. We transduced NMuMG cells (26) with a MYB retrovirus. Normally NMuMG cells grow attached to the substratum in 2D tissue culture but show very little growth in agarose. By

contrast, *MYB*-transduced cells displayed increased anchorage-independent growth (**Figures 6A-B**). Under sub-optimal growth conditions (FCS 0.1%) NMuMG cells attach but show relatively poor growth in 2D culture, whereas *MYB*-transduced cell displayed robust growth (**Figure 6C**) associated with enhanced survival as assessed by AnnexinV staining (**Figure 6D**). The expression of two MYB-target genes associated with cell survival that are over-expressed and regulated by MYB in colorectal cancer and BRCA, *Grp78/BIP* and *BCL-2* (10) were elevated (**Figures 6E-F**). These data extend our previous findings that *MYB* suppresses differentiation and apoptosis of human BRCA cell lines (*10*).

Discussion

The role for *MYB* in hematopoietic malignancies has overshadowed its important role in epithelial cancers (9). RNA profiling studies collectively support the view that high *MYB* tracks with ERα+ve BRCA compared to most other epithelial cancers with the notable exception of colorectal cancers where *MYB* is not only over-expressed but also of prognostic importance (34). Accordingly our focus has been on ERα+ve BRCA as this is the predominant subtype and we have made an important functional leap to show here the requirement for sustained *MYB* expression in established BRCA xenografts. We also showed that HER2+ve BRCA are MYB+ve.

To explore early events in mammary carcinogenesis, we turned our attention to mouse cell lines and transgenic models initially to evaluate MYB expression. In most instances we identified expression in excess of that found in immortalized mammary cells and mammary glands. With these data in mind we then had a rational basis to manipulate MYB expression in the mouse during mammary gland development and tumorigenesis.

The *MMTV*-promoter has been exploited extensively to drive gene expression and here we used the *MMTV-Cre* transgene to ablate MYB expression to assess its potential role in mammary gland development. We found that loss of MYB led to a delay in gland development, but most this difference was resolved in adult mice without a demonstrable consequence to fecundity or lactation. In the context of mouse mammary carcinogenesis, the earliest and a substantive advance made by Muller and colleagues was to drive the *NEU* gene with the *MMTV* promoter (13). Here we have shown that MYB is required for NEU-driven tumorigenesis in these mice.

Previously we focused our attention on MYB in the context of ERα+ve BRCA but now we have to consider MYB in the broader context of HER2+ve BRCA. Thus this role in HER+ve BRCA which is the next most clinically tractable group to treat after ERα+ve BRCA further raises the importance of MYB more generally in BRCA. Indeed, recent analyses of BRCA expression profiling indicates that *MYB* is associated with high survival, particularly in patients with ERα+ve tumors (35) but that *MYB* did not track specifically with identified molecular subtypes such as basal, HER2, luminal A or B or normal-like BRCA. High *MYB* was also associated with improved survival in another study most obviously in luminal type BRCA bearing patients (36). Together with our IHC data it seems that *MYB* expression arises in a range of BRCA sub-types. However, in contrast to an earlier pioneering report by Guerin et al (23) that emphasised an inverse relationship between *HER2* and *MYB* where they still observed co-expression in 56% (24/56) and discordant expression in 77% (53/69) of non-inflammatory BRCA we found by IHC that 92% HER+ve BRCA co-expressed MYB (12/13). ERα expression was present in 9/13 HER+ve BRCA that we examined however MYB and HER2 can be co-expressed in the absence of ERα (4/13 HER2+ve tumors).

This relationship between MYB and mammary carcinogenesis is not without complexities as the *MMTV-PyMT* mouse data suggested. In these mice we found that although MYB was required for the normal kinetics of tumorigenesis its ablation did not stop tumor formation in three-quarters of female mice and indeed the tumors that arose were both *MYB* high and low. Conversely, in the absence of this genetic selection most tumors that arose in *MMTV-PyMT* mice had detectable MYB expression but the levels were variable. These data suggested that MYB was required for the timely development of mammary tumors but was not

ultimately essential for their progression. Certainly we and others have reported that MYB is not in all BRCA cell lines; MDA-MB-231 being a notable ERα-ve and MYB-ve cell line (6) and 15/133 BRCA were MYB-ve in the TMAs (**Supplementary Figure 1**). However, the link between these models might be that mammary tumorigenesis may proceed through an ERα-dependent phase where PyMT but not NEU expression may more readily progress in the absence of MYB.

In vitro studies were used to demonstrate that MYB modifies the growth of immortalized mammary cells and activates genes associated with cell survival. To probe the functional role of MYB during mammary gland tumorigenesis, the normal mammary epithelial cell line NMuMG expressing full-length MYB was assessed for the expression of established MYB target genes, *Grp78,* and *BCL-2,* which were found to be consistently up-regulated. NMuMG cells transduced with *MYB* showed enhanced proliferation and reduced apoptosis likely to be due to the elevated expression of these pro-survival genes (37). However elevated MYB did not alter cell migration as measured by the scratch assay (***data not shown***).

Although many studies have documented MYB target genes (9) less is known about the mechanisms that regulate MYB expression itself. Certainly the *MYB* promoter is subject to regulation by the PI3 kinase and AKT pathway in T-cells (38) and can be auto-regulated by MYB itself (39-40). Beyond promoter activation a principle mechanism of *MYB* regulation is the ability of exon-1 transcripts to be extended by transcriptional elongation (41-44) and in the case of colon cancer, we have shown that mutations in a region that regulates this process are relatively common in the human gene (9). However similar mutations were not detected in human BRCA or cell lines (42), rather we found that the ERα when bound by estradiol

controlled elongation through this region (6). We have not as yet established the same relationship between *MYB* transcription and ERα in mouse mammary cells perhaps because most mouse mammary cancer lines are typically ERα low or negative by the time they are in culture. It would seem that there is a strong case that most tumors also progress through an ERα+ve stage. This has been shown most convincingly with *MMTV-PyMT* mice that develop adenoma/MIN that closely resembles the ductal epithelial breast hyperplasia observed in humans. These lesions progress to mimic ductal carcinoma *in situ*. The primary tumors are typically 30-40% ERα+ve and this may increase to 80%. As the carcinoma progresses, ERα-ve cells emerge becoming the predominant category. Approximately 30% of early carcinoma may have 70-80% PR+ve nuclei but this expression also disappears in later stages of carcinoma progression (45). Similarly, measures that modify ERα status and/or function can delay or block tumorigenesis in the *MMTV-NEU* model (46).

IHC studies revealed MYB and ERα+ve cells in the ductal epithelium of mouse mammary glands but as there are more MYB+ve, than ERα+ve cells (**Supplementary Figure 5**), the question remains as to whether the former is regulated by the latter. However it is clear that without MYB, tumorigenesis is markedly inhibited. Similarly the relationship between *NEU* and *MYB* regulation has been intriguing. It is notable that *NEU* expression is evident in both the normal epithelia and early stage tumors and increases with progression while there is a commensurate reduction that tracks with ductal and TEB maturation. At the protein level NEU increases 3-5 fold in tumors compared to Adenoma/MIN. Although relatively high during mammary gland development NEU expression declines as gland maturation is near complete (45). Whether these events occur in a parallel fashion in human breast development and

BRCA are less clear. Similarly it is unclear whether HER2 can drive *MYB* expression in breast epithelial cells and if so, this regulation may have to take into account the observation that MYB has been reported to repress *HER-2* transcription in non-BRCA cells (47).

The mouse studies used here show that established human BRCA xenografts do not advance when *MYB* is knocked down using shRNA, and that NEU mammary tumors are rarely formed without *MYB*. Taken together with our previous observations made on the direct role of *MYB* human BRCA cell growth and progression (10), we have now provided evidence across a spectrum of models that argue for a role for MYB in mammary carcinogenesis. Finally, these findings may contribute to the understanding and treatment of human BRCA. For example, the KO data have implications for the etiological timing of BRCA initiation in young women (48), while the expression pattern and essential role of *MYB* in multiple forms of mammary carcinogenesis strongly encourages the pursuit of therapeutic approaches that directly or indirectly target *MYB* (10, 49).

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References

1. Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. Nat Rev Cancer. 2007;7:659-72.
2. Blackshear PE. Genetically engineered rodent models of mammary gland carcinogenesis: an overview. Toxicol Pathol. 2001;29:105-16.
3. Lund MJ, Trivers KF, Porter PL, Coates RJ, Leyland-Jones B, Brawley OW, et al. Race and triple negative threats to breast cancer survival: a population-based study in Atlanta, GA. Breast Cancer Res Treat. 2009;113:357-70.
4. Schneider BP, Winer EP, Foulkes WD, Garber J, Perou CM, Richardson A, et al. Triple-negative breast cancer: risk factors to potential targets. Clin Cancer Res. 2008;14:8010-8.
5. Campeau PM, Foulkes WD, Tischkowitz MD. Hereditary breast cancer: new genetic developments, new therapeutic avenues. Hum Genet. 2008;124:31-42.
6. Drabsch Y, Hugo H, Zhang R, Dowhan DH, Miao YR, Gewirtz AM, et al. Mechanism of and requirement for estrogen-regulated MYB expression in estrogen-receptor-positive breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America. 2007;104:13762-7.
7. Zhao L, Glazov EA, Pattabiraman DR, Al-Owaidi F, Zhang P, Brown MA, et al. Integrated genome-wide chromatin occupancy and expression analyses identify key myeloid pro-differentiation transcription factors repressed by Myb. Nucleic Acids Res. 2011.
8. Quintana AM, Liu F, O'Rourke JP, Ness SA. Identification and Regulation of c-Myb Target Genes in MCF-7 Cells. BMC Cancer. 2011;11:30.
9. Ramsay RG, Gonda TJ. MYB function in normal and cancer cells. Nat Rev Cancer. 2008;8:523-34.
10. Drabsch Y, Ramsay RG, Gonda TJ. MYB suppresses differentiation and apoptosis of human breast cancer cells. Breast Cancer Research. 2010;12.
11. Foulkes WD, Stefansson IM, Chappuis PO, Begin LR, Goffin JR, Wong N, et al. Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J Natl Cancer Inst. 2003;95:1482-5.
12. Kauraniemi P, Hedenfalk I, Persson K, Duggan DJ, Tanner M, Johannsson O, et al. MYB oncogene amplification in hereditary BRCA1 breast cancer. Cancer Res. 2000;60:5323-8.
13. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proceedings of the National Academy of Sciences of the United States of America. 1992;89:10578-82.
14. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol. 1992;12:954-61.
15. Emambokus N, Vegiopoulos A, Harman B, Jenkinson E, Anderson G, Frampton J. Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. Embo J. 2003;22:4478-88.
16. Wagner KU, Wall RJ, St-Onge L, Gruss P, Wynshaw-Boris A, Garrett L, et al. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res. 1997;25:4323-30.
17. Zorbas M, Sicurella C, Bertoncello I, Venter D, Ellis S, Mucenski ML, et al. c-Myb is critical for murine colon development. Oncogene. 1999;18:5821-30.

18. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res. 1992;52:1399-405.
19. Masibay A, Mozer TJ, Sprecher C. Promega Corporation reveals primer sequences in its testing kits. J Forensic Sci. 2000;45:1360-2.
20. Rayner JR, Gonda TJ. A simple and efficient procedure for generating stable expression libraries by cDNA cloning in a retroviral vector. Mol Cell Biol. 1994;14:880-7.
21. Ramsay RG, Ciznadija D, Mantamadiotis T, Anderson R, Pearson R. Expression of stress response protein glucose regulated protein-78 mediated by c-Myb. Int J Biochem Cell Biol. 2005;37:1254-68.
22. Ramsay RG, Ishii S, Nishina Y, Soe G, Gonda TJ. Characterization of alternate and truncated forms of murine c-myb proteins. Oncogene Res. 1989;4:259-69.
23. Guerin M, Sheng ZM, Andrieu N, Riou G. Strong association between c-myb and oestrogen-receptor expression in human breast cancer. Oncogene. 1990;5:131-5.
24. Miller BE, Roi LD, Howard LM, Miller FR. Quantitative selectivity of contact-mediated intercellular communication in a metastatic mouse mammary tumor line. Cancer Res. 1983;43:4102-7.
25. Lelekakis M, Moseley JM, Martin TJ, Hards D, Williams E, Ho P, et al. A novel orthotopic model of breast cancer metastasis to bone. Clin Exp Metastasis. 1999;17:163-70.
26. David G, Van der Schueren B, Bernfield M. Basal lamina formation by normal and transformed mouse mammary epithelial cells duplicated in vitro. J Natl Cancer Inst. 1981;67:719-28.
27. Malaterre J, Carpinelli M, Ernst M, Alexander W, Cooke M, Sutton S, et al. c-Myb is required for progenitor cell homeostasis in colonic crypts. Proceedings of the National Academy of Sciences of the United States of America. 2007;104:3829-34.
28. Richert MM, Schwertfeger KL, Ryder JW, Anderson SM. An atlas of mouse mammary gland development. J Mammary Gland Biol Neoplasia. 2000;5:227-41.
29. Mucenski ML, McLain K, Kier AB, Swerdlow SH, Schreiner CM, Miller TA, et al. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell. 1991;65:677-89.
30. Toscani A, Mettus RV, Coupland R, Simpkins H, Litvin J, Orth J, et al. Arrest of spermatogenesis and defective breast development in mice lacking A-myb. Nature. 1997;386:713-7.
31. Yuan T, Wang Y, Pao L, Anderson SM, Gu H. Lactation Defect in a Widely Used MMTV-Cre Transgenic Line of Mice. PLoS One. 2011;6:e19233.
32. Jeselsohn R, Brown NE, Arendt L, Klebba I, Hu MG, Kuperwasser C, et al. Cyclin D1 kinase activity is required for the self-renewal of mammary stem and progenitor cells that are targets of MMTV-ErbB2 tumorigenesis. Cancer Cell. 2010;17:65-76.
33. Watkin H, Richert MM, Lewis A, Terrell K, McManaman JP, Anderson SM. Lactation failure in Src knockout mice is due to impaired secretory activation. BMC Dev Biol. 2008;8:6.
34. Ramsay RG, Malaterre J. Insights into c-Myb functions through investigating colonic crypts. Blood Cells Mol Dis. 2007;39:287-91.
35. Nicolau M, Levine AJ, Carlsson G. Topology based data analysis identifies a subgroup of breast cancers with a unique mutational profile and excellent survival. Proceedings of the National Academy of Sciences of the United States of America. 2011;108:7265-70.
36. Thorner AR, Parker JS, Hoadley KA, Perou CM. Potential tumor suppressor role for the c-Myb oncogene in luminal breast cancer. PLoS One. 2010;5:e13073.

37. Zhang GJ, Kimijima I, Tsuchiya A, Abe R. The role of bcl-2 expression in breast carcinomas (Review). Oncol Rep. 1998;5:1211-6.
38. Lauder A, Castellanos A, Weston K. c-Myb transcription is activated by protein kinase B (PKB) following interleukin 2 stimulation of Tcells and is required for PKB-mediated protection from apoptosis. Mol Cell Biol. 2001;21:5797-805.
39. Ciznadija D, Tothill R, Waterman ML, Zhao L, Huynh D, Yu RM, et al. Intestinal adenoma formation and MYC activation are regulated by cooperation between MYB and Wnt signaling. Cell Death Differ. 2009.
40. Nicolaides NC, Gualdi R, Casadevall C, Manzella L, Calabretta B. Positive autoregulation of c-myb expression via Myb binding sites in the 5' flanking region of the human c-myb gene. Mol Cell Biol. 1991;11:6166-76.
41. Bender TP, Thompson CB, Kuehl WM. Differential expression of c-myb mRNA in murine B lymphomas by a block to transcription elongation. Science. 1987;237:1473-6.
42. Hugo H, Cures A, Suraweera N, Drabsch Y, Purcell D, Mantamadiotis T, et al. Mutations in the MYB intron I regulatory sequence increase transcription in colon cancers. Genes Chromosomes Cancer. 2006;45:1143-54.
43. Toth CR, Hostutler RF, Baldwin AS, Jr., Bender TP. Members of the nuclear factor kappa B family transactivate the murine c-myb gene. J Biol Chem. 1995;270:7661-71.
44. Watson RJ. A transcriptional arrest mechanism involved in controlling constitutive levels of mouse c-myb mRNA. Oncogene. 1988;2:267-72.
45. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. Am J Pathol. 2003;163:2113-26.
46. Yang X, Edgerton SM, Kosanke SD, Mason TL, Alvarez KM, Liu N, et al. Hormonal and dietary modulation of mammary carcinogenesis in mouse mammary tumor virus-c-erbB-2 transgenic mice. Cancer Res. 2003;63:2425-33.
47. Mizuguchi G, Kanei-Ishii C, Takahashi T, Yasukawa T, Nagase T, Horikoshi M, et al. c-Myb repression of c-erbB-2 transcription by direct binding to the c- erbB-2 promoter. J Biol Chem. 1995;270:9384-9.
48. Hamilton AS, Mack TM. Puberty and genetic susceptibility to breast cancer in a case-control study in twins. N Engl J Med. 2003;348:2313-22.
49. Williams BB, Wall M, Miao RY, Williams B, Bertoncello I, Kershaw MH, et al. Induction of T cell-mediated immunity using a c-Myb DNA vaccine in a mouse model of colon cancer. Cancer Immunol Immunother. 2008;57:1635-45.

Figure legends

Figure 1

MYB is required for human breast tumor progression (**A**) ZR-75-1 cells (stably expressing either the *MYB* or *SCR* shRNA) were injected into NOD/SCID mice mammary fat pads. Estrogen pellets were implanted 3 days before tumor cell injection. At two weeks after transplantation, mice were imaged for eGFP. After confirmation of the presence of tumors, mice were divided into two groups with equal sized tumors. To induce shRNA expression, one group was given chow supplemented with Doxycycline. (**B & C**) Tumor growth was measured by eGFP intensity.

Figure 2

MYB and HER2 co-expression without ERα **(A)** Serial sections of four breast tumor samples (i-iv) are represented where MYB is evident in the regions of relatively robust HER2 staining but where ERα is essentially absent. Brown arrows denote regions that co-express MYB and HER2 but are ERα–ve (white arrow). **(B)** In sample (iv) a region that is histologically normal HER2-ve and ERα+ve is also positive for MYB **(C)**.

Figure 3

Mammary tumors show elevated MYB expression and MMTV-NEU tumor-prone mice lacking MYB in the mammary gland display prolonged survival (**A**) MYB protein IHC in a *MMTV-NEU* tumor (ii and iv). IgG control (i and iii); Scale bar = 50μm (**B**). *MYB* levels were determined in four tumors (T1-4) harvested from *MMTV-NEU* mice compared to normal

mammary glands (Mg1-4). **(C)** Kaplan-Meier analysis of tumor-free survival in female *MMTV-NEU;MYB$^{f/f}$* (n=28) and *MMTV-NEU;MYB$^{f/f}$;MMTV-Cre* (n=14) mice. (**D**) Analysis of the three tumors (T1a-3a) that developed in *MMTV-NEU;MYB$^{f/f}$;MMTV-Cre* mice revealed modest *MYB* expression indicating incomplete *MYB* deletion. (****p = 0.0001; Scale bar = 50μm)

Figure 4

MYB KO delays MMTV-PyMT tumorigenesis **(A)** MYB protein detected by IHC (ii) [(i) IgG antibody control] and **(B)** mRNA expression in *MMTV-PyMT;MYB$^{f/f}$* mammary tumors (T1-4) and unaffected mammary glands (Mg1-4). **(C)** Tumor-free survival of mice bearing the *MMTV-PyMT$^{f/f}$* transgene: mice were assigned to 3 groups; Thirteen *MMTV-PyMT;MYB$^{f/f}$* (wt), 14 *MMTV-PyMT;MYB$^{f/+}$;MMTV-Cre* (*heterozygous for MYB*) and 15 *MMTV-PyMT;MYB$^{f/f}$;MMTV-Cre* (*KO)*. (****p = 0.0001; Scale bar = 50μm).

Figure 5

MYB is required for their timely mammary gland development (**A**) RT-qRT-PCR analysis of *MYB* and *A-MYB* mRNA expression was assessed at different stages of mammary gland development. Data are presented as the mean ± SEM for 3 mice at each time point. (**B-D**) Representative images of glands harvested from mice at different developmental stages. MYB IHC in virgin mammary glands at 6, 13 and 20 weeks of age, in the mammary glands of pregnant mice on day 1, 10 and 15 of pregnancy as well as on days 1 and 7 of lactation and finally, during gland involution 14 days post-lactation. Nuclear MYB was found in the rudimentary ducts of virgin glands harvested at 6 weeks of age and rarely in glands remodeling

during involution (red arrow). Intense staining was mainly localised to the luminal epithelium of the ducts, indicating that the spatial expression of MYB is associated with the luminal epithelial cells (*also see* ***Supplementary Figure 5***). (**E**) Whole mounts of adult glands from wt (*MYB*$^{f/f}$*)* and *MYB*KO (*MYB*$^{f/f}$*;MMTV-Cre)* mice harvested at different developmental stages. (**F**) Ductal branch morphology quantified using Metamorph^{R} as well as the enumeration of terminal end buds (TEB). The number of ducts was quantified as a percentage of the area occupied by all ductal branches. (**G**) The number of TEB was measured in both wt and KO glands. (*p = 0.05; Scale bars = 50μm)

Figure 6

MYB-transduced NMuMG cells show enhanced survival and growth (**A**) NMuMG cells transduced with either control or full length *MYB* retroviruses were seeded into six well plates (15,000 cells/well) containing 0.35% agarose and were allowed to grow for 21 days before colonies were photographed and (**B**) counted; n=3; mean +/- SEM. Enhanced anchorage-independent growth was observed in *MYB*-transduced NMuMG cells as well as improved adherent growth when plated under serum-deprived conditions (0.1% FCS) for 48 hr (**C**). (**D**) Levels of surface AnnexinV expression measured by FACS in serum-deprived cells. (**E&F**) Expression of MYB targets *Grp78/BIP* and *BCL-2* in *MYB*-transduced NMuMG cells from 2D cultures (3 experiments in triplicate; mean +/- SEM; *p = 0.05).

Figure 1 Miao et al

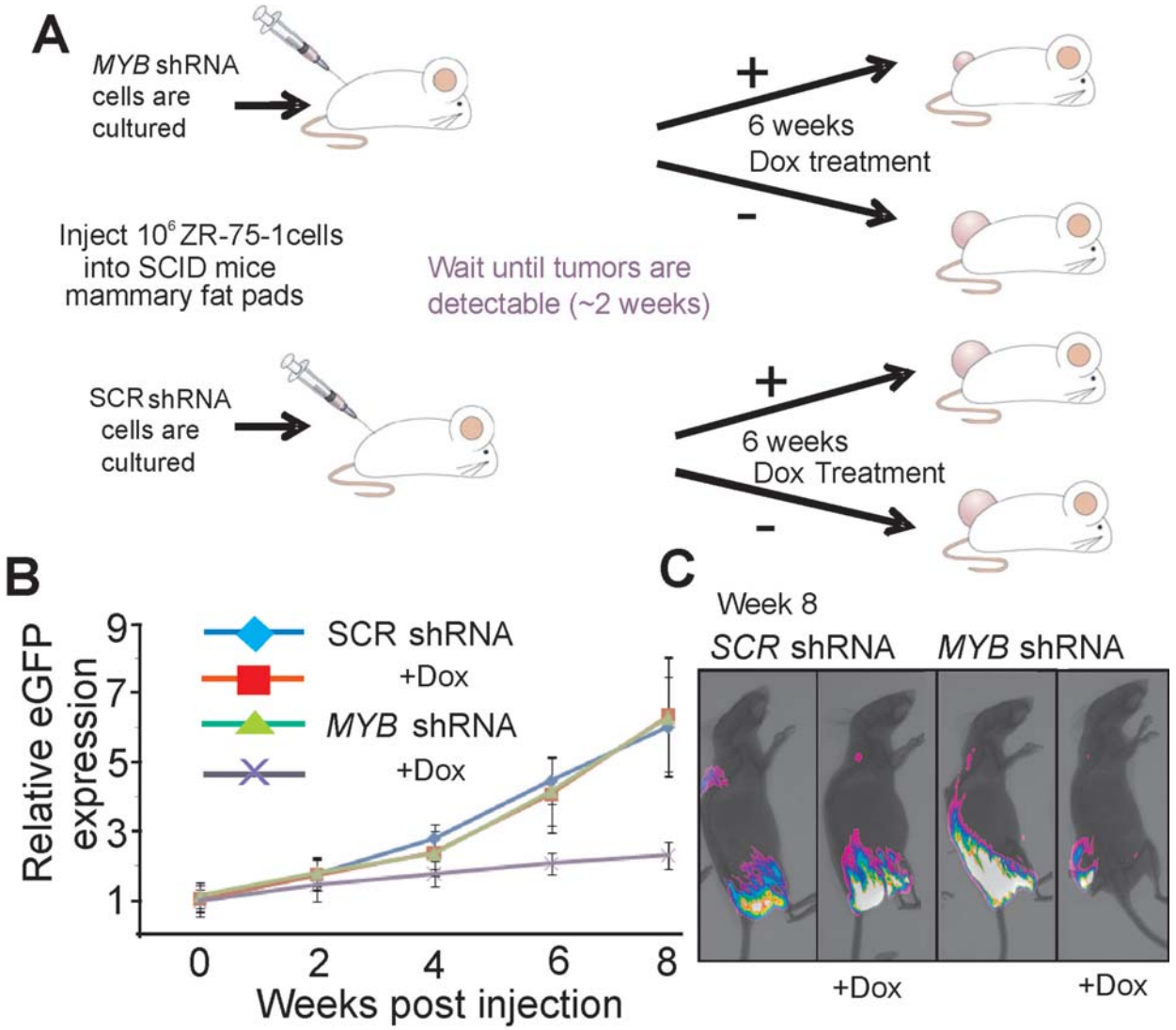


Figure 2 Miao et al

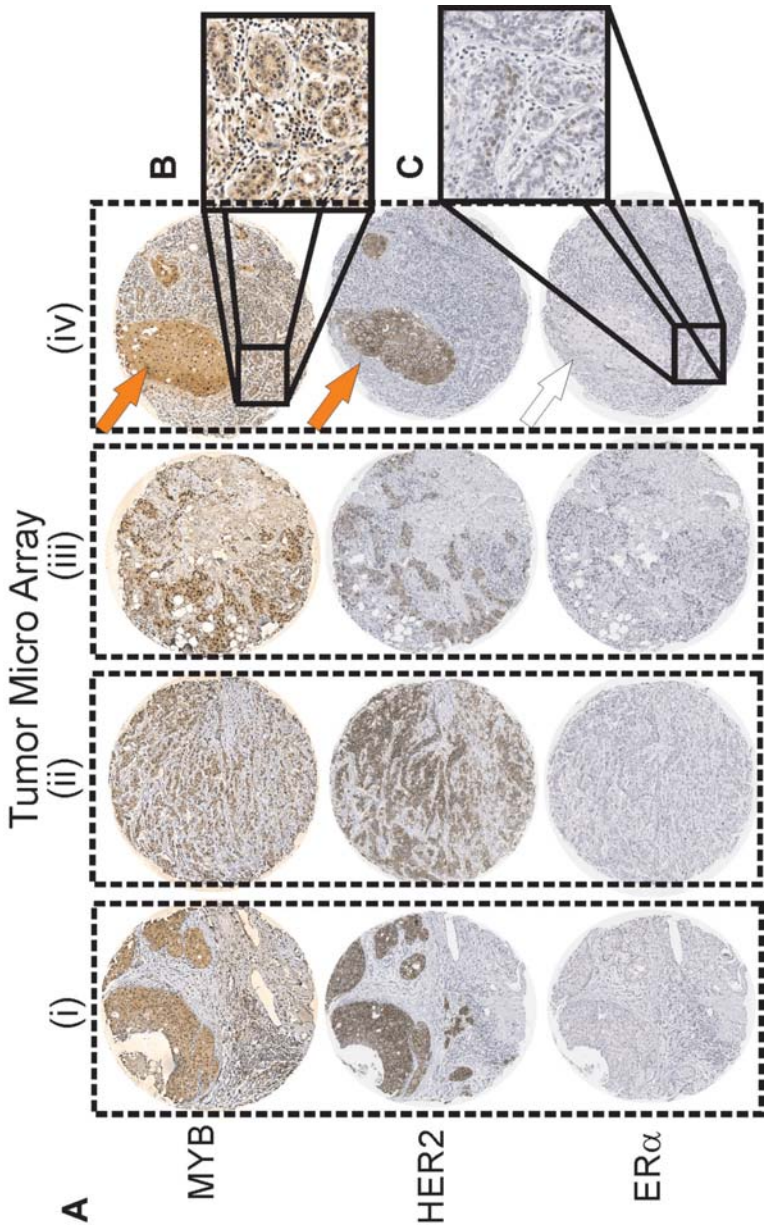


Figure 3 Miao et al

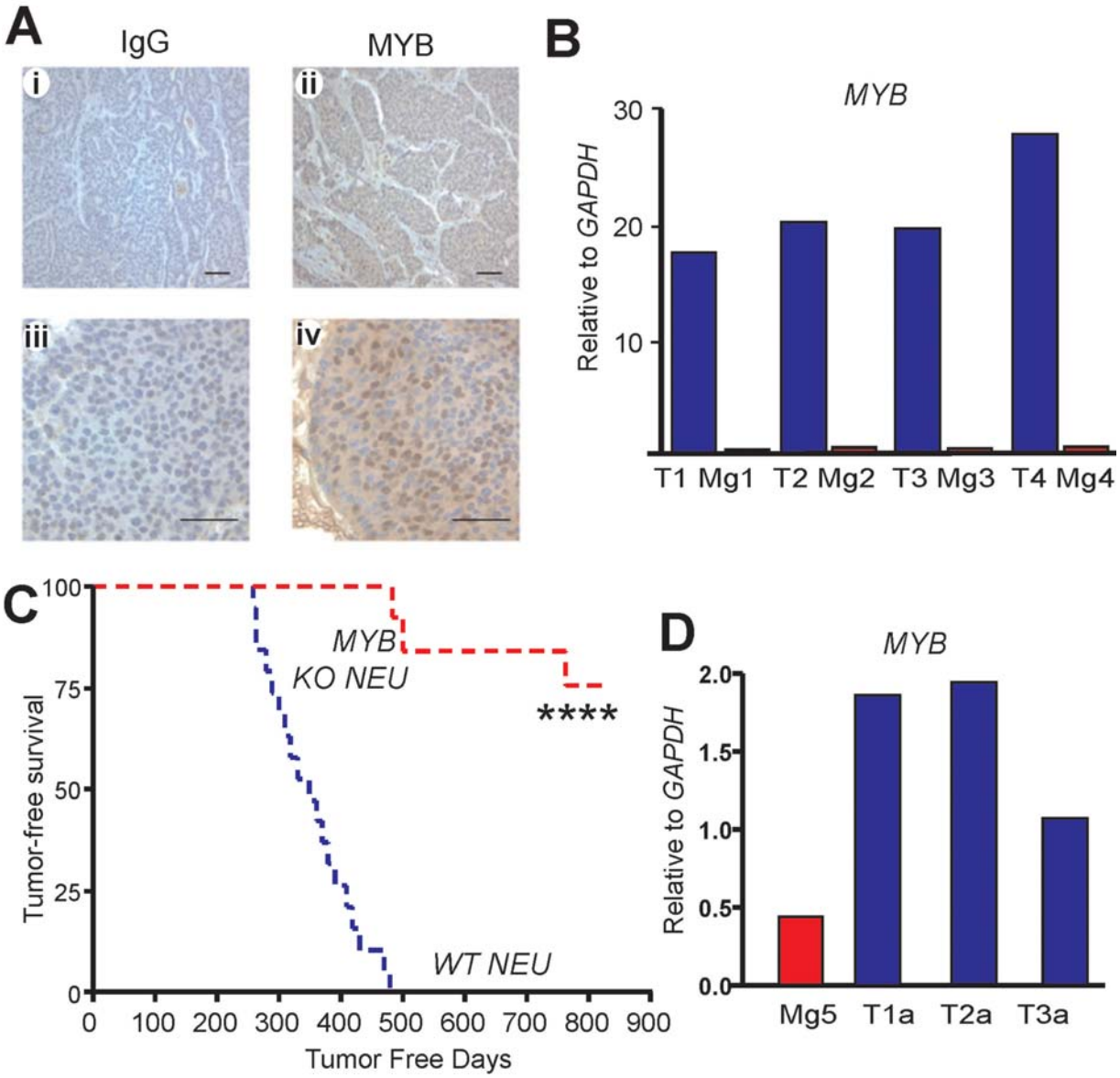


Figure 4 Miao et al

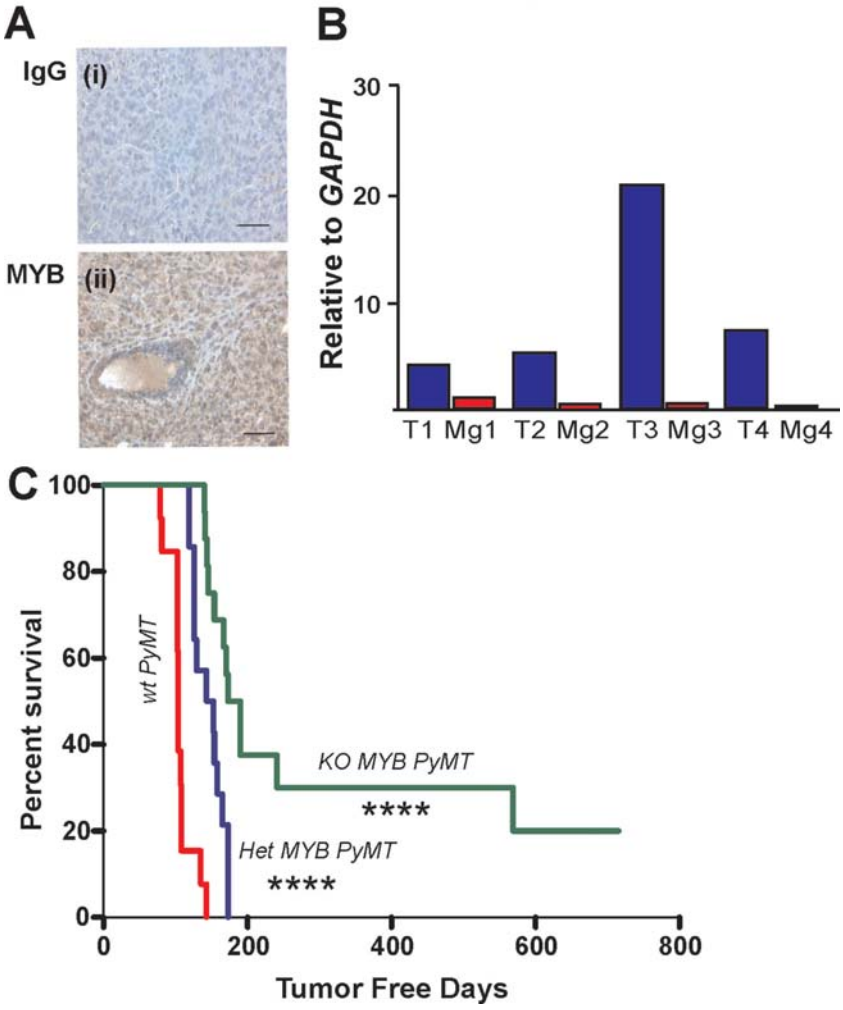
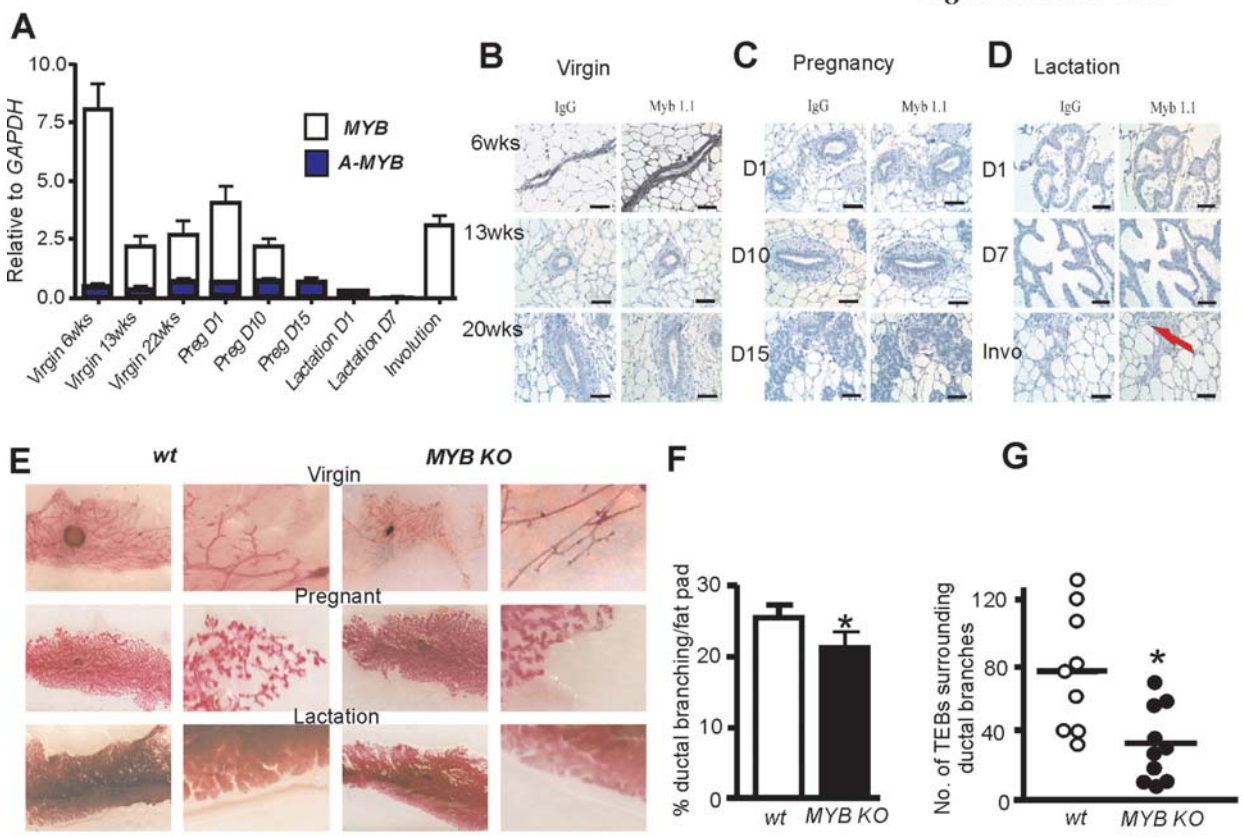


Figure 5 Miao et al



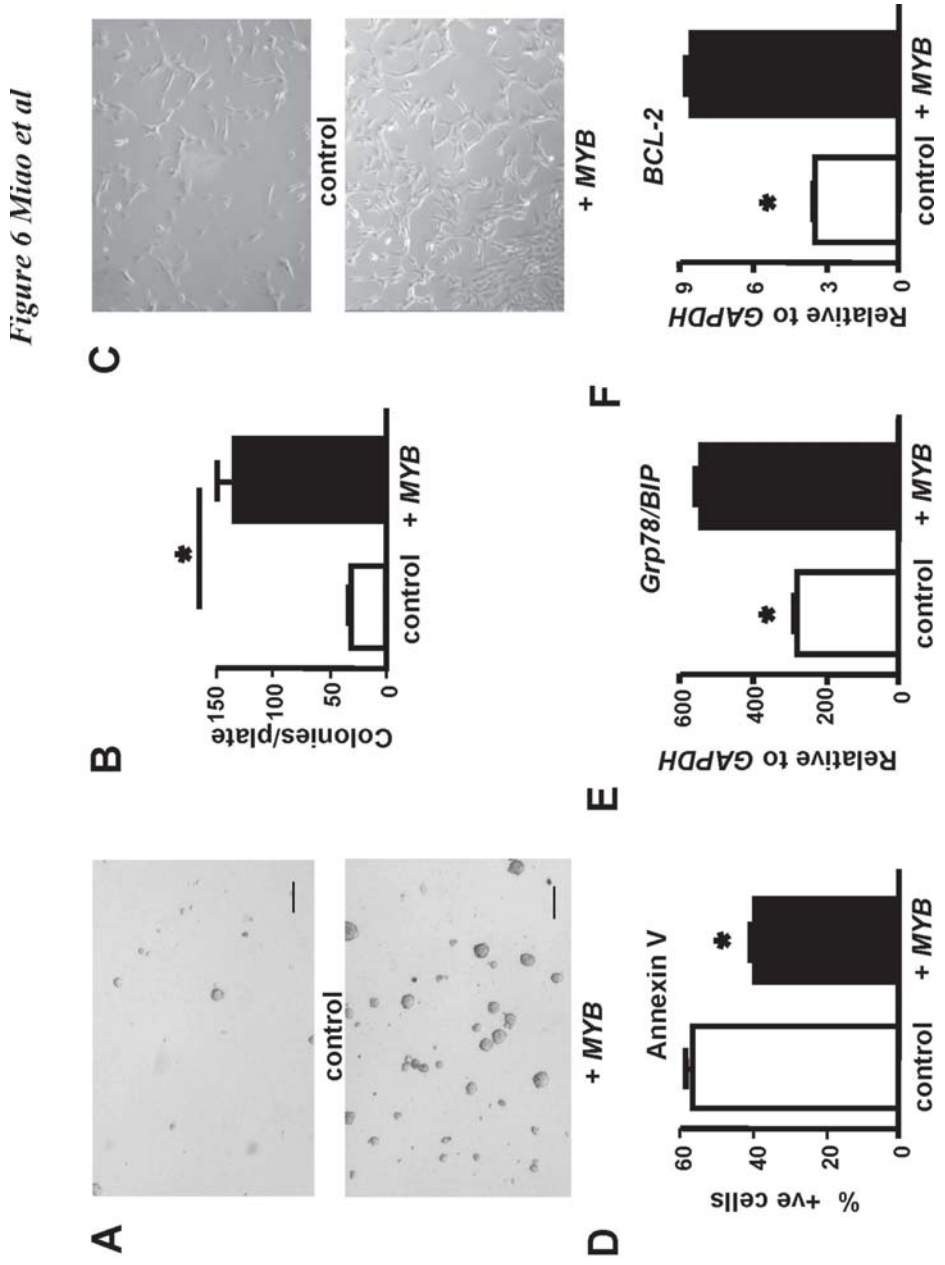


Figure 6 Miao et al

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MYB is essential for mammary tumorigenesis

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