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# Repression of SRF target genes is critical for Myc-dependent apoptosis of epithelial cells

Katrin E Wiese<sup>1,†</sup>, Heidi M Haikala<sup>2,†</sup>, Björn von Eyss<sup>1,†</sup>, Elmar Wolf<sup>1</sup>, Cyril Esnault<sup>3</sup>, Andreas Rosenwald<sup>4,5</sup>, Richard Treisman<sup>3</sup>, Juha Klefström<sup>2</sup> & Martin Eilers<sup>1,5,\*</sup>

## Abstract

Oncogenic levels of Myc expression sensitize cells to multiple apoptotic stimuli, and this protects long-lived organisms from cancer development. How cells discriminate physiological from supraphysiological levels of Myc is largely unknown. Here, we show that induction of apoptosis by Myc in breast epithelial cells requires association of Myc with Miz1. Gene expression and ChIP-Sequencing experiments show that high levels of Myc invade target sites that lack consensus E-boxes in a complex with Miz1 and repress transcription. Myc/Miz1-repressed genes encode proteins involved in cell adhesion and migration and include several integrins. Promoters of repressed genes are enriched for binding sites of the serum-response factor (SRF). Restoring SRF activity antagonizes Myc repression of SRF target genes, attenuates Myc-induced apoptosis, and reverts a Myc-dependent decrease in Akt phosphorylation and activity, a well-characterized suppressor of Myc-induced apoptosis. We propose that high levels of Myc engage Miz1 in repressive DNA binding complexes and suppress an SRF-dependent transcriptional program that supports survival of epithelial cells.

#### Keywords Akt; apoptosis; Miz1; Myc

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### Introduction

Deregulated and enhanced expression of Myc contributes to the genesis of a large fraction, potentially the majority, of all human tumors (Dang, 2012). Paradoxically, high expression levels of Myc not only promote transformation, but also sensitize cells against a broad range of pro-apoptotic stimuli and enhance the cellular

dependence on trophic factors provided by the cell's environment (Askew *et al*, 1991; Evan *et al*, 1992; Murphy *et al*, 2008). Like the induction of senescence by oncogenic *RAS*, sensitization to apoptosis by enhanced expression of Myc is a form of 'intrinsic tumor suppression' that protects long-lived organisms from tumorigenesis (Lowe *et al*, 2004). The ability of cells to respond in this manner has been reported from a multitude of experimental systems, suggesting that it is a central part of Myc biology (Nilsson & Cleveland, 2003).

How cells can discriminate between different levels of Myc is only beginning to emerge. Myc proteins bind broadly to enhancers and promoters of RNA polymerase II genes with an open chromatin structure (Guccione et al, 2006; Zeller et al, 2006; Chen et al, 2008). Comparing genome-wide occupancy profiles at different Myc levels shows that the apparent affinity of Myc for individual target sites varies widely, such that Myc highly occupies some promoters and enhancers in normal proliferating cells, whereas others are occupied to a detectable degree only in cells expressing oncogenic levels of Myc (Lin et al, 2012; Sabo et al, 2014; Walz et al, 2014). GO-term analyses show that differences in occupancy correlate with the function of encoded genes, such that functionally related genes have Myc binding sites with similar apparent affinity (Walz et al, 2014). These findings suggested that oncogenic levels of Myc 'invade' lowaffinity binding sites and that genes with low-affinity promoters encode proteins with functions that are distinct from proteins encoded by genes that are regulated by physiological Myc levels. Consistent with this model is the identification of a group of genes that is regulated specifically by oncogenic Myc levels in a B-cell line, which expresses a tetracycline-inducible allele of Myc and which has been used to model Myc function in Burkitt lymphomas (Yustein et al, 2010).

Myc binds DNA together with a partner protein, Max, and Myc/ Max heterodimers activate transcription (Meyer & Penn, 2008; Dang, 2012). Consistent with the broad binding of Myc to open promoters and enhancers, Myc is required for upregulation of virtually all induced genes during the mitogenic stimulation of primary

<sup>1</sup> Biocenter, Theodor Boveri Institute, University of Würzburg, Würzburg, Germany

<sup>2</sup> Faculty of Medicine, Cancer Cell Circuitry Laboratory, Translational Cancer Biology Research Program and Institute of Biomedicine Biomedicum Helsinki, University of Helsinki, Helsinki, Finland

<sup>3</sup> Cancer Research UK, London Research Institute, Lincoln's Inn Fields Laboratories, Transcription Laboratory, London, UK

<sup>4</sup> Institute of Pathology, University of Würzburg, Würzburg, Germany

<sup>5</sup> Comprehensive Cancer Center Mainfranken, University of Würzburg, Würzburg, Germany

<sup>\*</sup>Corresponding author. Tel: +49 931 318 4111; E-mail: martin.eilers@biozentrum.uni-wuerzburg.de <sup>†</sup>These authors contributed equally to this work

B-cells, demonstrating that Myc can act as a 'general amplifier' of transcription (Lin *et al*, 2012; Nie *et al*, 2012). Surprisingly, Mycdriven tumor cells differ from their normal counterparts not only as they show enhanced expression, but also due to decreased expression of directly bound Myc target genes (Yustein *et al*, 2010). One reason for this is that Myc/Max heterodimers can repress transcription when they associate with Miz1 (Wiese *et al*, 2013; Walz *et al*, 2014). Complex formation with Miz1 is detectable in transformed, but not in primary cells, suggesting that it is a stress response to oncogenic Myc levels (Wolf *et al*, 2013; Walz *et al*, 2014).

Collectively, the available data suggest that transcriptional changes occurring specifically in response to oncogenic Myc levels —either due to invasion of low-affinity binding sites, complex formation with Miz1 or both—enable cells to discriminate physiological from oncogenic levels of Myc and sensitize cells toward apoptosis. Consistent with this model, RNA- and ChIP-Sequencing experiments in U20S cells that express a doxycycline-inducible allele of Myc had shown that genes encoding proteins involved in cell survival and suppression of apoptosis are occupied only at oncogenic levels of Myc and that these genes are repressed by Myc (Walz *et al*, 2014). Here, we tested this hypothesis in mammary epithelial cells, a well-established model of Myc-induced apoptosis (Partanen *et al*, 2007; Zhan *et al*, 2008).

### Results

## Myc promotes apoptosis of mammary epithelial cells in a Miz1-dependent manner

To test which aspects of Myc function require the interaction with Miz1 in epithelial cells, we used lentiviruses to stably express wild-type Myc (termed WT in the figures) and MycV394D (MycVD), a point mutant that is unable to bind to Miz1 and repress transcription via Miz1 (van Riggelen *et al*, 2010). We used lentiviruses carrying two different promoters: the phosphoglycerate kinase (PGK) promoter to express moderate levels of Myc and the spleen focus-forming virus (SFFV) promoter to express high levels of Myc in

MCF10A, a non-tumorigenic mammary epithelial cell line (Fig 1A). MCF10A cells carry a deletion of the *CDKN2A/B* locus that encodes Arf and have three copies of *MYC*; hence, endogenous Myc levels are slightly higher than in primary cells (Worsham *et al*, 2006). Comparison to endogenous levels showed that overall levels of Myc in cells expressing ectopic Myc from the PGK promoter were about twofold elevated relative to parental MCF10A cells (Fig 1A; Supplementary Fig S1A). Levels of Myc driven from the SFFV promoter were higher and similar to those found in HeLa and HCT116 colon carcinoma cells, which are known to express high levels of endogenous Myc (Fig 1A and shown for Myc-ER in Supplementary Fig S1A).

At moderate expression levels (PGK), neither Myc nor MycVD significantly affected the growth of MCF10A cells (Supplementary Fig S1B). However, both Myc and MycVD accelerated cell cycle re-entry of serum-starved cells upon re-stimulation (Supplementary Fig S1C) and enhanced the sphere-forming (clonogenic) potential of these cells in non-adherent conditions (Fig 1B). Ectopic expression of Myc is known to sensitize cells to a range of pro-apoptotic stimuli including glutamine starvation and DNA damage (Rupnow *et al*, 1998; Yuneva *et al*, 2007). Myc and MycVD differed in their pro-apoptotic properties, since more cells expressing Myc than cells expressing MycVD underwent apoptosis, both in unstressed conditions and upon glutamine starvation or exposure to doxorubicin (Fig 1C).

Consistent with the effect of moderate Myc expression on sphere formation, high SFFV-driven expression levels of both wild-type Myc and MycVD induced a shift in a large percentage of the cell population to a CD44<sup>+</sup>/CD24<sup>low</sup> surface phenotype that is characteristic of mammary stem cells in MCF10A and in HMLE cells (Liu *et al*, 2009) (Fig 1D). We noted in these experiments that expression of Myc decreased progressively during culture of both infected MCF10A and HMLE cells, whereas expression of MycVD was better tolerated during prolonged culture, arguing that cells that express high levels of Myc, but not cells expressing MycVD, are rapidly counterselected (Fig 1E; Supplementary Fig S1D). In accord with this interpretation, elevated levels of Myc, but not of MycVD, suppressed colony formation of MCF10A cells

#### Figure 1. Induction of apoptosis by Myc requires association with Miz1.

- A Immunoblot analysis documenting different Myc expression levels. The left panel shows levels of Myc in pools of cells infected with a virus that carries a PGK promoter (moderate levels); the right panel shows expression levels when Myc is expressed under the control of the SFFV promoter (high levels). Vinculin was used as a loading control (*n* = 3; unless otherwise indicated, *n* indicates the number of biological repeat experiments in the following legends).
- B Myc and MycVD promote mammosphere formation. The indicated pools of MCF10A cells were grown under non-adherent conditions. The graph shows the number of secondary mammospheres formed per 1,000 plated cells. *P*-values were calculated using a Student's *t*-test (n = 3, ns: P > 0.05; \*\*\* $P \le 0.01$ ; \*\*\*\* $P \le 0.0001$ ).
- C Myc sensitizes MCF10A cells to apoptosis in a Miz1-dependent manner. The indicated pools of MCF10A cells expressing moderate Myc levels were grown under control or glutamine-deprived (–Gln) conditions for 24 h. Where indicated, doxorubicin was added (0.1  $\mu$ g/ml; 24 h). The graph shows the percentage of early apoptotic (annexinV<sup>+</sup>/Pl<sup>-</sup>) and late apoptotic (annexinV<sup>+</sup>/Pl<sup>+</sup>; PI: propidium iodide) cells. Error bars show standard deviation (n = 3). *P*-values were calculated using a Student's *t*-test (\* $P \le 0.05$ ; \*\*\* $P \le 0.001$ ).
- D High levels of Myc and MycVD induce a CD44<sup>high</sup>/CD24<sup>low</sup> surface phenotype in mammary epithelial cells. Pools of infected cells were analyzed by FACS. The upper panels show a representative experiment in HMLE cells. The lower panels show the quantification of CD44<sup>high</sup>/CD24<sup>low</sup> cells in HMLE and MCF10A cells. Error bars show standard deviation (n = 3). *P*-values were calculated using a Student's t-test (\*\* $P \le 0.001$ ; \*\*\*\* $P \le 0.0001$ ).
- E High expression levels of Myc WT are counterselected more rapidly than MycVD in MCF10A cells. Upper panel: Cells were infected with the indicated vectors and harvested for immunoblots at the indicated time points. Vinculin was used as a loading control. A representative result is shown. Lower panel: Quantification of relative MYC levels from three independent biological experiments. *P*-values were calculated using a Student's *t*-test.
- F Colony assays documenting growth of pools of MCF10A cells infected with the indicated SFFV promoter-based expression viruses. A representative result of crystal violet stainings is shown (*n* = 3).
- G Photographs documenting growth of pools of MCF10A cells infected with the indicated SFFV promoter-based expression viruses in three-dimensional culture. Pictures were taken at day 4 of acinar culture. Scale bar, 50 μm.



and induced overt apoptosis in two-dimensional culture (Fig 1F; Supplementary Fig S1E). Culturing MCF10A cells in matrigel also resulted in rapid death of cells expressing high levels of Myc, but not MycVD, arguing that death is not due to the twodimensional culture conditions (Fig 1G). We concluded that both sensitization to apoptosis by moderate levels of Myc and overt apoptosis induced by high levels of Myc require association with Miz1.

#### Characterization of Myc/Miz1-mediated apoptosis

To characterize the underlying molecular mechanisms and identify and compare genes directly regulated by Myc and MycVD, we generated MCF10A cells that express elevated levels of the steroid activatable Myc-ER (Myc-ER) and MycVD-ER chimeric proteins under the control of the SFFV promoter (Eilers et al, 1989; Littlewood et al, 1995). MCF10A cells do not express endogenous ERα (Lane *et al*, 1999). Immunoblots confirmed that the amounts of both proteins were identical in both cell pools (Fig 2A). Incubation with increasing amounts of 4-OHT resulted in a concentrationdependent increase in nuclear Myc-ER and MycVD-ER (Supplementary Fig S2A). This was paralleled by a concentration-dependent increase in the percentage of both early (annexinV-positive) and late apoptotic (annexinV/propidium iodide-double positive) cells that express Myc-ER (Fig 2B). While the relative proportion of early and late apoptotic cells varied between different experiments, addition of 4-OHT at any concentration elicited only a small increase in the percentage of either early or late apoptosis in cells expressing MycVD-ER (Fig 2B). Consistently, 4-OHT-mediated activation of Myc-ER, but not MycVD-ER, induced a robust accumulation of p53 and cleavage of PARP at high Myc levels (Fig 2C). Activation of Myc-ER, but not MycVD-ER, also induced apoptosis in HMLE mammary epithelial cells (Supplementary Fig S2B and C).

We used a number of criteria to validate that Miz1-dependent induction of apoptosis shows characteristic features of Myc-induced apoptosis. Depletion of Max strongly attenuated Myc-induced apoptosis, validating that apoptosis was due to Myc-induced changes in transcription (Supplementary Fig S3A) (Amati *et al*, 1993). Analysis of two transcription-deficient mutants of Myc, Myc∆BR that lacks the basic region, and MycD (MycT358D/S373D/T400D), which is strongly compromised in its ability to bind to Max (Huang *et al*, 2004), confirmed that binding to DNA and to Max is required for apoptosis (Supplementary Fig S3B). Consistent with previous observations (Hermeking & Eick, 1994; Nieminen *et al*, 2013), shRNA-mediated depletion of p53 (*TP53*) abolished Myc-induced apoptosis (Supplementary Fig S3C). Previous work had established that Myc activates expression of Bim (*BCL2L11*) and that Bim is a critical mediator of Myc-induced apoptosis (Egle *et al*, 2004; Hemann *et al*, 2005; Muthalagu *et al*, 2014). Consistent with these data, depletion of Bim or ectopic expression of Bcl-2, which buffers Bim, attenuated Myc-induced apoptosis (Supplementary Fig S3D and E). Both Myc and MycVD induced Bim to a similar extent, suggesting that elevated Bim levels are tolerated in cells expressing MycVD (Supplementary Fig S3F).

#### Miz1-dependent transcriptional repression by Myc

Neither induction of Myc nor of MycVD in proliferating MCF10A cells led to significant alterations in cellular RNA content (Supplementary Fig S4A). We therefore performed microarray experiments from triplicate biological samples to identify Myc-dependent changes in gene expression. These experiments identified 993 genes that were significantly induced and 804 genes that were repressed in response to activation of Myc-ER and MycVD-ER (P < 0.05). The ability of MycVD-ER to activate these target genes was very similar to that of wild-type Myc-ER (r = 0.785 for all activated genes) (Fig 2D). In contrast, the ability of MycVD-ER to repress transcription was significantly compromised (r = 0.397;  $P < 2.2 \times 10^{-16}$  for the difference between activation and repression; ANCOVA) (Fig 2D). qRT-PCR analysis of individual genes confirmed the results of this analysis (Supplementary Fig S4B). Gene set enrichment analysis (GSEA) showed that genes activated by Myc and MycVD in MCF10A cells were similar to previously identified target genes of Myc (Supplementary Fig S4C). Importantly, GSEA confirmed that MycVD was compromised in its ability to repress known Myc targets and multiple other gene sets (Supplementary Fig S4C and D). GO-term analysis of genes that were differentially repressed by Myc and MycVD showed an enrichment of genes involved in apoptosis as well as in cell migration, cell adhesion, and angiogenesis (Fig 2E). For example, integrins alpha1 and beta4 as well as laminins A3, B3, and C2 are repressed by Myc, but not by MycVD. This analysis is consistent

#### Figure 2. Identification of genes repressed by Myc via association with Miz1.

- A Immunoblot documenting levels of Myc and Myc-ER in pools of infected cells. MCF10A cells were infected with lentiviruses expressing Myc-ER or MycVD-ER under control of the SFFV promoter. Empty vector is termed 'ctr'. Vinculin was used as a loading control (*n* = 3).
- B Induction of apoptosis by Myc-ER is dose and Miz1 dependent. FACS assays documenting the percentage of early (annexinV<sup>+</sup>/PI<sup>-</sup>) and late (annexinV<sup>+</sup>/PI<sup>+</sup>) apoptotic cells after incubation of MCF10A cells with the indicated concentrations of 4-OHT for 48 h. Error bars show standard deviation (n = 3). The symbols for *P*-values refer to the difference between Myc-ER and MycVD-ER and are calculated for the sum of apoptotic cells with a Student's *t*-test (\* $P \le 0.05$ ; \*\* $P \le 0.001$ ; \*\*\*\* $P \le 0.001$ ).
- C High levels of Myc, but not of MycVD, induce p53 and cleavage of PARP. The panels show immunoblots of MCF10A-Myc-ER and MycVD-ER whole-cell lysates after culture in the presence of 5 nM or 100 nM 4-OHT for 24 h. Cdk2 is used as a loading control.
- D Summary of microarray analyses of gene expression in MCF10A-Myc-ER and MycVD-ER cells upon incubation with 100 nM 4-OHT for 24 h. The *y*-axis shows change in gene expression in response to MycVD-ER (4-OHT versus control); the *x*-axis shows changes induced by activation of Myc-ER for significantly regulated probe IDs (*P* < 0.05). Arrays were performed from triplicate biological samples (*r* indicates Pearson's correlation coefficient for activated or repressed genes, respectively).
- E GO-term analysis of genes that are repressed by Myc-ER (log<sub>2</sub>FC  $\leq$  -0.5), but less by MycVD-ER (log<sub>2</sub>FC  $\geq$  0.5 for the difference between Myc-ER and MycVD-ER). The y-axis indicates significance as  $-\log_{10} q$ -value (Benjamini); only significant GO-terms are shown.
- F qRT-PCR assays documenting repression of the indicated genes in response to incubation of MCF10A-Myc-ER and MycVD-ER cells with 5 nM or 100 nM 4-OHT for 24 h. The *y*-axis shows fold repression relative to control cells. Bars represent mean plus SEM of at least 5 independent biological experiments. *P*-values were calculated using a Student's *t*-test (ns: P > 0.05; \* $P \le 0.05$ ; \*\* $P \le 0.01$ ).



Figure 2.

with previous observations showing that multiple integrin genes are targets for Myc-dependent repression (Wilson *et al*, 2004; Gebhardt *et al*, 2006; Liu *et al*, 2012). The microarray analysis had been performed on samples exposed to 100 nM 4-OHT, which reflects high levels of Myc. qRT-PCR analyses of individual genes that are targets for Miz1-dependent repression showed that elevated concentrations of 4-OHT were required for Miz1dependent repression since there was no significant repression at 5 nM 4-OHT (Fig 2F).

Depletion of Miz1 was highly toxic in MCF10A cells, precluding an independent confirmation that repression of cell adhesion genes requires Miz1 (K.E. Wiese, unpublished data). To address this issue, we made use of the finding that U2OS cells tolerate depletion of Miz1. GO-term analysis and analysis of individual genes showed that genes repressed by wild-type Myc, but not MycVD, were highly similar in both cell lines (compare Fig 2E with Supplementary Fig S5A). We have shown previously that depletion of Miz1 in U20S cells de-represses a large fraction of Myc-repressed genes and that for each gene, the degree of de-repression upon depletion of Miz1 parallels de-repression in cells expressing MycVD instead of wild-type Myc (Walz *et al*, 2014). This observation is recapitulated in Supplementary Fig S5B (left panel; each dot reflects expression upon depletion of Miz1 in U20S cells also paralleled the de-repression observed when MycVD-ER instead of Myc-ER is expressed in MCF10A cells for all genes that are repressed by Myc (Supplementary Fig S5B, right panel). The observation argues that Miz1-dependent repression by the VD mutant is due to its inability to form a complex with Miz1.



#### Figure 3. Miz1-dependent repression in human breast tumors.

A Left: Immunohistochemistry documenting levels of Miz1 and Myc in low-grade (400× magnification) and high-grade (triple negative, 200× magnification) breast cancer. Dashed lines in the right panels separate tumor from normal tissue. Representative examples are shown. Right: Table summarizing the results of all analyzed tumor cases.

B Heatmap of gene expression in mammary tumors. The graph shows association scores documenting similarity of gene expression in a given tumor to the indicated predefined gene sets: red color shows high expression (high association score); blue color indicates low expression of genes contained in a given gene set (low association score). Tumor grade and p53 mutation status are indicated on the sides of the heatmap. The *P*-value refers to the correlation of low levels of Myc/Miz1 repressed genes with p53 mutation status and was calculated with the use of a Fisher's exact test.

## Myc-dependent gene repression is detectable in human mammary tumors

Previous work has established that human breast tumors can be stratified according to the similarity between their gene expression pattern and genes regulated by Myc in tissue culture (Horiuchi et al, 2012). To test whether Miz1-dependent repression by Myc can be detected in mammary tumors, we initially showed that Miz1 is expressed both in triple negative breast tumors that express high levels of Myc and in low-grade tumors that express lower Myc levels (Fig 3A). We then analyzed publically available data sets of gene expression in human breast cancer, taking a set of target genes that are activated by Myc as an initial measure for Myc activity (Schuhmacher et al, 2001; Miller et al, 2005). Consistent with Myc protein expression, the fraction of tumors expressing high levels of Myc-activated target genes increased with increasing grade (Fig 3B). Notably, analyzing expression of a set of 50 genes that are repressed by wild-type Myc, but not by MycVD, showed the inverse relationship, with the fraction of tumors that express high levels of Myc/Miz1-repressed genes decreasing with increasing grade (Fig 3B). Over all tumors and particularly among the grade 2 tumors, tumors expressing high levels of Myc-activated genes expressed low levels of Myc/Miz1-repressed genes and vice versa (Fig 3B). Consistent with the tissue culture data, low-level expression of Myc/Miz1-repressed genes was significantly associated with mutation of p53 (Fig 3B, P = 0.0066). Taken together, the data argue that Myc/Miz1-mediated repression occurs in human mammary tumorigenesis.

#### Chromatin binding of Myc/Miz1 complexes

In order to test which effects on gene expression are direct, we performed ChIP-Sequencing experiments using antibodies directed against Miz1 and the ER-moiety of the Myc-ER fusion protein. We analyzed chromatin isolated from Myc-ER, MycVD-ER, and control cells grown in the presence and absence of 4-OHT (see Supplementary Table S1 for all statistics, Fig 4A for example tracks and Supplementary Fig S6A for control immunoblots). Consistent with multiple published data on the association of Myc with chromatin, active Myc-ER proteins bound to thousands of promoters of RNA polymerase II-transcribed genes (Fig 4B). In parallel, we performed ChIP-Sequencing for endogenous Miz1 under all experimental conditions (Fig 4A–D). We have previously shown that Miz1 binds to limited number of core promoters (129) in primary neuronal progenitor cells, which carry a direct binding site for Miz1 in their promoter

(Wolf *et al*, 2013). Miz1 occupied almost all of these sites in MCF10A cells (Supplementary Fig S6B). Consistent with results obtained in other cell types, genes with a consensus Miz1 binding sites were not regulated by Myc (Walz *et al*, 2014).

Similar to observations made in human and mouse tumor cell lines, Miz1 bound a large number of sites that included between 1,288 and 3,718 core promoters (numbers given for a false discovery rate FDR  $\leq$  0.1) of RNA polymerase II-transcribed genes (Fig 4B; Supplementary Fig S6B and Supplementary Table S1) (Walz et al, 2014). Chromatin binding of Miz1 to core promoters was not identical between the different experimental conditions, since activation of Myc-ER globally enhanced binding of Miz1 to core promoters (Fig 4B and C). In cells expressing active Myc-ER, Miz1 bound to 3,718 core promoters and the binding site overlapped with a Myc binding site in 2,282 promoters ( $P < 2.2 \times 10^{-308}$ ). The overall binding pattern of MycVD-ER was very similar to that of Myc-ER; however, promoter occupancy appeared lower, leading to a decrease in the number of promoters for which significant binding could be detected (Fig 4B and D; Supplementary Table S1). In contrast to wild-type Myc-ER, activation of MycVD-ER did not increase binding of Miz1 to core promoters, arguing that wild-type Myc-ER and Miz1 bind co-operatively to core promoters (compare Fig 4C with D).

Sites that are bound by both Myc and MycVD were enriched for the presence of consensus E-boxes (Fig 4E, upper panel). In contrast, no significant enrichment of either Myc or Miz1 consensus binding sequences was observed at binding sites that were bound selectively by Myc-ER together with Miz1, but not by MycVD-ER (Fig 4E, lower panel). The overall occupancy of Myc on activated promoters was higher than on repressed target genes, whereas the occupancy of both classes of promoters by Miz1 was similar (Fig 4F). As a consequence, the ratio of Myc/Miz1 bound to Mycrepressed promoters is lower than on Myc-activated promoters. This is consistent with our previous observation that the ratio of Myc/ Miz1 bound to a promoter correlates closely with the direction of the transcriptional response to Myc (Walz et al, 2014). Taken together, the data suggest that a fraction of active Myc-ER proteins, but not of MycVD-ER proteins, invades core promoters in a complex with Miz1, leading to transcriptional repression.

## Myc/Miz1-repressed genes are enriched for genes regulated by serum-response factor (SRF)

To better understand how Miz1-dependent transcriptional repression sensitizes cells to apoptosis, we performed a second GSEA

#### Figure 4. Identification of direct target genes of Myc and Miz1.

- A Example for ChIP-Sequencing tracks at the *MRPS23* locus. ChIP-Seq was performed with the indicated antibodies from control MCF10A cells and from cells expressing either Myc-ER or MycVD-ER in the presence of 100 nM 4-OHT.
- B Heatmap documenting binding of Myc-ER and endogenous Miz1 proteins to core promoters transcribed by RNA polymerase II at all human genes (UCSC RefSeq list) in a window of  $\pm$ 5 kb surrounding the transcription start site (TSS). Genes are ranked according to Myc occupancy.
- C Distribution of Miz1 (top panel) and Myc-ER tags (bottom panel) in control cells (vector) and cells expressing Myc-ER in the presence or absence of 100 nM 4-OHT. Binding is shown for a window of  $\pm 5$  kb around Miz1 peaks (found  $\pm 1.5$  kb from a TSS) as reference coordinates.
- D Distribution of Miz1 (top panel) and MycVD-ER tags (bottom panel) in control and MycVD-ER manipulated conditions. Binding is shown as in (C).
- E Enrichment of consensus (CACGTG) and non-consensus (CANNTG) E-box sequences in binding sites that are bound by both Myc-ER and MycVD-ER (top panel) or in genes that are specifically bound only by Myc-ER jointly with Miz1 (bottom panel). The plots show enrichment over a defined background data set (Myc peaks in 4-OHT situation shifted by 500 bp).

F Occupancy of Myc-ER (left panel) and Miz1 (right panel) at Myc-activated and Myc-repressed genes in Myc-ER cells exposed to 100 nM 4-OHT.



Figure 4.

#### Figure 5. Myc/Miz1-repressed genes overlap with target genes of serum-response factor (SRF).

- A Promoters of Myc-repressed genes contain SRF motifs. Example plots for two SRF gene sets found in the GSEA analysis of motif gene sets (C3, see Supplementary Fig S7A for a list). The analysis was performed comparing cells expressing Myc-ER versus MycVD-ER. For one of these sets, the genes regulated in response to induction of Myc-ER are included as red dots in Supplementary Fig S7B. ES = enrichment score; NES = normalized enrichment score; FDR = false discovery rate.
- B Plots documenting repression of murine Srf and Mrtf-A targets by Myc-ER versus MycVD-ER. Direct target genes of Srf and Mrtf-A in MEFs were used to generate custom gene sets and subjected to GSEA analysis.
- C Venn diagrams documenting overlap of Srf- (left panel) or Srf/Mrtf-A (right panel)-bound genes with Myc-ER-regulated genes. *P*-values show significance of overlap between target genes (based on hypergeometric distribution).
- D GO-term analysis demonstrating functional categories of direct Srf/Mrtf-A target genes (left panel) or target genes overlapping with Myc-repressed genes (right panel).
- E Venn diagram showing overlap of Srf-bound genes with genes bound by Myc-ER and Miz1 in cells expressing active Myc-ER. *P*-value shows significance of overlap between target genes (based on hypergeometric distribution).
- F Myc/Miz1 and SRF bind independently from each other. The distance between SRF (MCF7 cells, GSM1010839) and Myc binding sites (peak middle) at repressed target genes is plotted as the density estimate of a histogram. The Myc/Miz complex is shown for comparison.

analysis searching for transcription factor binding sites that are enriched in promoters of Myc-repressed genes (Supplementary Fig S7A). Promoters of genes that are repressed by wild-type Myc-ER, but not by MycVD-ER, were enriched in binding sites for the serumresponse factor (SRF), the structurally related myocyte enhancer factor 2 (Mef2) and c/EBP $\delta$  (Fig 5A; Supplementary Fig S7A). Analysis of a set of genes that have SRF sites in their promoter confirmed that the majority of genes in this set are repressed by Myc-ER and less repressed by MycVD-ER (Supplementary Fig S7B). The identification of c/EBP $\delta$  as a regulator of Myc/Miz1-repressed genes is consistent with the observation that Miz1 represses the *CEPD* gene itself (Do-Umehara *et al*, 2013).

Analysis of an experimentally identified set of direct target genes of murine Srf (Esnault et al, 2014) confirmed that Srf target genes are significantly repressed by Myc in a Miz1-dependent manner (Fig 5B). SRF binds to two functional classes of genes, one of which encodes proteins involved in chromatin function. The other class encodes genes involved in cell adhesion, migration and apoptosis; SRF uses an actin-regulated co-activator, MRTF-A, to activate this class of genes (Posern & Treisman, 2006; Esnault et al, 2014). Mycrepressed genes are enriched in the latter class of SRF target genes (Fig 5B-D). Consistent with the mRNA expression data, the set of promoters bound by both Myc and Miz1 overlapped significantly with SRF-bound promoters (Fig 5E;  $P = 1.55^{-7}$ ). On promoters bound by SRF and by Myc/Miz1, individual binding sites usually do not overlap (Fig 5F; Supplementary Fig S7C for examples) arguing that SRF and Myc/Miz1 bind independently of each other. Consistent with this notion, extensive co-immunoprecipitation revealed no evidence that Myc or Miz1 interact with SRF. gRT-PCR analysis of three SRF target genes showed that all three were repressed upon activation of Myc-ER (Supplementary Fig S7D). Importantly, repression by Myc was also observed in the presence of constitutively active RhoA, a major upstream activator of SRF, demonstrating that Myc does not regulate SRF indirectly via effects on Rho activity (Posern & Treisman, 2006) (Supplementary Fig S7D). We concluded that Myc represses multiple SRF target genes in a Miz1-dependent manner.

#### Restoration of SRF activity alleviates Myc-dependent apoptosis

As mentioned above, activation of target genes involved in cell adhesion and migration by SRF depends on an actin-regulated co-activator, MRTF-A.  $\Delta$ N-MAL, an amino-terminally truncated

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allele of MRTF-A, lacks a domain that binds actin and regulates nucleocytoplasmic shuttling of MRTF-A and is hence constitutively active (Miralles et al, 2003; Vartiainen et al, 2007). Using lentiviral infection, we expressed  $\Delta$ N-MAL in a doxycycline-inducible manner (Fig 6A). qRT-PCR assays showed that  $\Delta$ N-MAL activated SRF target genes and was able to restore normal expression in cells with active Mvc (Fig 6B). To test whether transcriptional repression of SRF target genes contributes to the pro-apoptotic properties of Myc, we used MCF10A cells that express moderate levels of Myc-ER; in the presence of 4-OHT, these cells displayed enhanced sensitivity to deprivation of glutamine and to DNA damage (doxorubicin) like their constitutive counterparts (Fig 6C and D). In addition, they displayed enhanced sensitivity to TRAIL, a hallmark of cells expressing deregulated Myc (Fig 6C and D) (Wang et al, 2004). Using activation of caspase-3 as an indicator of apoptosis, we found that induction of  $\Delta$ N-MAL expression by addition of doxycycline had little effect on apoptosis in the absence of 4-OHT and in unstressed cells, but significantly attenuated cell death in response to deprivation of glutamine, to addition of TRAIL and to DNA damage in the presence of active Myc (Fig 6C and D). Identical results were obtained using caspase-3/7 activity assays (Fig 6E and F; Supplementary Fig S8A). AnnexinV/propidium iodide-staining confirmed that expression of  $\Delta N$ -MAL had little effect on cell viability in control conditions, but reduced induction of apoptosis in the presence of 4-OHT (Fig 6G; Supplementary Fig S8B). Expression of a constitutive active allele of SRF, SRF-VP16, mimicked the effects of  $\Delta N$ -MAL on Myc-dependent apoptosis, arguing that SRF is indeed the critical target of  $\Delta$ N-MAL (Fig 6H).

#### Akt activity is downstream of Myc and SRF

Multiple genes that are repressed by Myc/Miz1 and activated by SRF encode proteins that participate in signal transduction pathways and promote survival of epithelial cells (see Discussion). Integrins, for example, activate focal adhesion kinase (Fak) and integrin-linked kinase (Ilk), enhancing Akt phosphorylation at S473 and Akt activity as a downstream effector (Khwaja *et al*, 1997; Danen & Yamada, 2001; Troussard *et al*, 2003; Hannigan *et al*, 2005; Hehlgans *et al*, 2007). We confirmed that Fak and Ilk promote phosphorylation of Akt at S473 in MCF10A cells (Supplementary Fig S8C). Since Akt limits Myc-induced apoptosis (Kauffmann-Zeh *et al*, 1997), this raises the possibility that Myc might limit growth-factor-dependent survival signals and Akt activity via repression of SRF target genes.



#### Figure 5.

We therefore probed MCF10A cells for Akt activity in the presence of active Myc-ER,  $\Delta$ N-MAL or both (Fig 7A). In support of this model, activation of Myc attenuated phosphorylation of AktS473 and of the

Akt downstream target, Gsk3S9. Induction of  $\Delta$ N-MAL restored both AktS473 and Gsk3S9 phosphorylation in the presence of active Myc. Neither Myc nor  $\Delta$ N-MAL had consistent effects on phosphorylation



Figure 6.

#### Figure 6. Restoring SRF transcriptional activity attenuates Myc-dependent apoptosis.

- A Immunoblots documenting doxycycline-inducible expression of GFP-tagged ΔN-MAL (Dox) in MCF10A-Myc-ER cells. Cells were treated with 200 nM 4-OHT where indicated. Vinculin was used as a loading control. + = 0.025 µg/ml Dox, ++ = 1 µg/ml Dox.
- B qRT-PCR assays documenting expression of SRF targets under the indicated experimental conditions. + = 1  $\mu$ g/ml Dox (to induce  $\Delta$ N-MAL) or 100 nM 4-OHT, respectively (n = 2). Bars represent mean + SD of technical triplicates (n = 2).
- C Immunofluorescence assays using antibodies directed against cleaved caspase-3 in either control cultures or cultures exposed to TRAIL (50 ng/ml, 1.5 h), doxorubicin (0.1 µg/ml, 24 h) or deprived of glutamine (48 h). 100 nM 4-OHT and doxycycline (Dox, 1 µg/ml) were added where indicated. Nuclei were counterstained with Hoechst. Results are shown for a representative experiment.
- D Quantification of (C). Error bars show SD of triplicate biological assays (n = 3). P-values were calculated using a Student's t-test (\* $P \le 0.05$ ; \*\* $P \le 0.01$ ).
- E Caspase-3/7 activity assays documenting attenuation of Myc-dependent apoptosis after glucose or glutamine deprivation in response to expression of  $\Delta$ N-MAL. Error bars show SD of triplicate biological assays (n = 3). *P*-values were calculated using a Student's *t*-test (\*\* $P \le 0.01$ ).
- F Same as (E), except that cells were treated with doxorubicin. P-values were calculated using a Student's t-test (\* $P \le 0.05$ ).
- G FACS assays documenting percentage of early apoptotic (annexinV<sup>+</sup>/PI<sup>-</sup>) and late apoptotic (annexinV<sup>+</sup>/PI<sup>+</sup>) cells under the indicated experimental conditions. Error bars show SD of triplicate biological assays (n = 3). P-values were calculated using a Student's t-test (\* $P \le 0.05$ ).
- H Caspase-3/7 activity assays documenting attenuation of Myc-dependent apoptosis after glutamine deprivation in response to expression of SRF-VP16. Error bars show SD of triplicate biological assays (n = 3). P-values were calculated using a Student's t-test (\*\*\* $P \le 0.001$ ).

of Akt at T308, which is phosphorylated by Pdk1, or on phosphorylation of Mek (Fig 7A). To probe the relevance of this regulation, we used MK-2206, a selective inhibitor of Akt kinases (Hirai *et al*, 2010). Consistent with previous observations (Kauffmann-Zeh *et al*, 1997), chemical inhibition of Akt had no significant effect on apoptosis in the absence of 4-OHT, but enhanced apoptosis in cells grown in the presence of 4-OHT (Fig 7B). Furthermore, Akt inhibition diminished the ability of  $\Delta$ N-MAL to alleviate Myc-induced apoptosis in glutamine-deprived cells (Fig 7B).

Depletion of both Akt1 and Akt2 using a siRNA targeting both isoforms also blunted the ability of SRF to alleviate Myc-induced apoptosis (Fig 7C; Supplementary Fig S8D). Since siRNA-mediated depletion of Akt also reduced cell growth in these assays, the total caspase activity shown here, which is normalized to cell number at the start of the experiment, slightly underestimates the apoptotic activity in wells treated with siAkt1/2. Conversely, expression of constitutively active Akt suppressed Myc-induced apoptosis in glutamine-deprived conditions (Fig 7D). We concluded that repression of SRF activity by high Myc levels limits anti-apoptotic signaling via Akt and that this contributes to sensitizing MCF10A cells toward multiple apoptotic stimuli (see Fig 7E for a model).

## Discussion

Enhanced and oncogenic levels of Myc sensitize cells to stressinduced apoptosis and can even induce frank apoptosis (Nilsson & Cleveland, 2003). A large body of evidence validates Myc-induced apoptosis as a tumor-suppressive mechanism in vivo (Eischen et al, 1999; Pelengaris et al, 2002; Egle et al, 2004; Hemann et al, 2005). Downstream effectors that mediate Myc-induced apoptosis include BIM, which is a direct target gene that is transcriptionally upregulated by Myc, and the Arf-protein that is stabilized by Myc (Zindy et al, 1998; Egle et al, 2004; Chen et al, 2013; Muthalagu et al, 2014). In addition, several previous observations suggest that transcriptional repression via Miz1 contributes to Myc-induced apoptosis in tissue culture and in vivo (Patel & McMahon, 2006, 2007; van Riggelen et al, 2010). In this report, we addressed the question why cells induce apoptosis specifically in response to supraphysiological levels of Myc, using mammary epithelial cells as model. We show that oncogenic levels of Myc repress genes that encode signaling proteins required to maintain the activity of Akt, a kinase that provides a critical survival signal for epithelial cells and for cells expressing elevated Myc levels (Kauffmann-Zeh et al, 1997; Altman et al, 2011).

Previous work has established that both variations in DNA sequence and chromatin structure affect the apparent affinity of Myc for different promoters (Blackwell *et al*, 1993; Guccione *et al*, 2006; Sauve *et al*, 2007; Rodrik-Outmezguine *et al*, 2011; Guo *et al*, 2014). ChIP-sequencing studies in human tumor cells and in mouse models show that oncogenic levels of Myc 'invade' low-affinity binding sites, allowing a discrimination between genes bound and regulated by physiological and by high levels of Myc (Lin *et al*, 2012; Sabo *et al*, 2014; Walz *et al*, 2014). In tumor cells, high levels of Myc and

#### Figure 7. AKTS473 phosphorylation correlates with survival of mammary epithelial cells.

- A Immunoblots documenting levels and phosphorylation status of the indicated proteins in MCF10A Myc-ER cells treated with Dox (1 µg/ml) and/or 4-OHT (200 nM) for 24 h.
- B Effect of Akt inhibition on Myc-dependent sensitization to apoptosis and rescue by  $\Delta$ N-MAL. Data show caspase-3/7 activity in Myc-ER cells treated with 4-OHT and/ or doxycycline in control or glutamine-deprived (–Gln) conditions. Where indicated, cells were incubated with 250 nM Akt inhibitor MK-2206 for 24 h. Error bars show SEM (n = 3). *P*-values were calculated with a Student's t-test (ns: P > 0.05; \* $P \le 0.05$ ).
- C Effect of siRNA-mediated depletion of Akt1/2 on Myc-dependent sensitization to apoptosis and rescue by  $\Delta$ N-MAL. The experiment was performed as in (B), except that cells were transfected with either 100 nM control siRNA or siRNA targeting Akt1/2 for 72 h. qRT-PCR assays showed an average 40% reduction in Akt1 and Akt2 mRNA levels (see Supplementary Fig S8D). Error bars show SD (n = 3); *P*-values were calculated with a Student's t-test (ns: P > 0.05; \* $P \le 0.05$ ).
- D Constitutively active Akt reverts Myc-dependent sensitization to apoptosis. MCF10A Myc-ER cells were infected with either control or retrovirus expressing v-akt. The upper panel shows caspase-3/7 activity in Myc-ER cells treated with 4-OHT in control or glutamine-deprived conditions. Error bars show SEM (n = 3). The lower panel shows an immunoblot documenting expression of v-akt. *P*-value was calculated using a Student's t-test (\*\*\*\*P  $\leq$  0.0001).
- E Model linking SRF/MRTF-A promoted adhesion signaling to phosphorylation of Akt S473 and survival of mammary epithelial cells. High levels of Myc and formation of the Myc/Miz1 complex inhibit SRF/MRTF-A target genes, adhesion and survival signaling, leading to the induction of apoptosis. Constitutive SRF/MRTF-A activity is expected to have little effect on Akt activity in control cells, but restores Akt activity in cells expressing high Myc levels.

Miz1 bound to each promoter correlates with the transcriptional response to changes in Myc levels (Walz *et al*, 2014). Consistent with these previous findings, we showed here that activation of

wild-type Myc-ER, but not of MycVD-ER, enhances binding of Miz1 to core promoters. Only a small proportion of promoters that are specifically bound by Myc, but not by MycVD, had consensus



Figure 7.

E-boxes and all lacked consensus Miz1-binding sites. Since the presence of consensus Myc binding sequences enhances binding affinity *in vitro* (Sauve *et al*, 2007) and correlates with enhanced occupancy *in vivo* (Guo *et al*, 2014), we suggest that these promoters have a low affinity for Myc. Notably, the Arf-protein promotes assembly of Myc/Miz1 complexes, suggesting that Myc/Miz1-dependent repression is itself a stress-regulated pathway (Herkert *et al*, 2010; Inoue *et al*, 2013; Peter *et al*, 2014).

Joint target genes of Myc and Miz1 overlapped significantly with genes that are upregulated by several transcription factors. A significant overlap was observed with genes bound and activated by the serum-response factor, SRF (Posern & Treisman, 2006). The use of an amino-terminally truncated and constitutively active allele of the SRF-co-activator MRTF-A ( $\Delta$ N-MAL) (Vartiainen *et al*, 2007), allowed us to override Myc-dependent repression of SRF target genes. Restoring SRF activity significantly attenuated sensitization to three paradigmatic inducers of Myc-induced apoptosis, exposure to DNA damaging agents, deprivation of glutamine and exposure to the TRAIL death receptor ligand. We obtained no evidence that Myc or Miz1 interacts with SRF directly and the binding sites of both factors on jointly bound promoters do not overlap, arguing that repression is not due to a direct interaction of these factors. Transcriptional activation by SRF requires histone acetylation (Alberts et al, 1998); conversely, repression by Myc correlates with histone de-acetylation at target promoters (Walz et al, 2014). Most likely, therefore, Myc/Miz1 complexes repress SRF target genes by recruiting histone deacetylases to joint target promoters.

Myc-induced apoptosis is inhibited by the Akt protein kinase (Kauffmann-Zeh et al, 1997). Multiple integrins, including those that are repressed by Myc via Miz1 in mammary epithelial cells, are upstream activators of PI3-kinase and Akt (King et al, 1997; Velling et al, 2004; Dennis et al, 2012). In addition, Myc, but not MycVD, represses multiple additional genes directly involved in signal transduction, including Sos2, neuregulin-1 (NRG1; a ligand of Erbb3), Neuropilin-1 (VEGF receptor), TGFbeta receptor II, and the Styk1 kinase that complexes with Akt. Akt activity requires phosphorylation at two sites, threonine 308 (T308) and serine 473 (S473) (Fayard et al, 2010). We found that high levels of Myc reduced phosphorylation of AktS473 and Gsk3S9 and activation of SRF restores phosphorylation of both sites. We therefore propose that one mechanism by which Myc/Miz1-mediated repression sensitizes epithelial cells to apoptosis is via reducing adhesive interactions with the microenvironment that normally provide Akt-dependent pro-survival signals; in this model, Myc-induced apoptosis is mechanistically similar to anoikis (Fig 7E) (Khwaja et al, 1997). Akt protects cells from apoptosis in part since it phosphorylates and thereby inactivates the pro-apoptotic Bad protein (Datta et al, 1997). Myc also induces expression of Bim, a BH3-only pro-apoptotic protein (Muthalagu et al, 2014). Downstream of Myc, both repression of SRF and induction of Bim may therefore alter the balance of pro- to anti-apoptotic proteins at the mitochondrial outer membrane and thereby sensitize cells to induction of apoptosis. In this model, induction of Bim is not sufficient to induce frank apoptosis as long as SRF is active as observed in MCF10A cells. A decrease in Akt is also expected to activate Gsk3 kinase that phosphorylates Myc at T58; phosphorylation at this site promotes Myc turnover and enhances Myc-induced apoptosis (Welcker et al, 2004; Yeh *et al*, 2004; Hemann *et al*, 2005; Wang *et al*, 2011). Repression of SRF target genes may therefore also provide a feedback signal that reduces Myc expression by ubiquitin-mediated proteolysis and that ensures that Myc-dependent transcription is geared toward apoptosis in tumor cells, in which degradation of Myc is compromised.

### Materials and Methods

#### Tissue culture and lentiviral transduction

HMLE and MCF10A cells were maintained in DMEM/F-12 supplemented with 1% penicillin/streptomycin, 10  $\mu$ g/ml insulin, 0.5  $\mu$ g/ml hydrocortisone and 20 ng/ml EGF. For MCF10A cells, 5% horse serum and 100  $\mu$ g/ml cholera toxin were added. HeLa, HCT116 and HEK293TN cells were grown in DMEM with 10% FCS and 1% penicillin/streptomycin.

Three-dimensional culture of MCF10A cells was performed as previously described (Debnath *et al*, 2003). To score mammosphere formation, single-cell suspensions of MCF10A cells were seeded into 24-well ultralow attachment plates at a density of  $2 \times 10^4$  cells/ml (primary culture) or  $5 \times 10^3$  cells/ml for secondary sphere formation, in DMEM/F-12 containing 1% penicillin/streptomycin, 1% methyl-cellulose, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 20 ng/ml EGF, 4 µg/ml heparin and 2% B27.

MCF10A cells transduced with pBabeMYC-ER have been described previously (Nieminen *et al*, 2007). Lentiviral particles were produced in HEK293TN cells with the 2<sup>nd</sup>-generation packaging plasmids psPAX2 and pMD2.G (provided by Didier Trono). To achieve stable lentiviral overexpression of MYC or MYC-ER fusion proteins, cells were transduced with pRRL-SFFV, pRRL-PGK or pLeGO-G/Puro vectors in the presence of 8 µg/ml protamine sulfate and selected with 85 µg/ml hygromycin B or 1 µg/ml puromycin 48 h later. Cells transduced with GFP-tagged  $\Delta$ N-MRTF in pInducer20 were selected with 200 µg/ml neomycin.

Cells were transfected with siRNA AKT1/2 (Cell Signaling Technology, #6211) or control siRNA (Qiagen) using HiPerFect transfection reagent (Qiagen) and incubated for 72 h before analysis.

Pools of cells were either treated with 4-OHT to induce MYC-ER activity (100 nM unless indicated otherwise), doxycycline (1  $\mu$ g/ml) to induce expression of  $\Delta$ N-MAL or ethanol as solvent control.

#### Flow cytometry and caspase-3/7 analysis

To determine the percentage of apoptotic cells, supernatants of respective cultures were harvested and pooled with adherent cells after trypsinization. Cell pellets were resuspended in annexinV-binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM CaCl<sub>2</sub>) and then stained with recombinant annexinV conjugated to Pacific Blue. Propidium iodide was added immediately prior to acquisition on a BD FACSCanto II flow cytometer. Cell surface stainings of HMLE or MCF10A cells were performed with fluoro-chrome-conjugated antibodies against CD44 (APC) and CD24 (FITC) in PBS, 10% FCS. Caspase activity was measured using the Caspase-Glo 3/7 luminescence kit (Promega) with VICTORTMX3 (PerkinElmer).

#### Gene expression and microarray analysis

Total RNA was extracted using peqGOLD TriFast Reagent (PEQLAB) according to the manual. M-MLV Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with random hexamer primers (Roche). qRT-PCRs were performed in technical triplicates using ABsolute SYBR Green Mix (Thermo Scientific) on an Agilent MX3000P system. Data were quantified with the comparative CT method using *RPS14* as reference for normalization. Primers are listed in Supplementary Table S3.

For microarray analysis, total RNA was extracted with QIAGEN RNeasy Kit and on-column DNAse-digested. Labeling and hybridization were performed following Agilent two-color microarray-based gene expression analysis protocol, and slides (Agilent Human Genome Microarray 4 × 44 K v2) were scanned using an Agilent DNA Microarray Scanner G2505C with Agilent Scan Control version A.8.1.3 software.

#### Immunoblotting

Adherent cells were lysed in sonication buffer (50 mM HEPES; 140 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% sodium deoxycholate; 0.1% SDS) containing protease and phosphatase inhibitor cocktails (Sigma). Preparation of nuclear extracts was achieved by incubating cells with swelling buffer (25 mM HEPES; 1.5 mM MgCl<sub>2</sub>; 10 mM KCl; 0.1% NP-40; 1 mM DTT) and subsequent lysis of the nuclear pellets in sonication buffer. Protein lysates were boiled in Laemmli sample buffer, separated by SDS–PAGE and transferred to a PVDF membrane (Millipore). Antibodies are listed in Supplementary Table S2.

#### **ChIP-Sequencing and statistical analyses**

Cells were induced with 100 nM 4-OHT for 1 h and cross-linked with 1% formaldehyde at room temperature. Nuclei were isolated as described above, and sonication was performed in a Branson sonifier to achieve fragment sizes < 300 bp. Prior to immunoprecipitation, anti-ERa or anti-Miz1 (10E2) antibodies were coupled to Protein A or G-magnetic beads, respectively (Dynabeads, Invitrogen). Bound chromatin was eluted with 1% SDS and decross-linked DNA was purified by phenol-chloroform extraction. ChIP-DNA library preparation was performed with NEBNext ChIP Prep Master Mix Set for Illumina. Fragments were size-selected on an agarose gel, purified with Qiagen gel extraction kit and PCR-amplified. Quality of the DNA library was analyzed with an Experion DNA Chip (Bio-Rad) and quantified using a picogreen assay. Sequencing was performed on an Illumina GAIIx sequencer. After quality control, fastq files were normalized to equal tag counts and aligned to the human genome (hg19) using Bowtie v0.12.8. SAMtools was used to generate binary files, and peaks were called with MACS-1.4.2 using a mixed-input sample as control. Annotated peak lists were generated by closestBed command (BEDTools-2.17.0) using a UCSC annotation file of RefSeq transcriptional start sites. Integrated Genome Browser software 8.0 was used to visualize ChIP-Seq tracks in wig format. seqMINER density array method was employed to generate distributions of tags around respective reference coordinates, and data were visualized as histograms using R (http://www.R-project.org) or as heatmap with Java Treeview software. Genomic Srf binding

data are taken from Esnault *et al* (2014) or downloaded from GEO (MCF7 data set, GSM1010839). Microarray and ChIP-Sequencing data have been deposited to the GEO repository and are available under series record GSE59001 (Muthalagu *et al*, 2014) and reference series GSE59147.

Expression data and additional phenotypic data for the Miller data set were downloaded from the Bioconductor homepage as part of the breast CancerUPP library. For calculation of association scores, we used a method that was previously used to determine the expression of an embryonic stem cell signature in breast cancer patients (Mizuno *et al*, 2010). All statistical analysis was performed using R environment. Unless indicated otherwise, *P*-values were calculated using an unpaired, two-sided Student's *t*-test. *P*-values for calculating the significance of overlaps between different lists are based on a hypergeometric distribution.

#### Supplementary information for this article is available online: http://emboj.embopress.org

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#### Author contributions

KEW, HMH, BvE, EW, and CE performed the experiments; KEW, BvE, EW, AR, and RT analyzed data; AR, RT, JK, and ME designed experiments; and ME wrote the paper.

#### **Conflict of interest**

The authors declare that they have no conflict of interest.

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