Supplemental Material for: Epithelial cell organization suppresses Myc function by attenuating Myc expression. Simpson *et al.*,

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1. SUPPLEMENTAL MATERIALS AND METHODS

Vectors

Adenoviral Myc and Myc mutant vectors were constructed by PCR amplification of pCGN-HA-Myc using primers with attB adapters. A stop codon was introduced at the end of the Myc ORF to express untagged Myc. Each adenoviral vector was transfected into 293A cells (purchased from *Invitrogen)* as described (*Invitrogen ViraPower Adenoviral Gateway Expression Kit*). Adenoviruses were purified twice by CsCl centrifugation, titered by plaque assay, and tested for expression in 2D and 3D MCF10A cultures.

Northern Analysis

The Myc template for the radiolabeled probe was amplified from pCGN-HA-Myc using nested PCR primers. *In vitro* transcription from a T7 promoter produced a 484 bp antisense ssRNA probe labeled with ^{32}P-UTP (*Perkin Elmer*) using the STRIP-EZ RNA kit (*Ambion*). The GAPDH probe was transcribed from the pTRI-GAPDH-Human antisense control template to produce a 316 bp ssRNA radiolabeled probe. Probes were DNAse treated and purified on a G-50 MicroSpin

column (*GE Healthcare*) prior to use. After pre-hybridization in 10 mL of Ultrahybridization buffer for an hour at 68˚C, the Myc probe was added to the pre-hybridization solution and hybridized overnight. Blots were then wash and revealed by phosphorimaging. Subsequently, blots were stripped and then re-probed for GAPDH.

Immunoblotting

In addition to using a Bradford reagent assay (*BioRad*) to quantify protein concentration, which can be affected by residual Matrigel in the harvested cells, 5% of the cells were removed prior to lysis and mixed with an equal volume of 2% SDS. The A_{260} of the crude lysate was measured to allow for normalization based on nucleic acid levels in each sample, to control for Matrigel contamination where problematic. For quantitative Western blots, bands were quantified by integrated intensity using the LI-COR Odyssey software. Localized background was subtracted by using the average of the top and bottom 3 pixels in each boxed area.

Immunofluorescence

For sequential labeling of Myc and cleaved Caspase-3 epitopes with rabbit polyclonal antibodies, an additional blocking step with unlabeled anti-rabbit F(ab) fragments was added after immunolabeling Myc with anti-Rabbit-IgG AlexaFluor488, but before incubating with cleaved Caspase-3 primary antibodies (and anti-Rabbit-IgG AlexaFluor568), to block cross-reaction during secondary labeling.

2. SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Quantification of proliferation and apoptosis in MCF10A acini expressing Myc and Myc mutants in 3D culture at day 16 (from Figure 1). (A) Quantification of the proportion of acini with ≥ 3 cells staining positive for cleaved Caspase-3 (CC3). (B) Quantification of the proportion of acini with ≥ 3 cells staining positive for Ki67. Error bars = ± 1 SEM (Chi-square test compared to WT Myc: *p-value < 0.0125).

Supplemental Figure 2. Timecourse of Myc induced apoptosis in MCF10A acini during morphogenesis. Images of cleaved Caspase-3 (CC3) immunofluorescence in MCF10A acini in 3D culture at days 8, 12, or 16. MCF10A acini expressing an empty vector (MLP), Myc (MLP Myc), a Myc box I deletion mutant (MLP ΔMbI Myc), a Myc box II deletion mutant (MLP ΔMbII Myc), or a Myc box III deletion mutant (MLP ΔMbIII Myc) are shown. Scale bars = 50 microns.

Supplemental Figure 3. Timecourse of Myc induced proliferation in MCF10A acini during morphogenesis. (A) Images and (B) quantification of Ki67 immunofluorescence in MCF10A acini in 3D culture at days 8, 12, or 16. MCF10A acini expressing an empty vector (MLP), Myc (MLP Myc), a Myc box I deletion mutant (MLP ΔMbI Myc), a Myc box II deletion mutant (MLP ΔMbII Myc), or a Myc box III deletion mutant (MLP ΔMbIII Myc) are shown. Scale bars = 50 microns. (B) Quantification of the proportion of acini with ≥ 3 cells staining positive for Ki67. Error bars = ± 1 SEM. (C) Western blot showing expression of E-cadherin, Myc, and Bim expression in 2D and 3D relative to Actin in MCF10A cell monolayers (2D) and MCF10A acini (3D day 16) transduced with the vectors analyzed in A and B.

Supplemental Figure 4. Comparison of mechanical and chemical separation methods for analysis of Myc expression. Western blots showing expression of Myc (A) or

MycER (B) relative to Actin. Untransduced (UN) MCF10A cells or MCF10A cells expressing an empty vector (MLP), Myc (MLP Myc), or MycER (pBabeMycER) were harvested by either scraping (-) or trypsinization (+), washed in PBS, and lysed in RIPA buffer. Myc proteins were detected by Western blotting with an antibody against Myc.

Supplemental Figure 5. Volcano dot plot of Myc target gene expression in 3D versus 2D. The 1,160 Myc target genes on the array were plotted by fold change (x-axis: $\log_2$[fold change]) versus statistical significance (y-axis: -log[P-value]) showing significantly up-regulated (red) and down-regulated (green) Myc target genes. See supplemental tables 3 and 4 for list of the Myc target genes, together with their fold change, and the corresponding P-values.

Supplemental Figure 6. (A) Quantification analysis of Western blot in Figure 5A, relative to endogenous Myc expression in 2D (left bar). (B) Quantitative Western blots were scanned for Adenoviral Myc (Adeno-Myc) and endogenous Myc (Adeno-lacZ) expression in 2D and 3D, and then compared to recombinant SUMO-Myc quantitative standards. Lanes 1 and 2 from Figure 5A in 3D (left) shown as an example relative to 2D (right). Myc blots were scanned at a higher intensity in 3D than in 2D and separate standard curves were used to calculate the amount of Myc in each sample (Supplemental Table 5). We calculated that there are approximately 28,000 molecules of endogenous Myc per cell, similar to what has been described in other cell types using other methods (35). (C) Colocalization of Myc (green) and cleaved-Caspase 3 (CC3; red) immunofluorescence in mature 3D acini infected with adenoviral Myc after sequential immunolabeling (secondary control: no primary CC3 antibody). Nuclei are counterstained in Hoechst 33342 (blue). (D) LacZ expression and immunofluorescence imaging of CC3 immunofluorescence (red) in MCF10A acini in 3D culture at day 20 with adenoviral vectors expressing lacZ, Myc (WT Myc), T58A Myc (TA Myc), S62A Myc (SA Myc), T58A S62A Myc (TASA Myc), a CPD deletion mutant missing $L_{56}P_{57}T_{58}P_{59}$ (ΔCPD Myc), a Myc box II deletion

mutant (ΔMBII Myc), or a Myc box III deletion mutant (ΔMBIII Myc). Scale bars = 50 microns.

3. SUPPLEMENTAL TABLE LEGENDS

Supplemental Table 1. Genes expressed higher in 3D culture than in 2D culture.

Supplemental Table 2. Genes expressed lower in 3D culture than in 2D culture.

Supplemental Table 3. Myc target genes expressed higher in 3D culture than in 2D culture.

Supplemental Table 4. Myc target genes expressed lower in 3D culture than in 2D culture.

Supplemental Table 5. Quantification of Adenoviral Myc expression in 3D relative to endogenous Myc 2D expression (from Figure 5A and Supplemental Figure 4A).

4. SUPPLEMENTAL FIGURES 1–6

(See separate images)

5. SUPPLEMENTAL TABLES 1–5

(See separate files)