Supplementary Information

Generation of the diced siRNA library

Total RNA from Hela and Jurkat cells was isolated using Qiagen Total RNA preparation kit or purchased. (human brain) and transcribed into a cDNA library using the Superscript III reverse transcription protocol. These three cDNA libraries were mixed together in equal amounts and were used as a template in the PCR reactions. Gene specific and nested PCR primers were designed against the last 500 base pairs of genes of interest using an in-house primer design program written in MATLAB (Supplementary Table 1). Gene specific PCR was carried out using 1μl of the cDNA library as a template. The PCR protocol was: 94 °C for 2minutes, 30 times 94 °C for 30 seconds, 57°C for 30seconds, 72°C for 45 seconds, followed by 10minutes at 72°C and then cooled to 4°C. The gene specific PCR products were diluted 1:40 and 2μl were used as templates for nested PCR. For the nested PCR the conditions were: 94 °C for 2minutes, 40 times 94 °C for 30 seconds, 55°C for 30seconds, 72°C for 45 seconds, followed by 10minutes at 72°C and then cooled to 4°C. The PCR products were checked on a 1% agrose gel with 100 wells to ensure they were pure and of the correct size (ie. 500base pairs). In vitro transcription was carried out and the remaining DNA was removed by following the protocol in the Ambion MEGASCRIPT *in vitro* transcription kit. The 500bp double stranded RNA obtained from *in vitro* transcription (total of 50μl) was incubated with 30ul of DICER (1U/μl) and 15ul of DICER buffer for 16-18hours at 37 degrees. The 21bp fragments were purified from any remaining 500bp fragments using a modified protocol from the Invitrogen RNA Cleanup kit (Invitrogen 12183-018). Specifically, a low isopropanol concentration (40%) step was done first too remove all of the large

RNA. The eluate from this purification was then brought to 70% isopropanol and the short interfering RNAs bound to the column. The columns were washed twice with Invitrogen Wash Buffer II. Once purified, the siRNA was run on a 3% Agarose gel to verify that the purification had been successful and that only small RNA remained in the samples. Concentration estimates of the purified siRNA were obtained using a spectramax 96-well plate reader and by comparison to synthetic siRNAs of known concentration on ethidium bromide stained gels. For the negative control, diced siRNA pools were generated from firefly luciferase cDNA (GL3, Accession number U47296, nucleotides 115-614 relative to the start codon).

Cellular Imaging and Hoescht staining analysis method

Cells were imaged with an Image Xpress 5000A automated epifluorescent microscope with a 4X S Fluor magnification objective and Chroma excitation /emission filter /dichoric sets: D360-4X/ D460-50m/400DCLP and S500/20X/ S535/30m/86002v2. Four different sites were imaged in each well. The images were analyzed using MATLAB.

For the Hoechst staining analysis method, centroids of cells were identified after applying a Gaussian filter. For each cell, the local background intensity was calculated in a box around the cell centroid. Any centroid that fell around the image edge was excluded from the analysis. In the box around each centroid, the two hundred lowest intensity pixels were considered local background. Any pixel that had an intensity greater than twenty standard deviations above the local background was considered part of the cell and was included in the calculations of cell area, Hoechst intensity and pRb intensity

(Supplementary Figure 1a). The values of 200 and 20% for the size of the background and standard deviation above the background to consider as part of the cell were determined empirically. This method of calculating the cell area reduced the standard deviation of each peak in the DNA content histograms (Supplementary figure 1b).   It also reduced the ratio of the G2/M peak and the G1/G0 peak from approximately 2.5 to 2.0 (which would be the expected ratio of the two peaks. To obtain clear histograms of the DNA content, it was necessary to use saturating levels of Hoechst stain and sufficient staining time (12hours). The Rb-antibody staining intensities were analyzed on a well-to-well basis to determine a threshold level to consider the cells Rb-positive. The threshold was set using a kmeans clustering algorithm that first excludes outliers by setting a minimum cluster size.   Use of this clustering algorithm minimized the effects of well-to-well variations in antibody staining intensities and was an unbiased method for setting the threshold for Rb-positive cells. For datasets with high percentages of cells in the G2/M phase such as those involving p53 knockdown, the original kmeans clustering algorithm often found an additional peak and determined an inappropriate limit for Rb positive cells. In these data sets, the threshold for Rb positive cells was determined using the kmeans clustering algorithm only for cells with total DNA content less than 2.2.

Optimization of the Cell cycle entry assay

The optimized transfection protocol yields approximately 75-90% knockdown efficiency for lamin A and lamin C (Supplementary Figure 2a). Sufficient cell confluency was a critical factor for optimal transfection efficiency, but the cell number was limited to 3000 cells per well to avoid contact inhibition and prevent the cells from entering into the

cell cycle due to cell density restrictions. We used PDGF as the growth factor stimulus since various isoforms of PDGF have been reported to cause ROS generation in a variety of cell types (Bae et al 2000, Meng et al 2002, Sundaresan et al 1995) and since PDGF-BB elicited a strong mitogenic response in HS68 cells. The PDGF-BB isoform of PDGF was used as the growth factor stimulus for all of the experiments in this manuscript except where it is explicitly stated. PDGF-AA did not stimulate proliferation in HS68 cells (Supplementary Figure 2b). A PDGF concentration of 10ng/ml was selected because it was the minimal concentration that consistently saturated the cell proliferation response (Supplementary Figure 2b). Choosing a saturating concentration of PDGF helped to minimize noise in the siRNA screening experiments. In HS68 cell cultures with approximately 3000 cells plated per well in a 96-well plate, Rb becomes phosphorylated on Ser807/811 starting approximately 9 hours after PDGF stimulation. The percentage of cells that stain positive for Rb increases from 9 to 27 hours. In the assay that we used to monitor cell cycle entry, in untransfected cells, approximately 40 to 50 percent of the cells are Rb positive at 27 hours after PDGF stimulation. This was the time point selected to conduct the cell cycle entry assay since there was the largest difference between the percent of Rb positive and non-Rb positive cells.

Measurement of CM-DCFDA Intensity

Cells were transfected, plated, serum starved and PDGF treated as described for the cell cycle entry assay. After 27hours of PDGF stimulation, Hoescht stain diluted in warm DMEM plus 0.1% Bovine serum albumin was added to a final concentration of 2ng/ml. Cells were returned to the incubator for fifteen to thirty minutes and were then

washed three times with PBS and returned to the incubator for five minutes. PBS was removed and cells were stained for five minutes in 5micromolar 5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-DCFDA, Invitrogen). Cells were then washed again with PBS three times and returned to the incubator for five more minutes. The plates were imaged immediately following this five minute incubation on an Image Xpress 5000A using a 4X S Fluor magnification objective and Chroma excitation /emission filter /dichoric sets: D360-4X/ D460-50m/400DCLP and S500/20X/ S535/30m/86002v2. The data were analyzed using MATLAB. Masks were calculated for both the Hoescht and CM-DCFDA stain by using the MATLAB graythresh command. The total mask intensity of CM-DCFDA was then divided by the number of objects identified in the Hoeschst stained image to get an estimate of the total CM-DCFDA intensity per cell. To normalize for variations between plates that may be due to differences in dye concentration or day to day fluctuations in the imaging conditions, the intensity per cell for each well was divided by the median intensity per cell for the unstimulated negative controls on each plate.

Analysis of p21 and p53 immunocytochemistry experiments:
Cell numbers were counted in 100 intensity ranges. The figure combines data from four (DUOX2) or six (NOX4) independent experiments with triplicate measurements on each day. To compare between different days, data were first put on a log scale and then normalized by dividing by the median of the negative control for each plate. P-values comparing the intensity distributions for DUOX2 or NOX4 knockdown to the negative control were calculated using the Wilcoxon rank sum test.

Supplementary Table 1: Complete list of genes that were targeted in the siRNA library with the sequences of the primers that were used to amplify the last 500 bp of each gene.

Supplementary Figure 1: Comparison of Cell Cycle Distribution Analysis Methods for images of Hoechst stained cells. a) Heat map of a cell with the region used to determine the cell area (pixels with an intensity greater than 20 times the standard deviation of the lowest 200 pixels in the box) labeled in dark red **b)** DNA content histograms are compared here for Hoechst stained cells from the same image using three different analysis methods. For the green line, the background was subtracted by subtracting a value equal to the lowest 20% of pixels in the whole image. Regions were defined using the bwlabel command in MATLAB. In red, data was analyzed in the same way as for the green line, but 2% of the edge cells were excluded. The blue line shows the final analysis method that we used Local background levels were calculated by the lowest 20% of pixels in a box surrounding each cell. Any pixel that exceeded the mean of this background level plus 20 standard deviations was considered to be part of the cell. This methods shows a significant improvement since the ratio of the 4N to 2N DNA content peak is closer to 2 and the standard deviation of each peak is significantly reduced.

Supplementary Figure 2: Optimization of cell cycle entry assay a) Western blot of HS68 cells transfected with 50 nM synthetic lamin siRNA from Dharmacon versus a negative control siRNA (diced GL3) stained with primary antibodies against Lamin A/C (05-714 1:5000, Upstate(Milipore, Billerica, MA, USA) . **b)** Titration of PDGF, bFGF

and PDGF-AA growth factors. **c)** Quantifications of the fraction of Rb phosphorylated cells obtained from cells transfected with various control synthetic siRNA pools. Knocking down the PDGF receptor, Grb2, and Rac, all known positive regulators in the PDGF proliferative response, significantly inhibited cell cycle entry. Knocking down either p53 or GSK-3, which play inhibitory roles in Rb-phosphorylation, enhanced cell cycle entry (Supplementary Figure 2d).

Supplementary Figure 3: Validation of siRNA knockdown a) Cell cycle entry assay using a Western Blot instead of immunofluorescence to measure Rb phosphorylation after 27 hours of PDGF stimuli. Cells were transfected with Dharmacon siGENOME synthetic pools of NOX4 and DUOX2 siRNA or with Dharmacon Negative Control siRNA. The lysis and western blotting were carried out as described in Materials and Methods. **b)** Quantification of the western blot shown in a. **c)** Measurements of CM-DCFDA intensity in live HS68 cells at the end of the cell cycle entry assay after 27hours of PDGF stimuli (dark grey) or after 72 hours of serum starvation (light grey bars). Data represents experiments on three different days and eight different 96-well plates. For the PDGF stimulated samples n=12 for the samples transfected with NOX4 or DUOX2 Dharmacon synthetic siRNA pools and n=24 for samples transfected with the Dharmacon negative control siRNA pool. For the unstimulated samples n=11 for NOX4 and DUOX2 transfected samples and n=23 for the negative controls. The mean CMDCFDA intensity per cell on each day was normalized by dividing by the median of the unstimulated negative controls on the same plate. Error bars represent standard error of the mean. **d)** Quantitiatve RT-PCR of cells transfected with diced NOX4 siRNA AND GL-3 negative

control siRNA. **e)** Quantitative RT-PCR data for cells transfected with NOX4-1, NOX4-2 and NOX4-3 siRNA. Cyclophilin was used as an endogenous control since GAPDH levels appeared to be affected by the siRNA treatment. **f)** Quantitative RT-PCR data for DUOX2 for cells transfected with DUOX2-1, DUOX2-2 and DUOX2-3 siRNA. GAPDH was used as an endogenous control .**g)** Quantification of cell number from the same experiments shown in Figure 3b and c.

Supplementary Figure 4: Effect of NOX4 and DUOX2 siRNA on ERK and Akt levels. a) Quantification of the ratio of total ERK1 or ERK2 protein to total GAPDH from western blots of lysates from cells transfected with synthetic pools of negative control siRNA or siRNA targeting NOX4 or DUOX2 (n=6, error bars represent ± standard error of the mean). **b)** Quantification of the ratio of total ERK protein or total Akt protein to total GAPDH from western blots of lysates from cells transfected with negative control siRNA or three different siRNA sequences targeting NOX4 (n=6, error bars represent ± standard error of the mean). **c)** Quantification of the ratio of total ERK1 or ERK2 protein to total GAPDH from western blots of lysates from cells transfected with negative control siRNA or three different siRNA sequences targeting DUOX2 (n=6, error bars represent ± standard error of the mean).

Supplementary Figure 5: Validation of the p53 and p21 antibodies Histogram of p21 antibody staining intensities (left) and p53 antibody staining intensities (right) from immunocytochemistry experiments of cells transfected with p21, p53 or negative control synthetic siRNA pools.

Supplementary Figure 6: a) DNA content scatter plots of CyclinD1 with negative control siRNA, p21 siRNA or p53 siRNA in which the color of the points represents the density of the points in a given region. The density is calculated by the reciprocal of the area of the voronoi region surrounding the centroid of a point. **b)** Co-knockdown of p21 or p53 with negative control siRNA or siRNA targeting cyclin D1 (error bars represent ± standard deviation for three wells of a 96-well plate experiment)

Supplementary Figure 7: NOX4 knockdown increases p53 and p21 levels a) Intensity distributions for p21 levels measured as described in Figure legend 6 for cells transfected with NOX4-UTR: CCAAAGAGACCCUGAAGAAUU NOX4V-3: GCAAUAAGCCAGUCACCAU or NOX4-V4: GAUGGGAUACAGAAGAUAA or negative control synthetic siRNA. The figure combines data from three independent experiments with triplicate measurements on each day (n=9). To compare between different days, data were first put on a log scale and then normalized by dividing by the median of the negative control for each plate. Using the rank sum test, the p-values comparing the p21 intensity distributions for each of the three NOX4 siRNA to the p21 intensity distribution for the negative control siRNA were less than .001. **b)** Same as a) except the intensity distributions are for p53 and the cells were transfected with either NOX4V-3, NOX4-UTR or negative control synthetic siRNA after 18 hours of PDGF stimulation. Using the rank sum test, the p-values comparing the p53 intensity distributions for both NOX4 siRNA to the p53 intensity distribution for the negative control siRNA were less than .001.
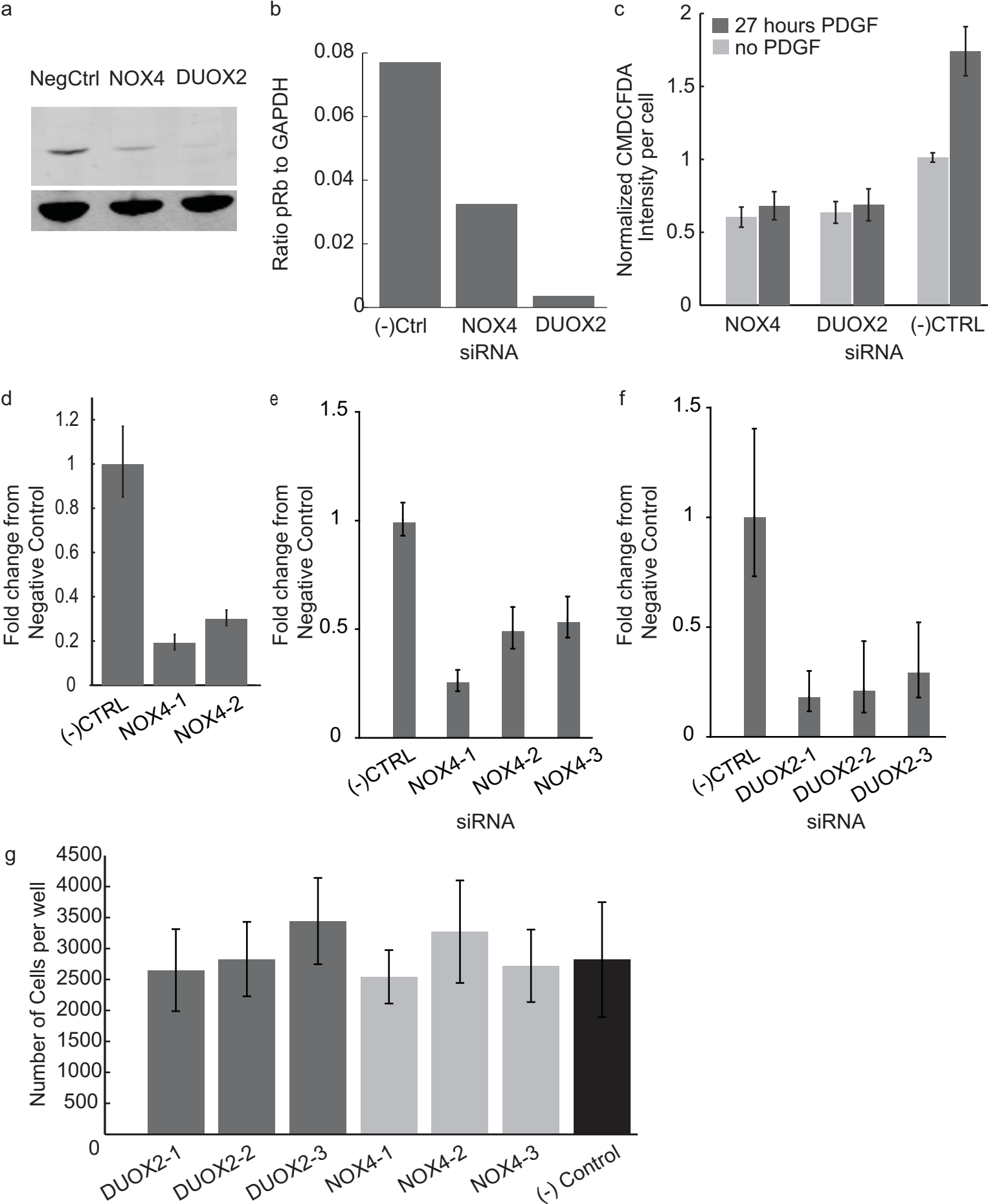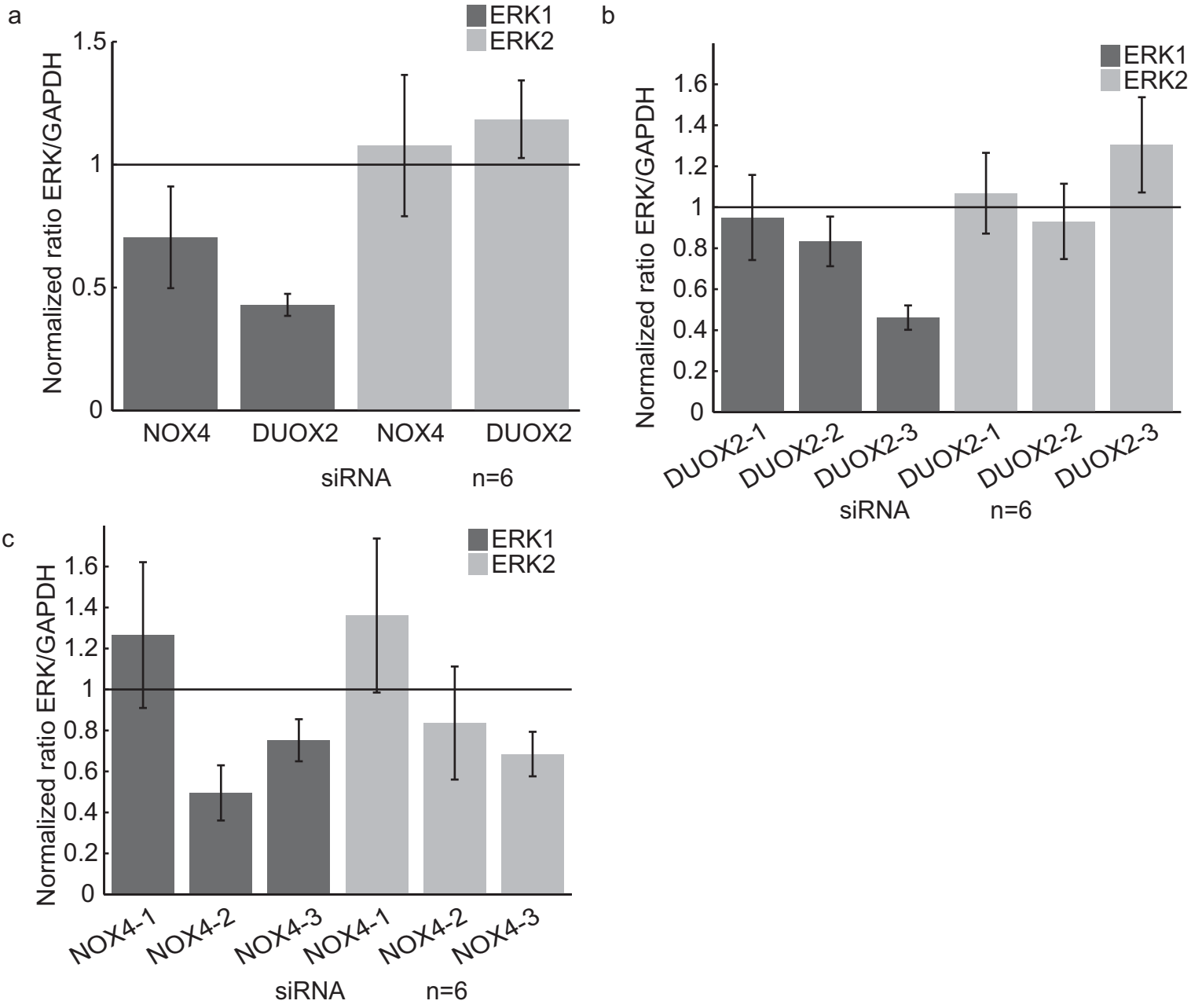
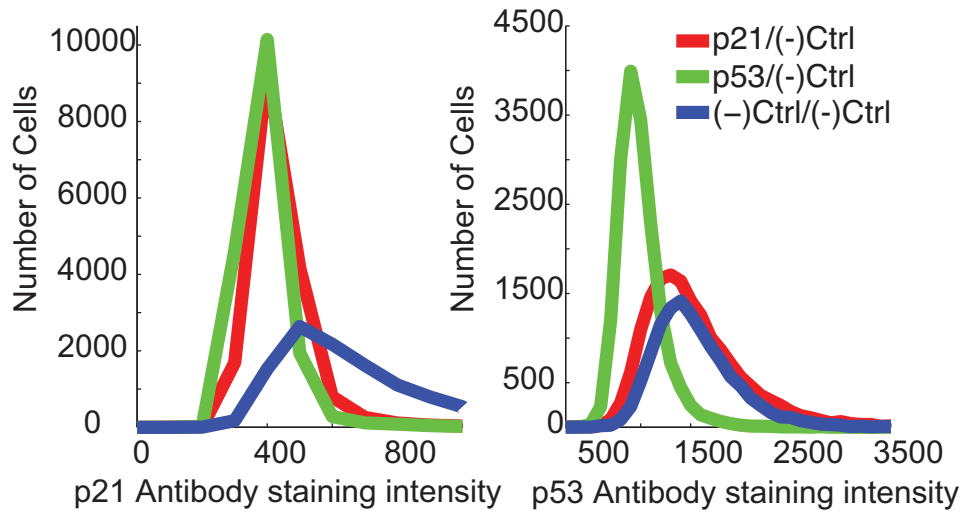# Supplementary Figure 1
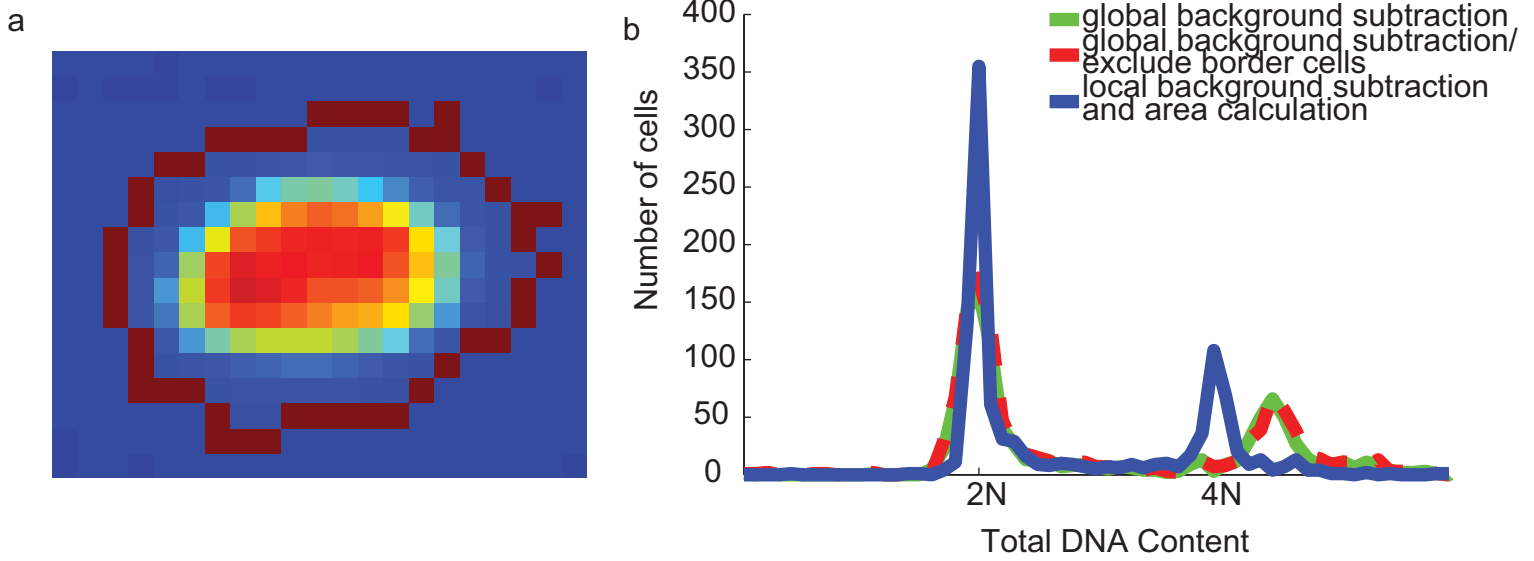
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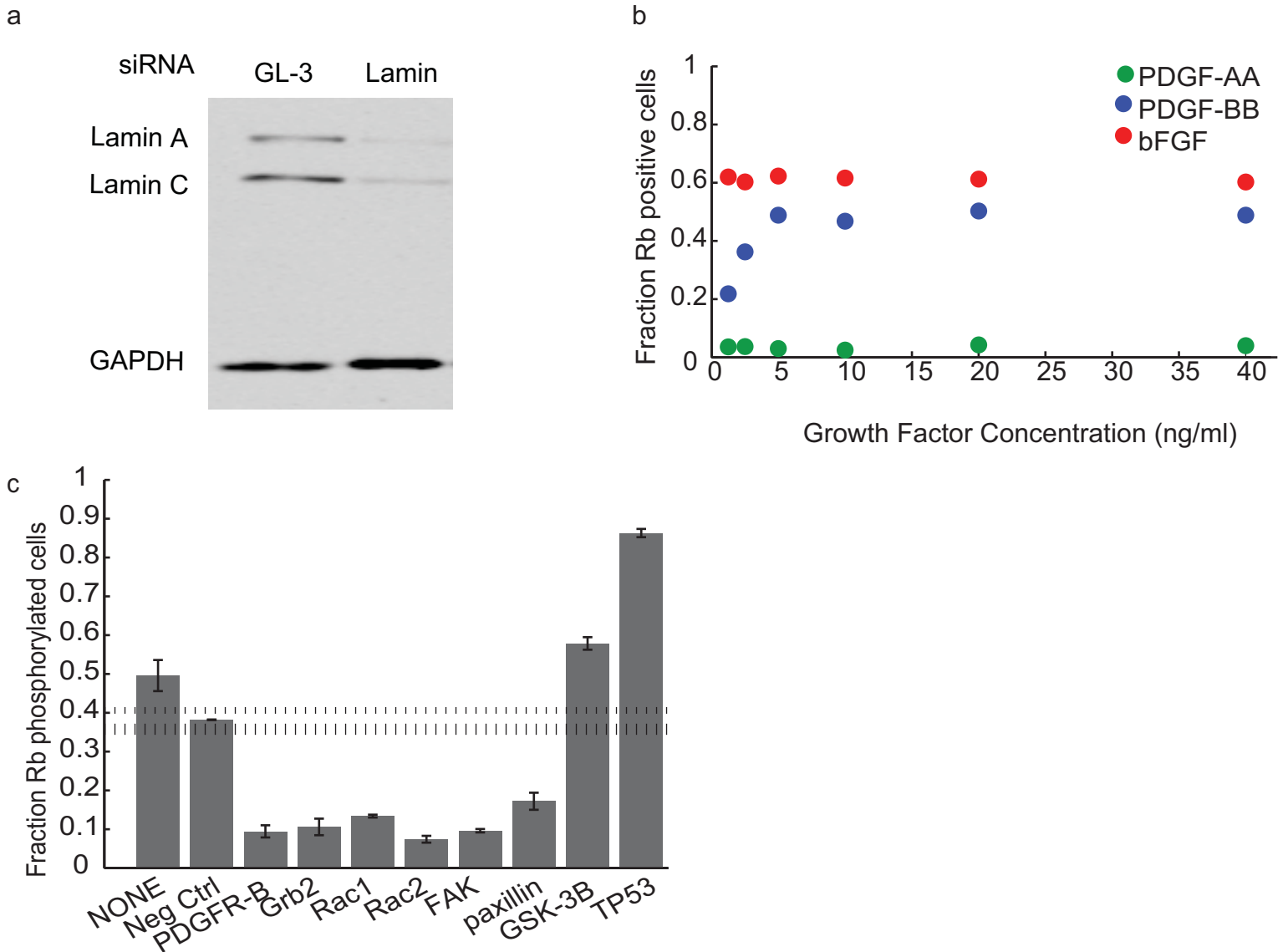
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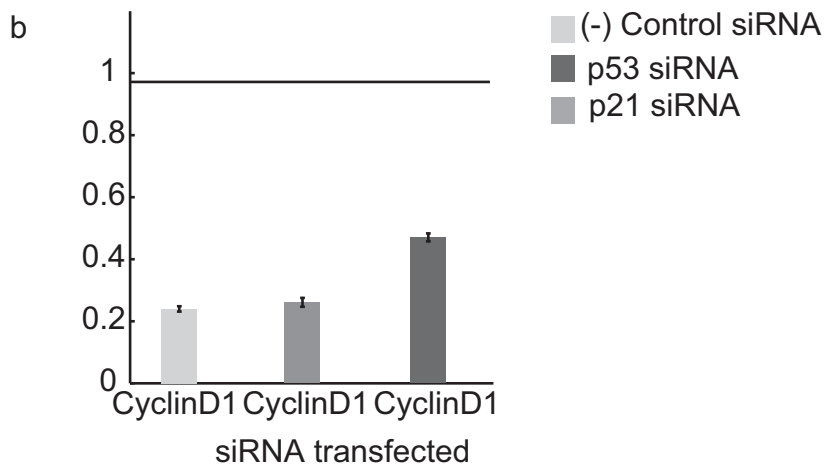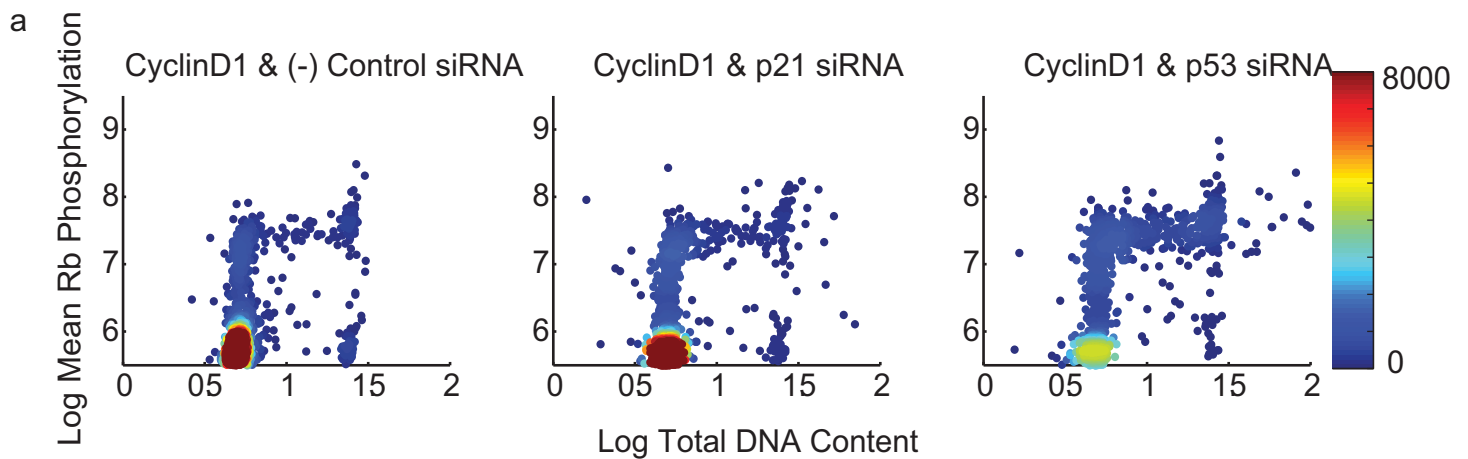
Supplementary Figure 4

Supplementary Figure 5

Supplementary Figure 6

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Supplementary Figure 7

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