Figure S1

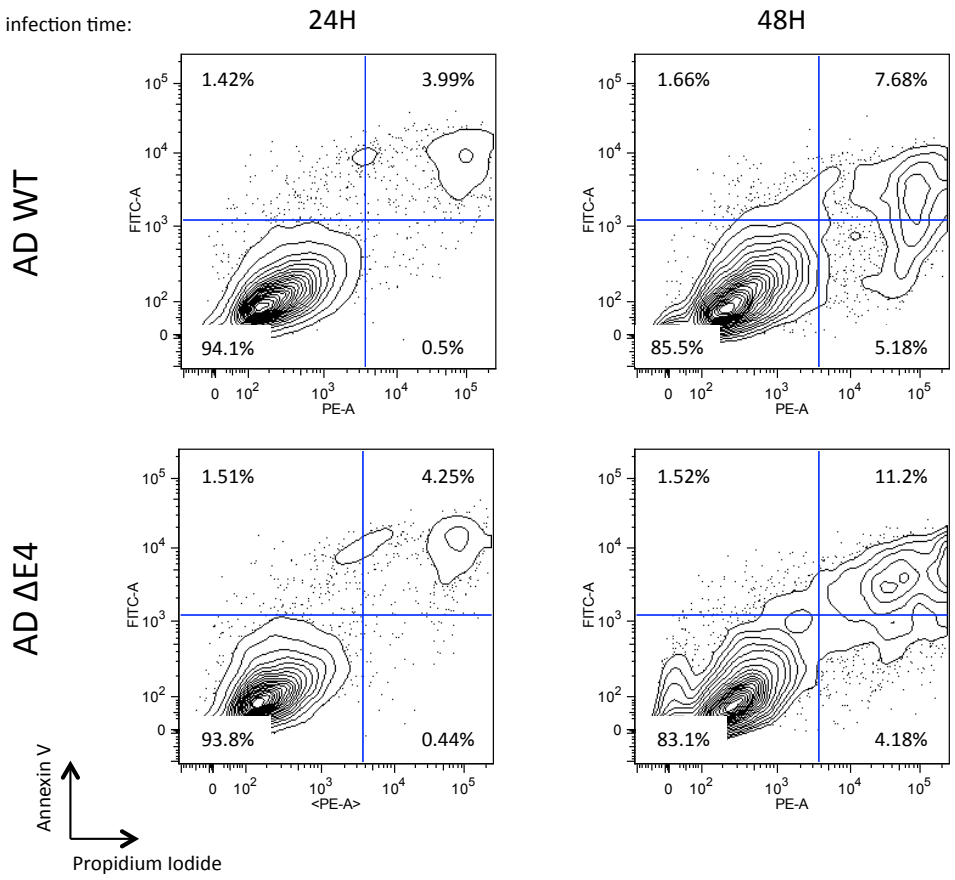
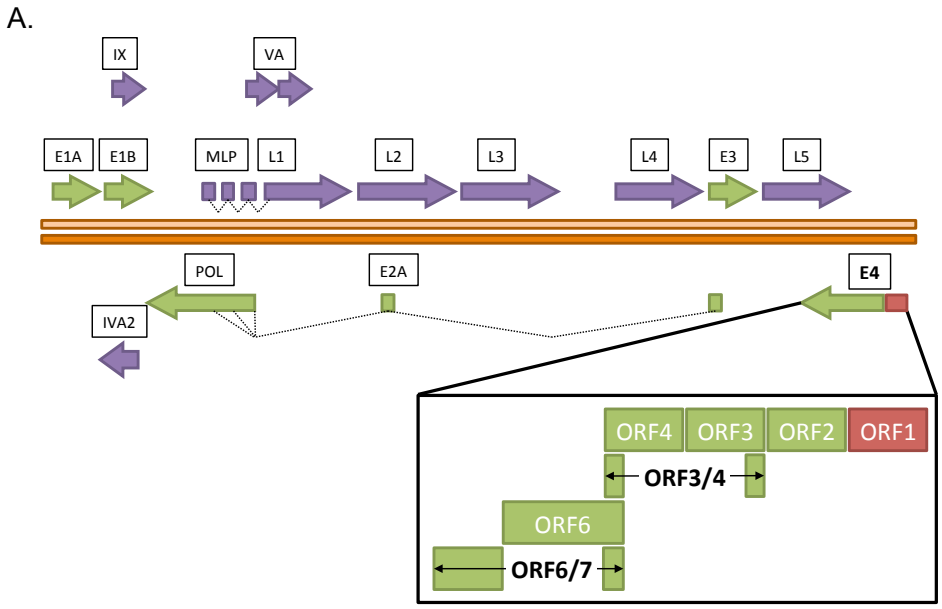

infection time:
24H
48H
AD WT
AD ΔE4
1.42%
3.99%
94.1%
0.5%
1.66%
7.68%
85.5%
5.18%
1.51%
4.25%
93.8%
0.44%
1.52%
11.2%
83.1%
4.18%
FITC-A
PE-A
<PE-A>
Annexin V
Propidium Iodide

Figure S1. Related to Figure 1A. The majority of cells are viable after 48 hr adenovirus infection. MCF10A cells infected with AD WT or AD ΔE4 for the indicated times. Cells were collected and stained with propidium iodide and Annexin V to determine populations that were viable (PI^-/A^-), early apoptotic (PI^-/A^+), or late apopotic/necrotic (PI^+/A^+).

Figure S2


A.
IX
VA
E1A
E1B
MLP
L1
L2
L3
L4
E3
L5
POL
E2A
E4
IVA2
ORF4
ORF3
ORF2
ORF1
ORF3/4
ORF6
ORF6/7

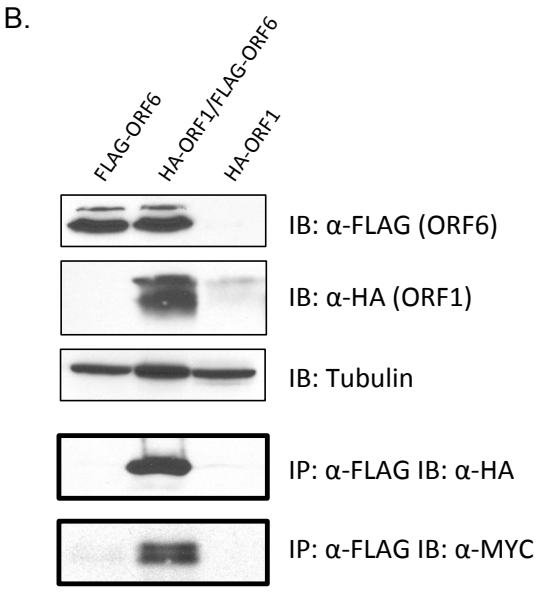

B.
FLAG-ORF6
HA-ORF1/FLAG-ORF6
HA-ORF1
IB: α-FLAG (ORF6)
IB: α-HA (ORF1)
IB: Tubulin
IP: α-FLAG IB: α-HA
IP: α-FLAG IB: α-MYC

Figure S2. Related to Figure 2 overexpression of E4 ORFs. (A) Map of adenovirus serotype 5 genome. Non-segmented, linear double-stranded DNA of 35-36kb. The genome has terminally redundant sequences which have inverted terminal repetitions (ITR). Early transcripts are represented by E1-E4 regions and late transcripts are represented by L1-L5 regions. MLP: Major late promoter. ORF: Open reading frame. **(B)** E4ORF6 binds to and stabilizes E4ORF1. Immunoblotting of lysates from MCF10A cells stably expressing flag tagged-E4ORF6, HA-tagged E4ORF1, or both (40µg total protein). The top panel was probed with FLAG antibody, the middle panel was probed with HA antibody, and the bottom panel was probed with tubulin antibody for loading control. To test for protein interaction, lysates (400µg total protein) were co-immunoprecipitated to flag-conjugated sepharose beads, and probed with antibody towards HA (ORF1) or MYC.

Figure S3

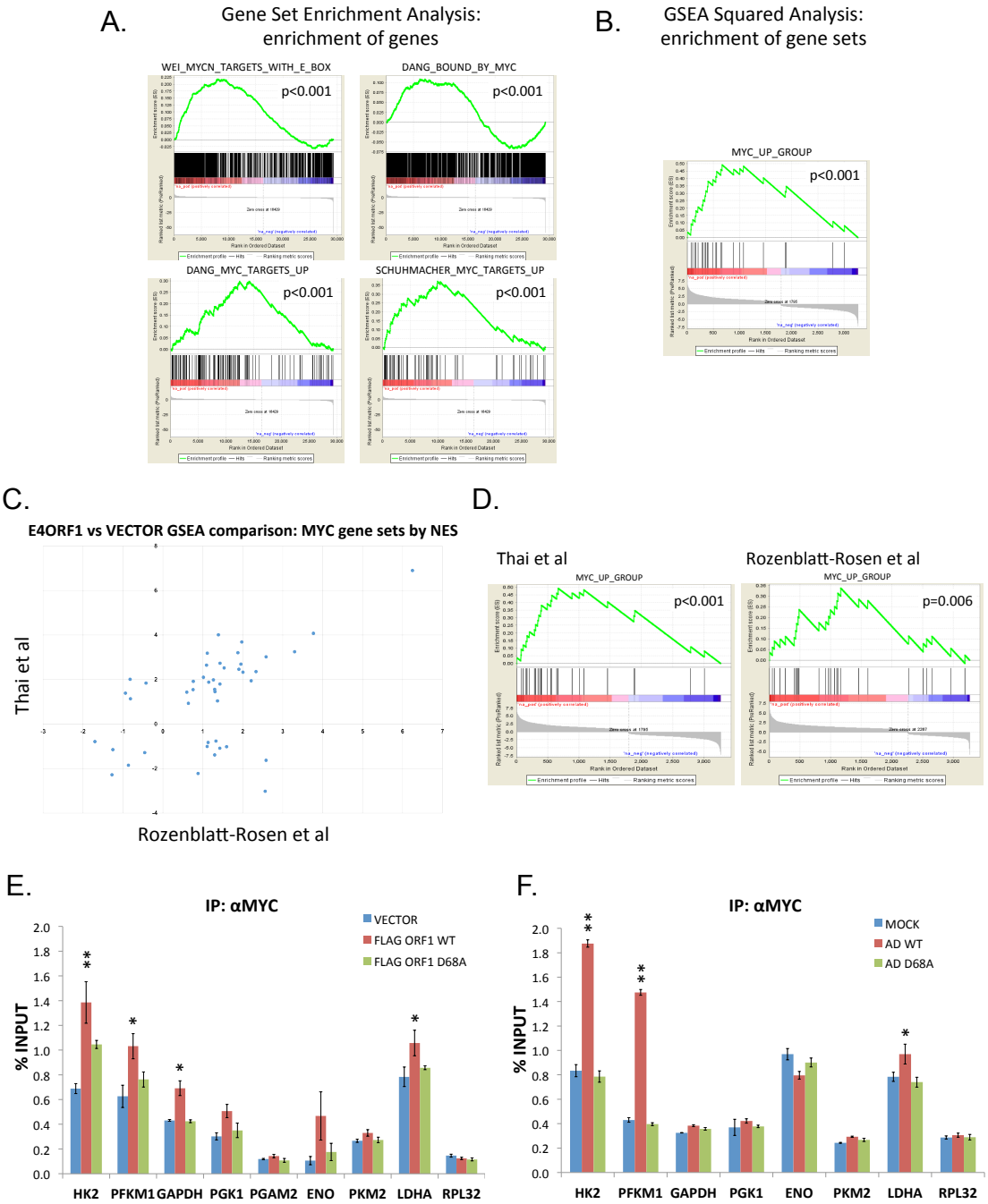

A.
Gene Set Enrichment Analysis:
enrichment of genes
WEI_MYCN_TARGETS_WITH_E_BOX
p<0.001
DANG_BOUND_BY_MYC
p<0.001
DANG_MYC_TARGETS_UP
p<0.001
SCHUHMACHER_MYC_TARGETS_UP
p<0.001
B.
GSEA Squared Analysis:
enrichment of gene sets
MYC_UP_GROUP
p<0.001
C.
E4ORF1 vs VECTOR GSEA comparison: MYC gene sets by NES
Thai et al
Rozenblatt-Rosen et al
D.
Thai et al
MYC_UP_GROUP
p<0.001
Rozenblatt-Rosen et al
MYC_UP_GROUP
p=0.006
E.
IP: αMYC
VECTOR
FLAG ORF1 WT
FLAG ORF1 D68A
% INPUT
HK2
PFKM1
GAPDH
PGK1
PGAM2
ENO
PKM2
LDHA
RPL32
F.
IP: αMYC
MOCK
AD WT
AD D68A
% INPUT
HK2
PFKM1
GAPDH
PGK1
ENO
PKM2
LDHA
RPL32

Figure S3. Related to Figure 3 E4ORF1 enhances MYC activity. E4ORF1 enriches expression of MYC target genes. Gene expression data from MCF10A cells expressing either E4ORF1 or the negative control vector was analyzed by Gene Set Enrichment Analysis (Subramanian et al., 2005). **(A)** Several individual MYC target gene sets were enriched in E4ORF1-expressing cells ($p < 0.001$ for each gene set). **(B)** Gene sets with increased MYC activity ("Myc_up_group") are enriched compared to all gene sets ($p < 0.001$). This "GSEA squared" analysis involves a second application of enrichment analysis – the initial GSEA results are subsequently analyzed by a second round of Kolmogorov-Smirnov-based statistical enrichment analysis to determine if the multiple 'MYC up' gene sets have higher rankings than would be expected at random. All gene sets from the MSigDB C2 ($n = 3,270$) were ranked by Normalized Enrichment Score and analyzed for enrichment of gene sets representative of increased MYC activity. Each tick mark represents a MYC up gene set ($n = 26$). **(C)** Comparison of the GSEA normalized enrichment scores (NES) for MYC gene sets (defined by association with increased MYC activity) in our Thai et al. breast epithelial system versus the Rozenblatt-Rosen et al. primary fibroblast system. The similarity has a correlation of $r = 0.52$ (two-tailed p-value = 0.0003). **(D)** GSEA-inferred MYC activity is upregulated by E4ORF1 in both breast epithelial and primary fibroblast cellular contexts. GSEA analysis of gene sets associated with increased MYC activity ("myc up group") relative to all gene sets demonstrates similar and statistically significant enrichment in both our Thai et al. breast epithelial system ($p < 0.001$) and the Rozenblatt-Rosen et al. primary fibroblast system ($p = 0.006$). **(E-F)** Related to Figure 3C and 3F. For direct quantitative comparisons, data from the chromatin immunoprecipitations are graphed as % input. The ChIP values represent the

amount of immunoprecipitated DNA in each sample relative to the total amount of input chromatin (equivalent to 1). Error bars denote standard errors of the mean (n=3). * denotes $p<0.05$; ** denotes $p<0.01$.

Figure S4

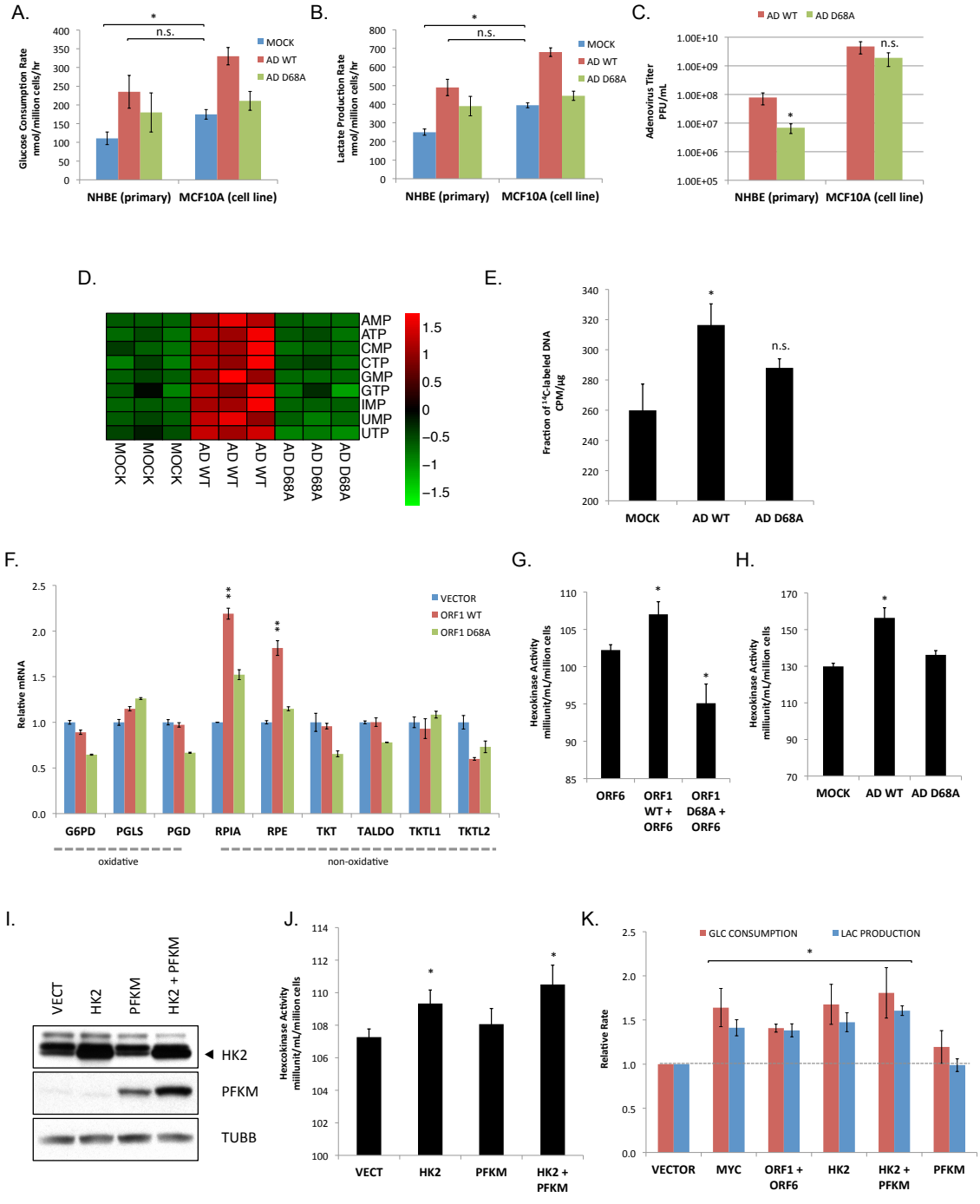

A.
Glucose Consumption Rate nmol/million cells/hr
MOCK
AD WT
AD D68A
n.s.
*
NHBE (primary)
MCF10A (cell line)
B.
Lactate Production Rate nmol/million cells/hr
C.
Adenovirus Titer PFU/mL
D.
AMP
ATP
CMP
CTP
GMP
GTP
IMP
UMP
UTP
MOCK
AD WT
AD D68A
E.
Fraction of 14C-labeled DNA CPM/µg
F.
Relative mRNA
VECTOR
ORF1 WT
ORF1 D68A
G6PD
PGLS
PGD
RPIA
RPE
TKT
TALDO
TKTL1
TKTL2
oxidative
non-oxidative
G.
Hexokinase Activity milliunit/mL/million cells
ORF6
ORF1 WT + ORF6
ORF1 D68A + ORF6
H.
I.
VECT
HK2
PFKM
HK2 + PFKM
TUBB
J.
K.
GLC CONSUMPTION
LAC PRODUCTION
Relative Rate
VECTOR
MYC
ORF1 + ORF6

Figure S4. Related to Figure 4. The Ad ORF1 D68A mutant virus exhibits replication deficiency in NHBE cells but not in the more glycolytic MCF10A cells. **(A and B)** MCF10A cells have higher basal glucose consumption and lactate production rates than NHBE cells (blue lines). **(C)** Functional viral titers of AD WT and AD D68A in NHBE versus MCF10A cells. The AD D68A mutant exhibits replication deficiency in the NHBE cells but not the MCF10A cells. **(D)** Heat map depicting relative abundance of nucleotides measured by LC-MS in extracts from NHBE cells mock infected or infected with AD WT or AD D68A. AD WT infection, but not AD D68A infection, increases total cellular levels of nucleotides. **(E)** E4ORF1 activation of MYC promotes increased incorporation of glucose carbons into DNA. NHBE cells mock infected or infected with AD WT or AD D68A were cultured in medium with 1μCi [U-^{14}C]-glucose for 24 hours, nucleic acids were purified, and ^{14}C activity was counted in a scintillation counter. The fraction of ^{14}C-labeled nucleotides is depicted as ^{14}C counts per minute (CPM) divided by total μg of DNA. **(F)** Related to Figure 4H. Relative mRNA levels of all pentose phosphate pathway genes from MCF10A cells induced to express empty vector, ORF1 WT, or ORF1 D68A. **(G)** Elevated hexokinase activity observed upon E4ORF1 expression and adenovirus infection is sufficient to drive increased glycolytic flux in MCF10A cells. Hexokinase activity is determined by a coupled enzyme assay, in which glucose is converted to glucose-6-phosphate by hexokinase, which is oxidized by glucose-6-phosphate dehydrogenase to form NADH. The resulting NADH reduces a colorless probe resulting in a colorimetric product proportional to the hexokinase activity present. Hexokinase activity in MCF10A expressing ORF1 WT or ORF1 D68A were normalized per million cells and compared. **(H)** Hexokinase activity in NHBE infected

by AD WT or AD D68A for 4 hours. **(I)** Immunoblot of lysates from MCF10A constitutively expressing HK2, PFKM, or both (20ug). **(J)** Hexokinase activity in MCF10A cells stably overexpressing HK2, PFKM, or both. **(K)** Relative glucose consumption rates and lactate production rates were measured and compared in MCF10A cells stably overexpressing HK2, PFKM, or MYC. Error bars denote standard errors of the mean (n=3). * denotes $p<0.05$; ** denotes $p<0.01$.

Table S1. Related to Figure S3. Gene Set Enrichment Analysis of E4ORF1 expressing MCF10A cells. RNA expression probsets from MCF10A cells expressing either E4ORF1 or the vector control (n = 3 for each condition) were ranked by the signal-to-noise ratio (SNR) and collapsed by the maximum absolute SNR for each gene. Gene set Enrichment Analysis was performed using the Molecular Signatures Database (MSigDB) C2 collection (version 2.5) of canonical signaling pathways, cellular processes, chemical and genetic perturbations, and human disease states. To assess the global enrichment of MYC-associated gene sets, MSigDB genes sets associated with increased or decreased MYC activity ("Myc_up_group" and "Myc_down_group", n = 26 and 13, respectively) were manually curated.

Supplemental Experimental Procedures

Plasmids and viruses

Genes constitutively expressed were either cloned into a modified pCCL lentivirus transfer vector containing CMV-HIV 5'LTR and vector backbones in which the SV40 polyadenylation and origin of replication sequences have been included downstream of the HIV 3'LTR with an additional blasticidin resistance gene ligated downstream the WPRE and cPPT enhancers, or cloned into the CMV-driven pLJM1 vector containing a puromycin resistance element. Infected cells were selected by blasticidin (10ug mL^{-1}) or puromycin (1ug mL^{-1}) for a minimum of 5 days. Inducible expressions of genes were cloned into the Tet-ON Gene Expression System (Clonetech). Genes were typically induced with 0.5 - 1ug mL^{-1} doxycycline for a minimum of 24 hours. The whole E4 transcription unit and individual E4ORFs were PCR amplified from pAd5, a 44kb cosmid containing the WT Ad5 genome between two PacI sites (od260).

H5wt300 (AD5 WT) and dl366 (AD ΔE4) were kind gifts from Dr. Frank McCormick. AD D68A was derived by subcloning the E4 region into pBluescript (SpeI-BamHI fragment), then performing site-directed mutagenesis, and subsequently recloning back into pAd5. The mutant viruses were propagated on the W162 cell line, which contain and express the region E4. Infection times of host cells are indicated in the figures at 37C with 10 PFU/cell.

Quantitative Real-Time PCR

RNA was purified with Qiagen RNeasy Kit. 1 μg of total RNA were used to synthesize cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) as per manufacturer's instructions. Quantitative PCR (qPCR) was conducted on the Roche LightCycler 480 using SYBR Green I Master Mix (Roche) and 0.5 μmol L^{-1} primers. Relative expression values are normalized to control gene (36B4) and expressed in terms of linear relative mRNA values.

Extract preparation, immunoprecipitation and immunoblot assays

MCF10A cells were obtained from American Type Culture Collection (ATCC) and the laboratory of Dr. Joe Gray (UC Berkeley) and cultured in Dulbecco's modified Eagle's medium and F12 (DMEM:F12) containing 5% Horse Serum, 50U/mL Penicillin-Streptomycin, 10μg mL^{-1} insulin, 0.5μg mL^{-1} hydrocortisone, 20ng ml^{-1} EGF, 10μg mL^{-1} cholera toxin. NBHE cells were obtained from Lonza and cultured in BEGM BulletKit (Lonza). Cells were lysed in either M-PER Mammalian Protein Extraction Reagent (Thermo) with 4 μg mL^{-1} aprotinin, 4 μg mL^{-1} leupeptin, 4 μg mL^{-1} pepstatin, 1mM PMSF, 1mM Na3VO4, and 5mM NaF or NP40 buffer containing 50 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 4 μg ml^{-1} aprotinin, 4 μg ml^{-1} leupeptin , 4 μg ml^{-1} pepstatin, 1mM PMSF, 1mM Na3VO4, and 5mM NaF. Nuclear and cytoplasmic fractions were prepared using NE-PER extraction reagents (Thermo). Western blot analysis was carried out according to standard methods. Protein concentrations of cell extracts were determined by the Bradford assay. Immunoprecipitations were carried out with 3XFlag conjugated sepharose beads (Sigma), or Protein A/G beads conjugated to the

bait antibody. The following commercial antibodies were used as probes: FLAG (Abcam), MYC (Cell Signaling), HA (Abcam), AKT and phospho-AKT (S473) (Cell Signaling), β-Tubulin (Sigma), Histone H3 (Cell Signaling), GFP (Abcam).

Measurement of oxygen consumption rates

Cellular oxygen consumption rates were measured using a water-jacked (37C) anaerobic chamber fitted with a fiber optic oxygen sensor (INSTECH Model 110 Optic oxygen Monitor). The fiber optic probe was calibrated with 15mM sodium hydrosulfite (Sigma) corresponding to 0% oxygen and cell culture media corresponding to 20.9% oxygen.

Cell viability measurements

Cell viability was measured using the FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen) according to the manufacturer's protocol. $1x10^5$ cells were incubated with Annexin V and propidium iodide (PI) for 15 min and then analyzed by flow cytometry (LSR II Becton Dickinson). Cells in early apoptosis were indicated by Annexin V positive, PI negative staining (lower right quadrant) while cells in late apoptosis were marked by Annexin V/PI double positive staining (upper right quadrant).

Chromatin Immunoprecipitation

Chromatin immunoprecipitations were performed using the SimpleChIP kit (Cell Signaling). Briefly, 5 x 15 cm plates of MCF10A cells (3×10^7) were induced to express ORF1 or ORF1 D68A with 1ug mL^{-1} doxycycline for 48 hours. Proteins were cross-

linked to DNA with 1% formaldehyde for 10 minutes at room temperature. Nuclei were pelleted, and DNA were digested with micrococcal nuclease for 10 minutes. Nuclear pellet was further sonicated in a water bath to shear the cross-linked DNA fragments to lengths between 200-1000 bp. For each ChIP, 100 µl of the cross-linked chromatin DNA was diluted in 400 µl of ChIP buffer and incubated with the immunoprecipitating antibody and magnetic Protein G beads overnight at 4 degrees with rotation. Magnetic beads were washed and eluted, and DNA was purified using spin columns. Real-time quantitative PCR was used to measure the amount of enrichment of a particular DNA sequence by a protein-specific immunoprecipitation. Additionally, primary NHBE cells were infected with adenovirus for 4 hours and cross-linked chromatin was prepared for performing chromatin immunoprecipitation.

Enzymatic Assay of Hexokinase

HK activity was measured using the Hexokinase Colorimetric Assay Kit from Sigma (MAK091). Briefly, 5 x 10^5 cells were plated in triplicate 6-well plates 24 hours prior to sample preparations. For assay reaction, cells were lysed in 400 µL HK assay buffer, and added to a mixture containing enzyme, developer, coenzyme, and substrate in 96-well plates. The plates were incubated at room temperature for 5 minutes to take an initial measurement at absorbance of 450 nm. Measurements were taken at every 5 minutes for 30 minutes. Background was corrected by subtracting the final measurement for the 0 (blank) NADH standard from the final measurement of the samples. HK activity was determined by the ΔA_{450} between the initial time and the final time.

Metabolite extraction and mass spectrometry-based metabolomics analysis

Cells were incubated in medium containing 1 g/L 1,2-^{13}C-glucose for 24 h. The following day, NHBE cells were rinsed with cold 150 mM ammonium acetate (NH_4AcO). Cells were carefully scraped off in 800 µL of 50% ice cold methanol. An internal standard of 10 nmol norvaline was added to the cell suspension, followed by 400 µL of cold chloroform. After vortexing for 15 min, the aqueous layer was transferred to a glass vial and the metabolites dried under vacuum. Metabolites were resuspended in 100 µL 70% acetonitrile (ACN) and 5 µL of this solution used for the mass spectrometer-based analysis. The analysis was performed on a Q Exactive (Thermo Scientific) in polarity-switching mode with positive voltage 3.0 kV and negative voltage 2.25 kV. The mass spectrometer was coupled to an UltiMate 3000RSLC (Thermo Scientific) UHPLC system. Mobile phase A was 5 mM NH4AcO, pH 9.9, B) was ACN, and the separation achieved on a Luna 3mm NH2 100A (150 x 2.0 mm) (Phenomenex) column. The flow was 300 µL / min, and the gradient ran from 15% A to 95% A in 18 min, followed by an isocratic step for 9 min and re-equilibration for 7 min. Metabolites were detected and quantified as area under the curve (AUC) based on retention time and accurate mass (≤ 3 ppm) using the TraceFinder 3.1 (Thermo Scientific) software. Relative amounts of metabolites between various conditions, as well as percentage of labeling was calculated, corrected for naturally occurring ^{13}C abundance as described in Yuan et al. (Yuan et al., 2008), and depicted in bar graphs.

Measurement of ^{14}C Incorporation into Nucleotides

Subconfluent NHBE cells were mock infected or infected with AD WT or AD D68A for 12 hours at 37C (n=3). Cells were subsequently cultured in medium with or without 1μCi [U-^{14}C]-glucose for 24 hours. Nucleic acids were purified with Qiagen DNeasy. ^{14}C activity was counted in a scintillation counter. The fraction of ^{14}C-labeled nucleotides is depicted as ^{14}C counts per minute (CPM) divided by the μg of DNA/RNA calculated from the corresponding non-radioactive sample.

Supplemental References

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U.S.a. *102*, 15545–15550.

Yuan, J., Bennett, B.D., and Rabinowitz, J.D. (2008). Kinetic flux profiling for quantitation of cellular metabolic fluxes. Nat Protoc *3*, 1328–1340.