www.sciencemag.org/cgi/content/full/1173288/DC1

Supporting Online Material for

Dependence of Mouse Embryonic Stem Cells on Threonine Catabolism

Jian Wang, Peter Alexander, Leeju Wu, Robert Hammer, Ondine Cleaver, Steven L. McKnight*

*To whom correspondence should be addressed.
E-mail: steven.mcknight@utsouthwestern.edu

Published 9 July 2009 on *Science* Express
DOI: 10.1126/science.1173288

This PDF file includes:

Materials and Methods:

Cell Culture and dropout media preparation.
Feeder-independent mouse E14Tg2A (BayGenomics) or CCE (Stemcell Technologies) ES cells were cultured on gelatinized dishes in the Glasgow minimum essential medium (GMEM, Sigma) supplemented with 10% FBS (Hyclone), 100μM MEM non-essential amino acids, 1mM sodium pyruvate, 2mM glutamine, 200ug/ml penicillin, 100ug/ml streptomycin (Stemcell Technologies), 50uM 2-mercaptoenthnol and 1000units/ml LIF (Chemicon). Mouse AOK-5P iPS cells were cultured on the feeder layer of γ-irradiated mouse MEF cells. For embryoid body formation, 2×10^6 cells were grown in suspension on 100mM low attachable dishes in 15 ml ES media without LIF. ES dropout media were prepared by omitting indicated amino acid from GMEM or non-essential amino acids. All component chemicals for dropout media preparation were obtained from Sigma.

Quantitative RT-PCR analysis.
Total RNA was extracted with RNA STAT-60 (Tel-Test) and converted into cDNA with oligo(dT) primer using the SuperScript first-strand synthesis kit (Invitrogen). PCR was performed on an AB 7900HT fast real-time PCR system (Applied Bioscience) using TDH specific primer pair with the sequences of 5'-CCTGGAGGAGGAACAACTGACTA-3' (forward) and 5'-ACTCGAATGTGCCGTTCTTTG-3' (reverse).

Measurement of TDH enzymatic activity.
TDH cDNA with an in-frame Flag tag sequence fused to the carboxyl terminus of the enzyme was cloned into mammalian expression vector pcDNA3.1 at EcoR1/Xho1 sites. The resultant vector was transfected into 293T cells using Lipofectamine 2000 reagent (Invitrogen). Stable transformants were selected in media containing 500μg/ml G418 and characterized by western blotting analysis using anti-Flag antibody (Sigma). To extract mitochondrial protein, cells were homogenized in the buffer containing 10mM Tris-HCl (pH 7.5), 250mM sucrose and 2mM EDTA on ice with a glass pestle douncer. Mitochondria were isolated from homogenates through a two-step differential centrifugation procedure at 600 and 11,000g for 10m each at 4°C. Mitochondrial protein was then extracted in 100mM potassium phosphate buffer (pH 7.4) containing 0.1% NP-40, 10mM DTT and 1× protease inhibitor cocktail (Sigma). TDH activity was determined by measuring the rate of formation of NADH at 25°C. The assay mixture contained 100mM Tris-HCl (pH 8.0), 25mM L-threonine, 5mM NAD^+ and 25mM NaCl in a final volume of 100μl. The reaction was initiated by the addition of mitochondrial protein extract, and absorbance of the reaction mixture at 340nM was recorded continuously on a microplate reader.

Generation of TDH antibody.
Rabbit polyclonal antibodies to the mouse threonine dehydrogenase enzyme were produced using the synthetic peptides NH2-PMILDDSNARKDWGWKHDFD-COOH and NH2-LSDIRKPPAHVFHSGPFVYAN-COOH. 5mg of each peptide was conjugated to

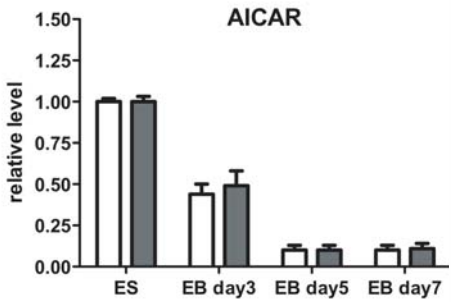
KLH carrier protein, mixed with complete Freund's adjuvant and injected into rabbits. Final bleeds containing TDH antibodies were taken after three additional immunizations in incomplete Freund's adjuvant.

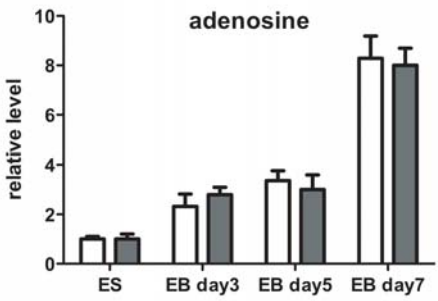
Thymidine incorporation assay.
After growth on 24-well plate for 1 day, mouse ES and HeLa cells were treated as indicated and metabolically labeled with [^{3}H]thymidine (2μCi per well) for 3 hours. Following three washes with PBS, cellular macromolecules were precipitated by a 15m incubation of cells on ice with 5% trichloroacetic acid, and then suspended in 0.5 N NaOH/0.5% SDS for liquid scintillation counting on a Beckman counter.

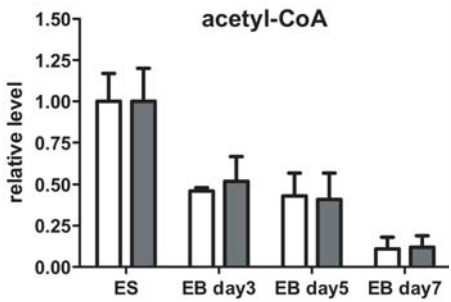
***in situ* hybridization and immunohistochemistry.**
Peri-implantation mouse embryos were subjected to *in situ* hybridization by use of the methods of C. Chazaud et al., (*Dev. Cell*, **10**, 615, 2006) and D. G. Wilkinson and M.A. Nieto, (*Methods Enzymol.* **225**, 361, 1999). Sense and anti-sense probes were prepared from a full length cDNA clone of the mouse TDH gene. For antibody staining, peri-implantation embryos were fixed for 2h in 4% paraformaldehyde in phosphate buffered saline (PBS). Following fixation embryos were rinsed 3 times for 5m in Tris-buffered saline supplemented by 0.5% Tween-20 (TBST). Blocking was carried out for 1h in 3% donkey serum in Tris-buffered saline supplemented with 0.1% Triton-X100. Primary antibody to the TDH enzyme (10) was diluted 1:500 in blocking buffer and incubated with embryos overnight at 4ºC. Embryos were rinsed 3 times for 5m in TBST. Secondary antibody, donkey anti-rabbit antibodies conjugated to Alexa488 was diluted 1:500 in blocking buffer and incubated with embryos for 2h at room temperature. Embryos were rinsed 3 times for 5m in TBST and visualized on an Axiovert fluorescence microscope (Zeiss) and photographed with a Hamamatsu Orca-ER camera.

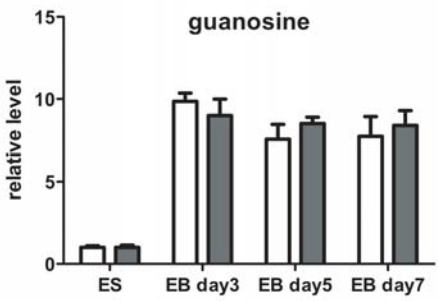
Culture of mouse embryos.
Eight-week old B6SJLF1 female mice were superovulated by a standard hormone regimen and mated to males of the same strain background. Fertilized one-cell eggs were cultured in microdrops of Brinster's medium for ovum culture (R.L. Brinster, *The Mammalian Oviduct*, University of Chicago Press, 419-444) under silicone oil in a 5% CO_2 humidified atmosphere for 48h. Pre-compacted morulae were separated into groups of 20-25 embryos and placed into microdrops of Brinster's medium containing 1mM, 300uM, 100uM and 30uM levels of 3-HNV with or without the addition of 4mM threonine. Embryos were visually scored for developmental stages at 24h and 48h following the initiation of drug administration and photographed by light microscopy.

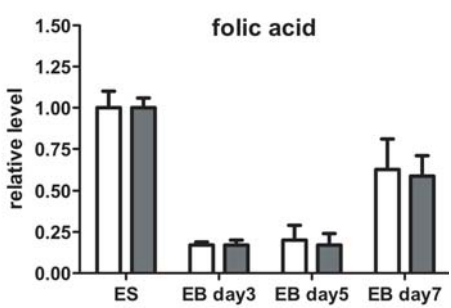
Supplemental Data
Figure S1

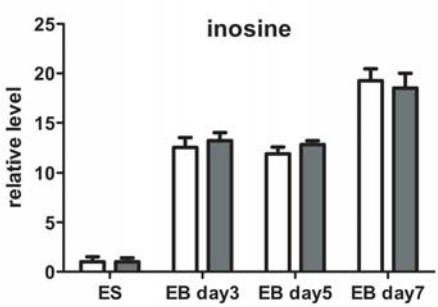


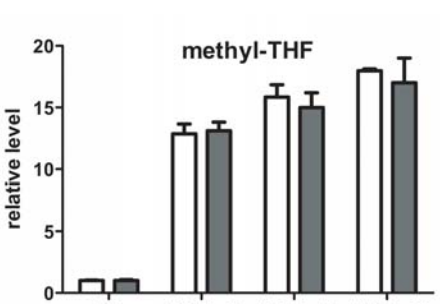


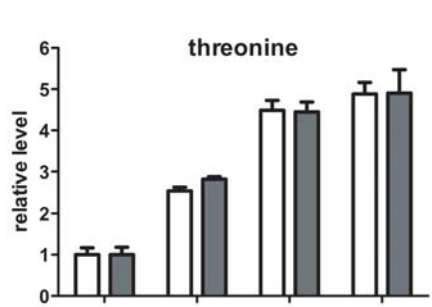




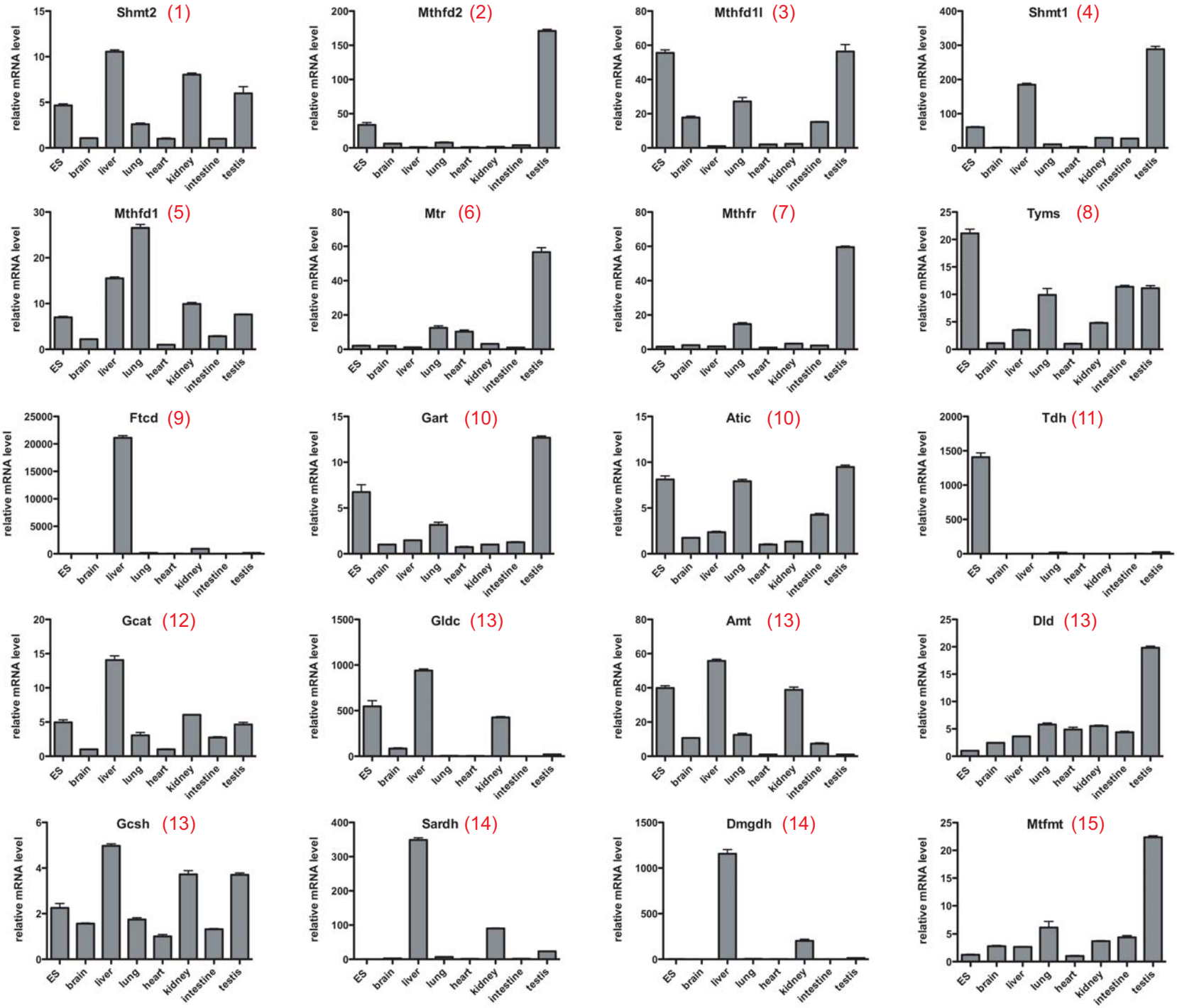




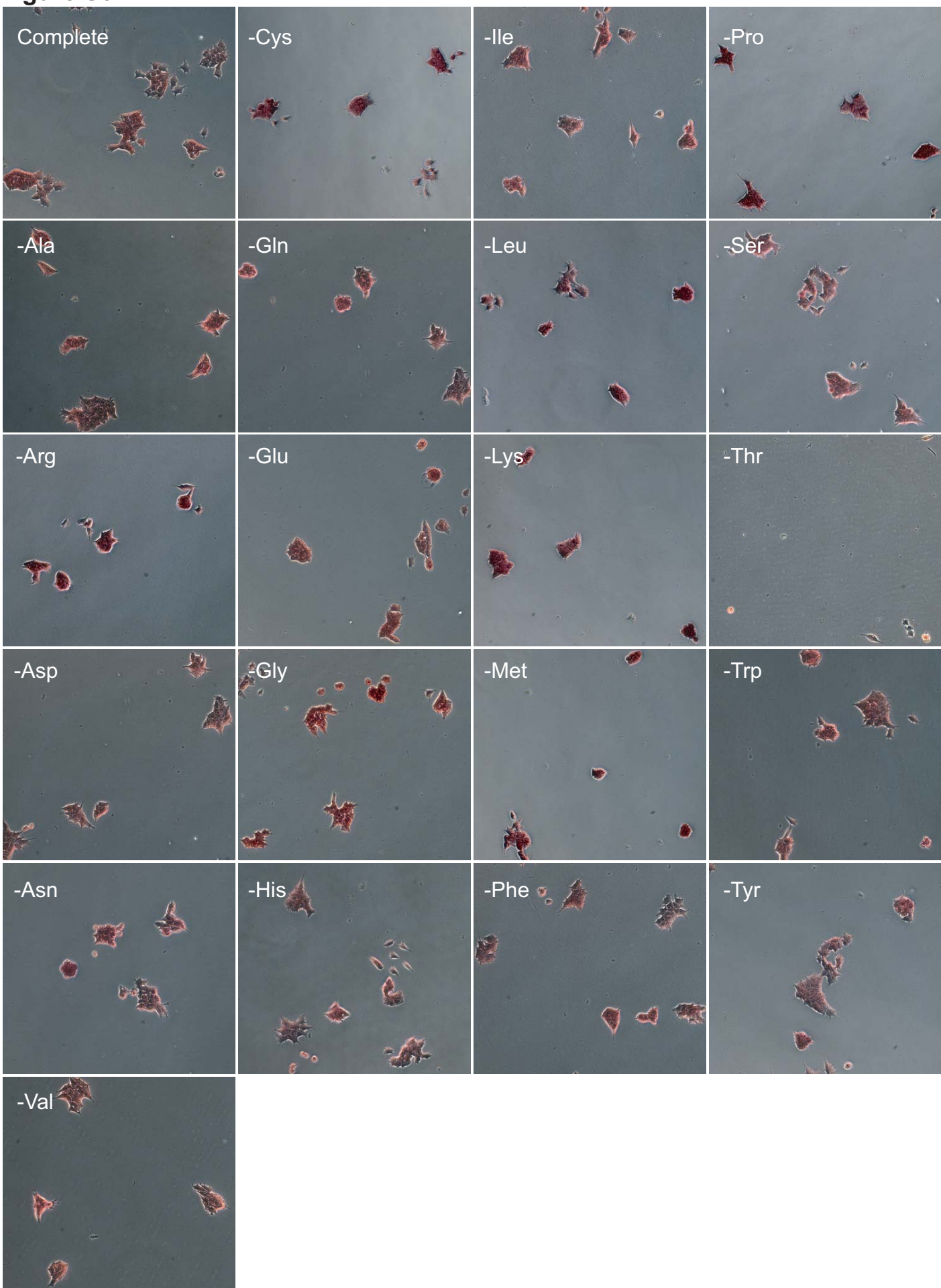




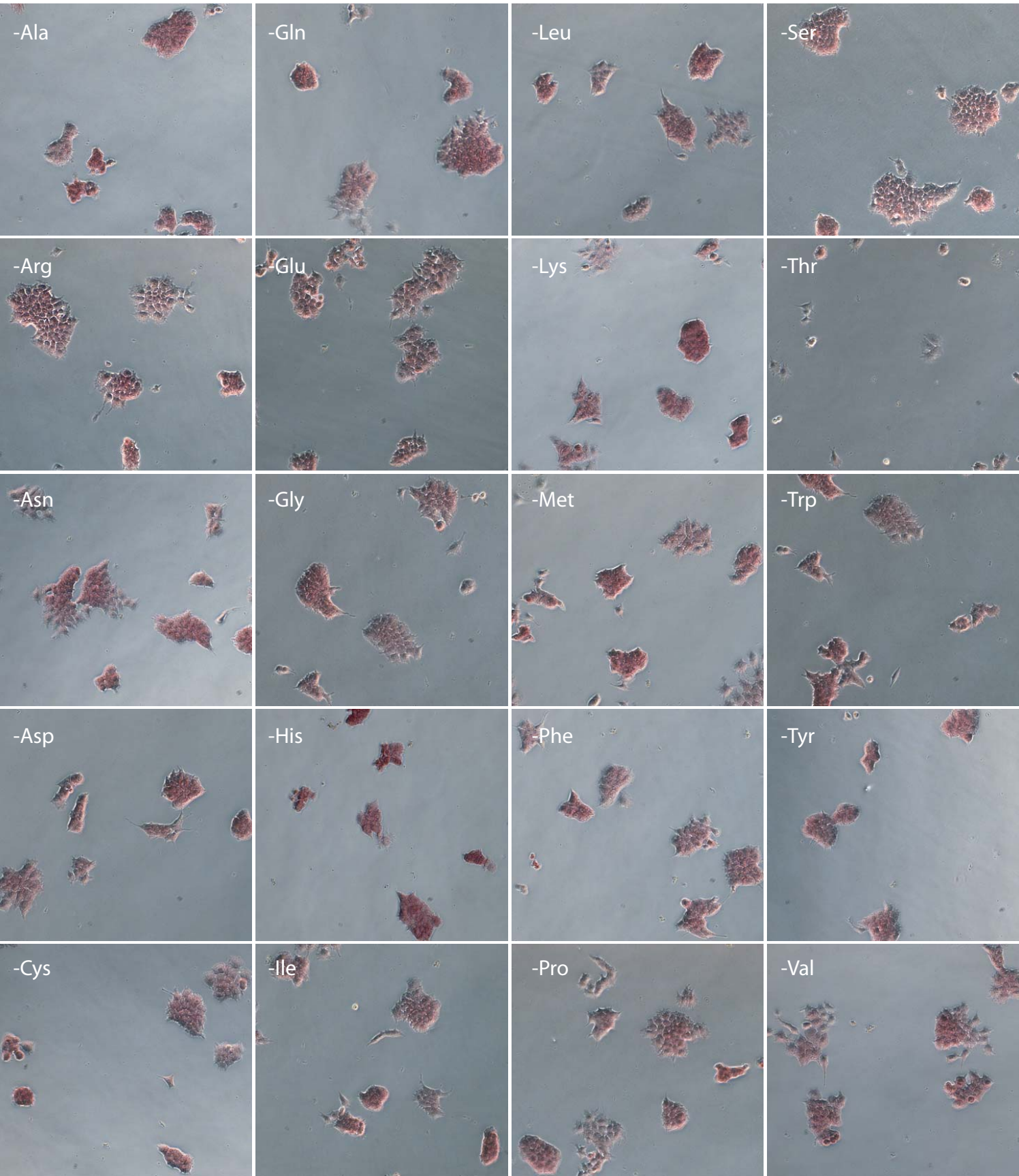
Supplemental Data
Figure S2



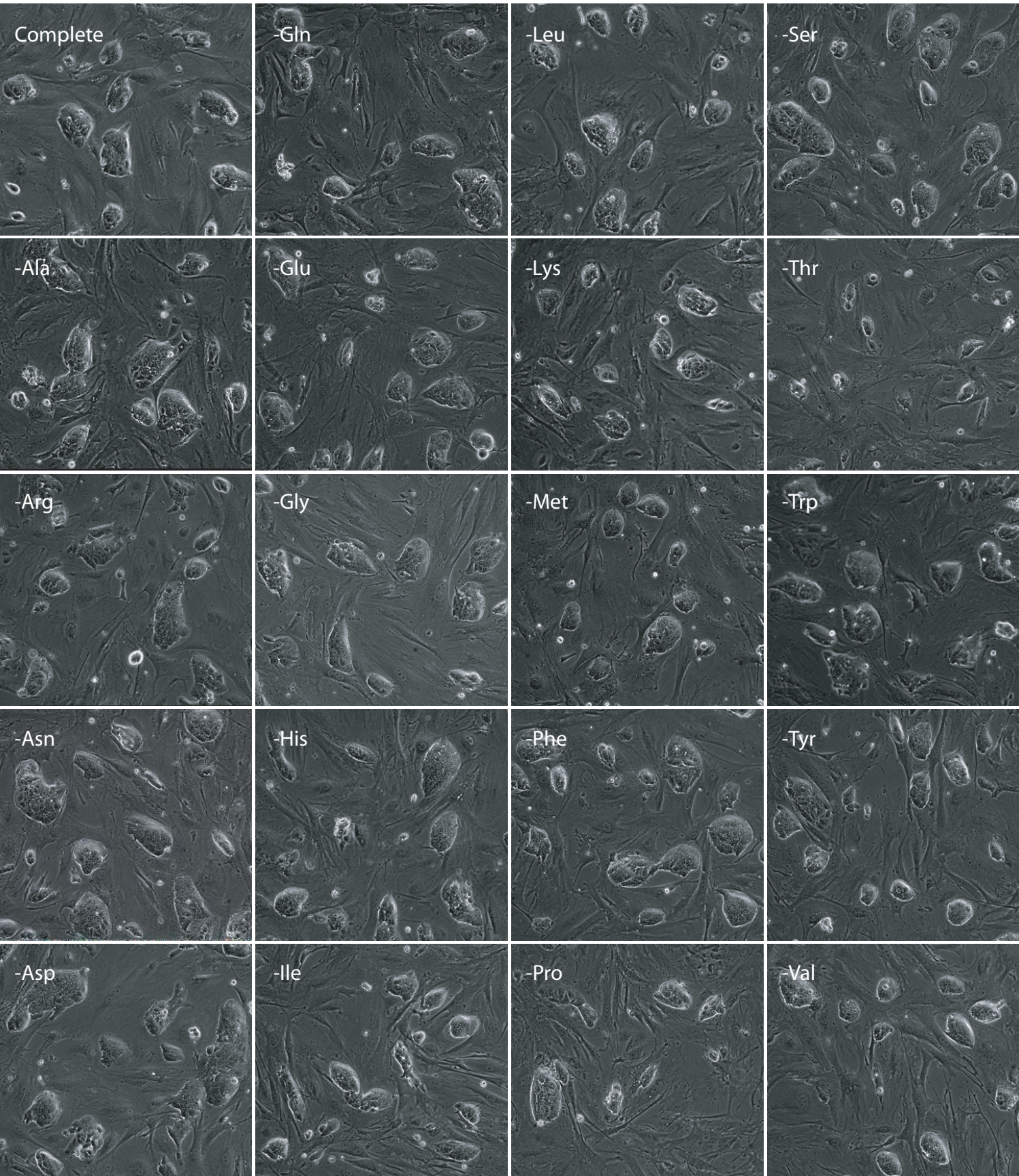
Supplemental Data
Figure S3A



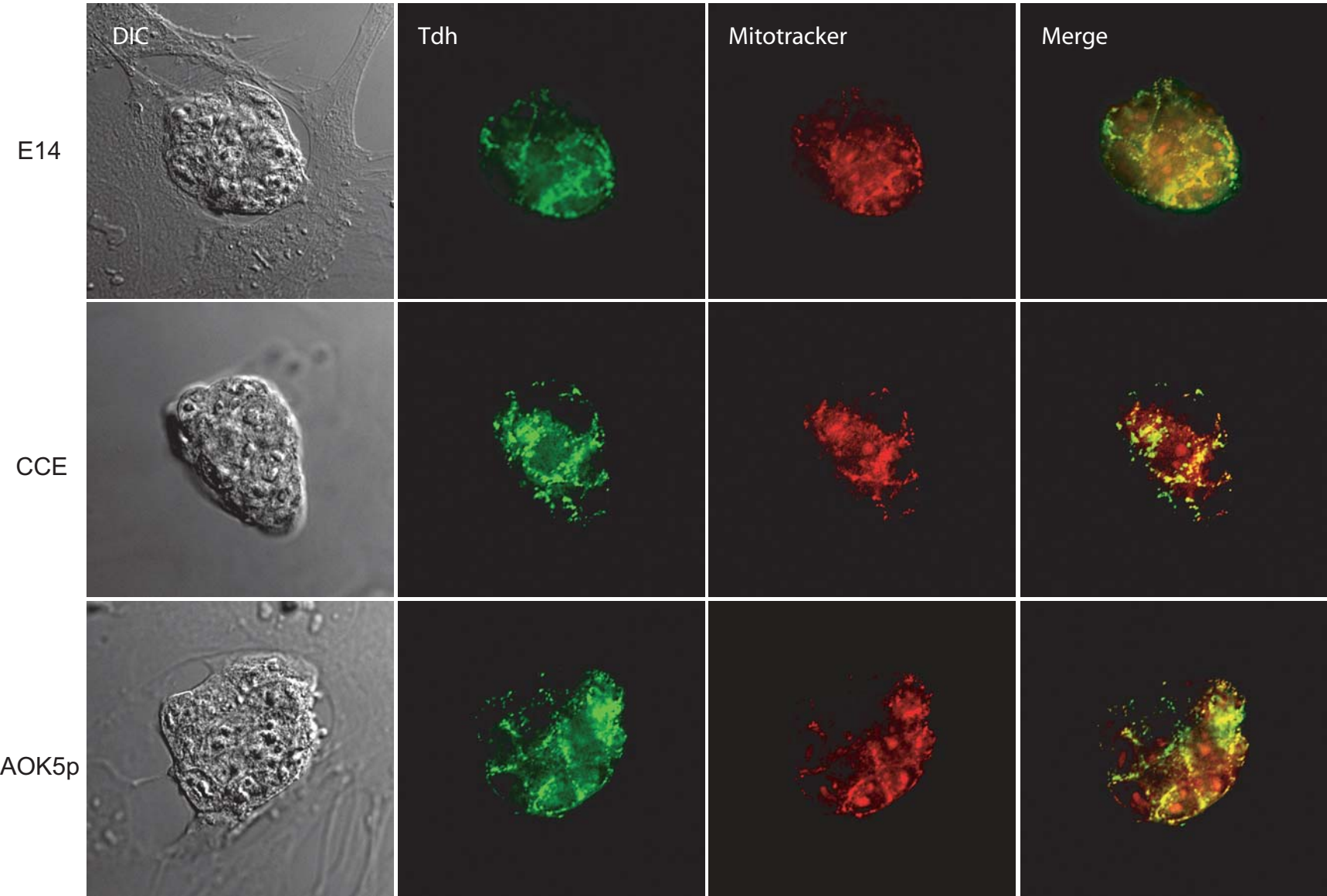
Supplemental Data
Figure S3B



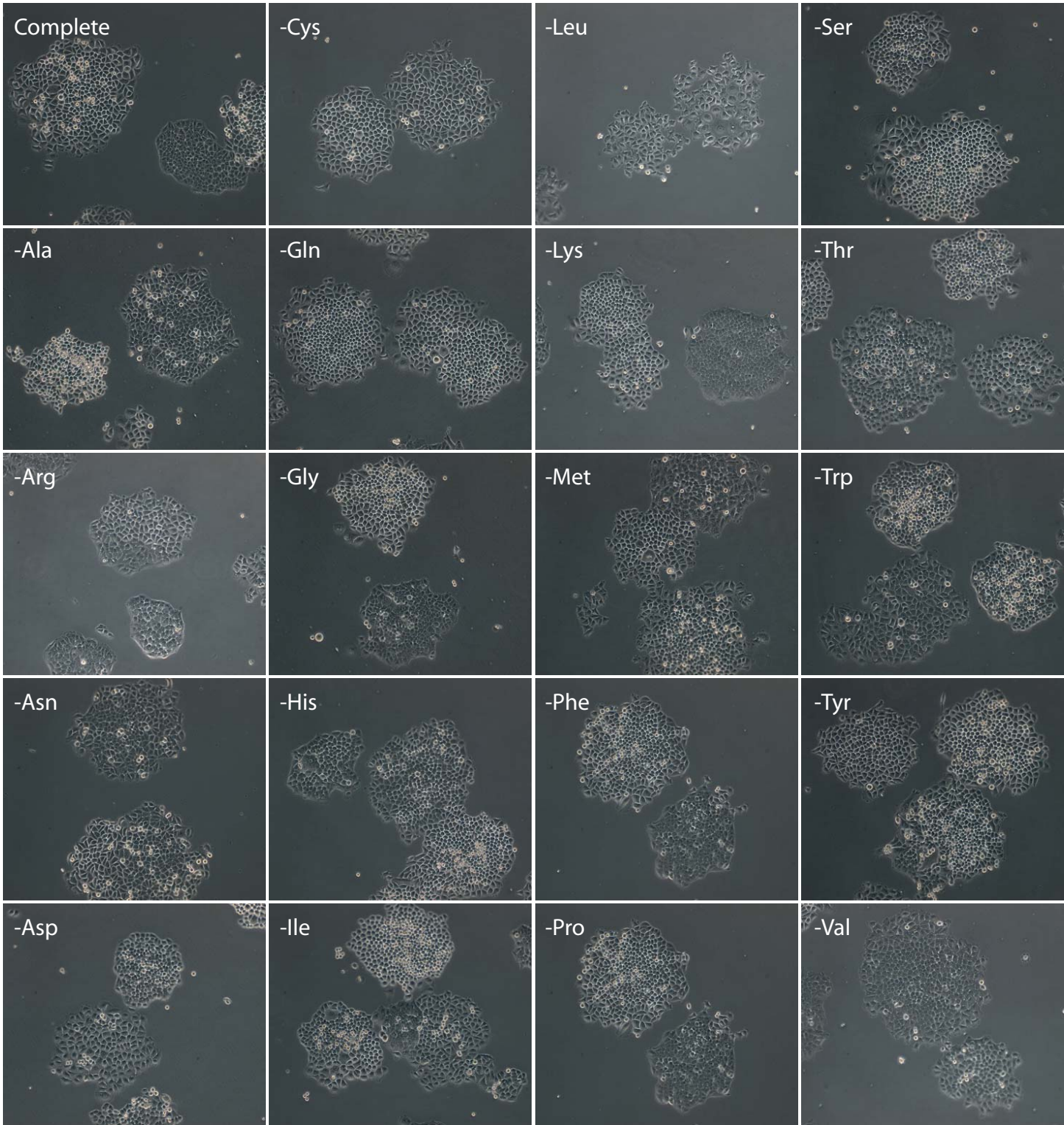
Supplemental Data
Figure S3C



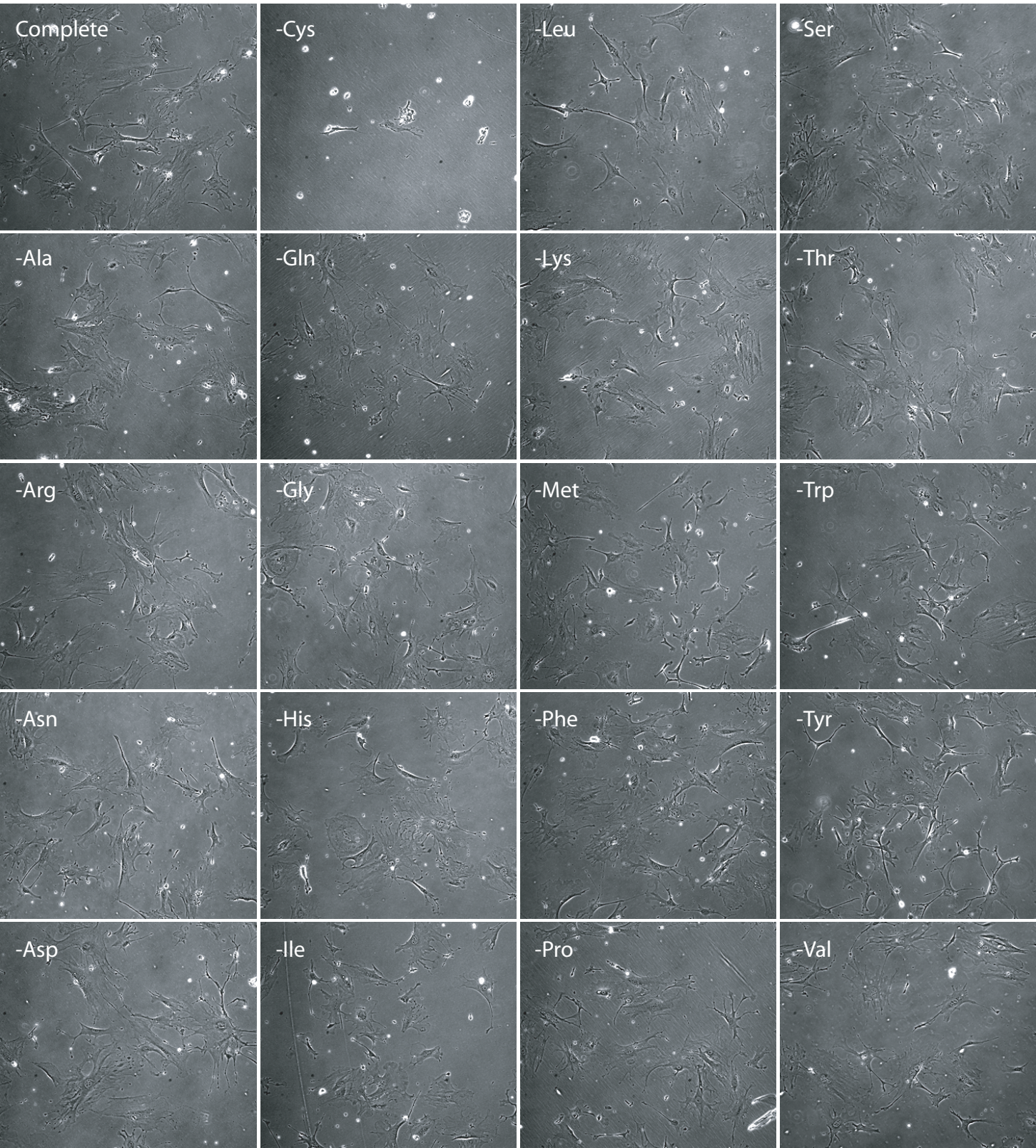
Supplemental Data
Figure S4



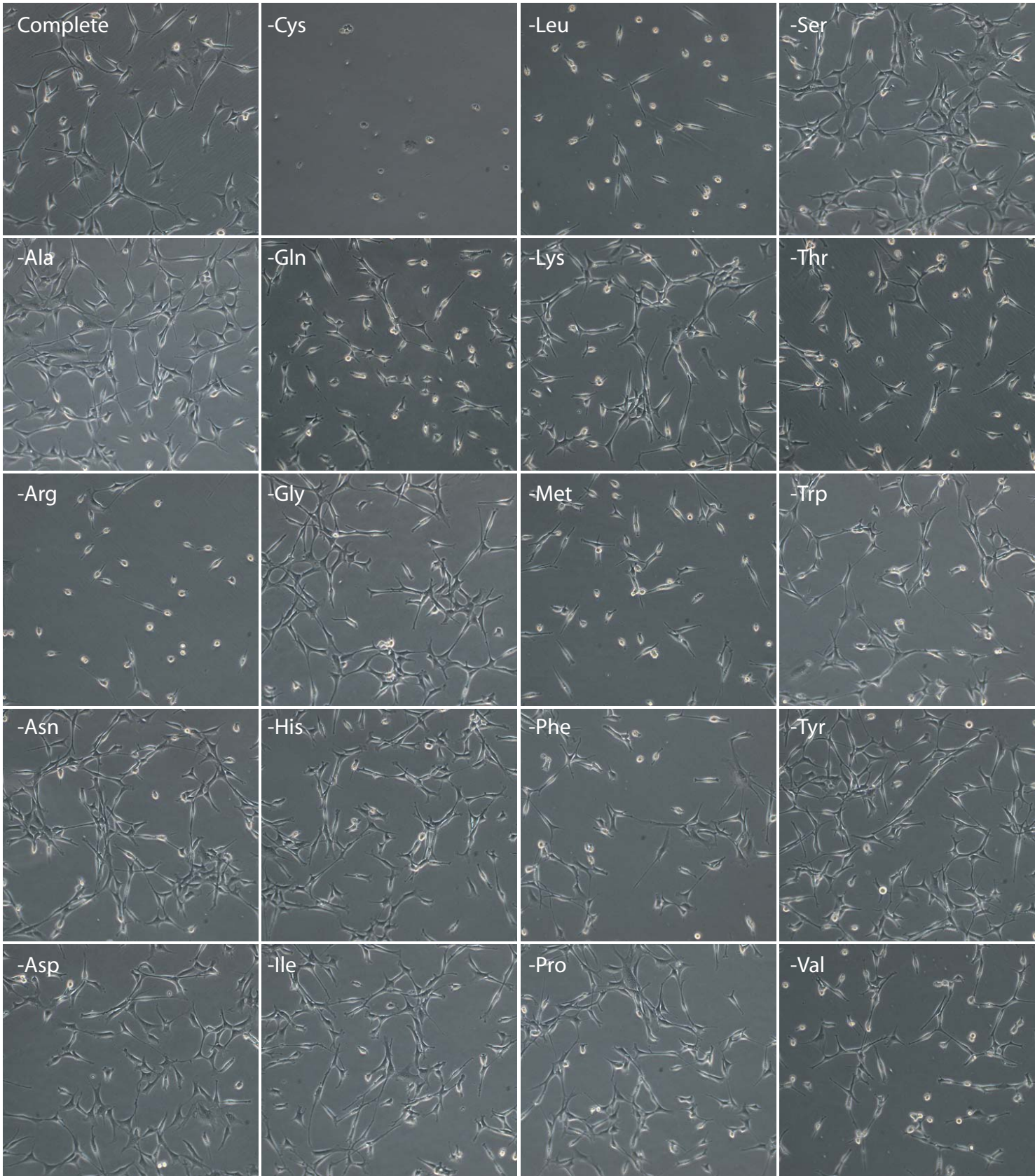
Supplemental Data
Figure S5A



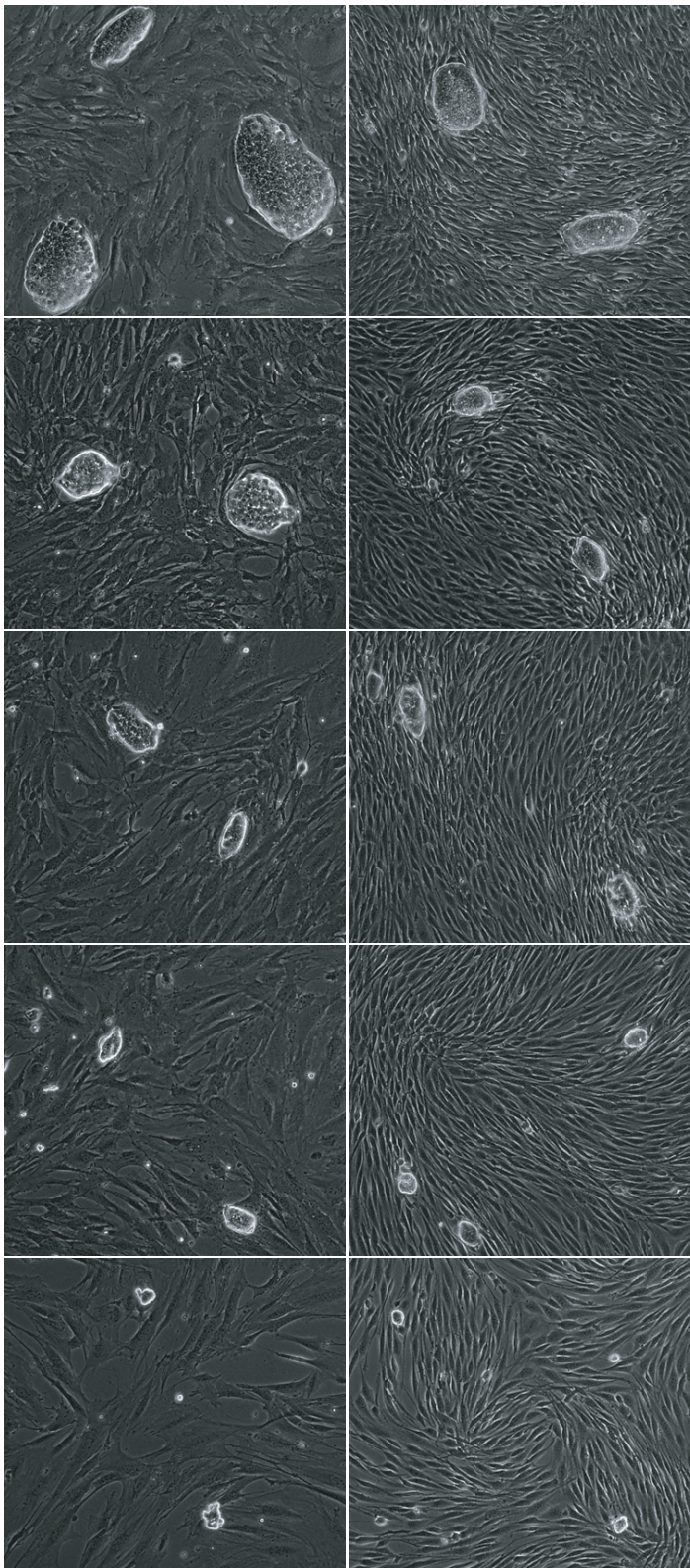
Supplemental Data
Figure S5B



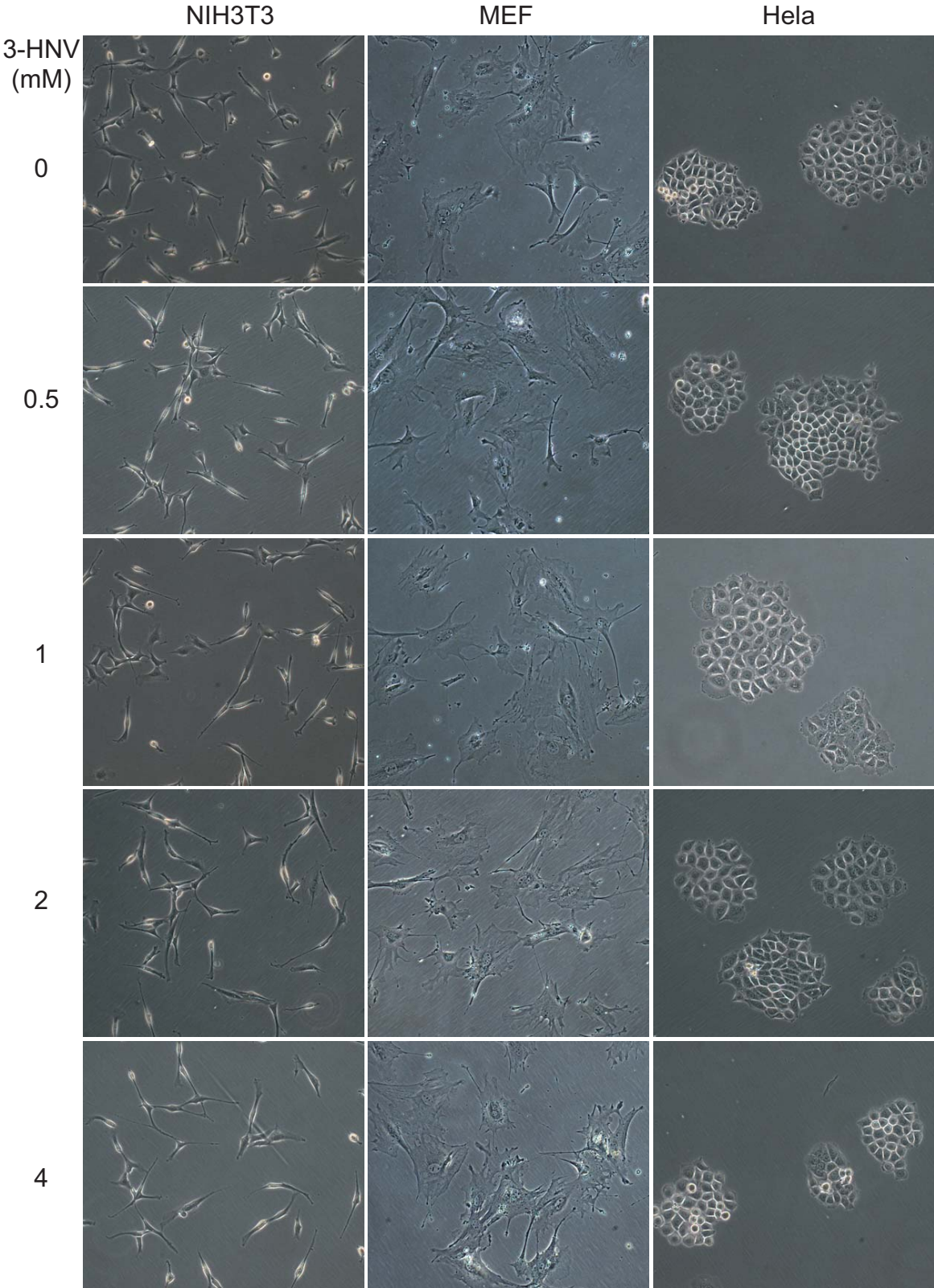
Supplemental Data
Figure S5C



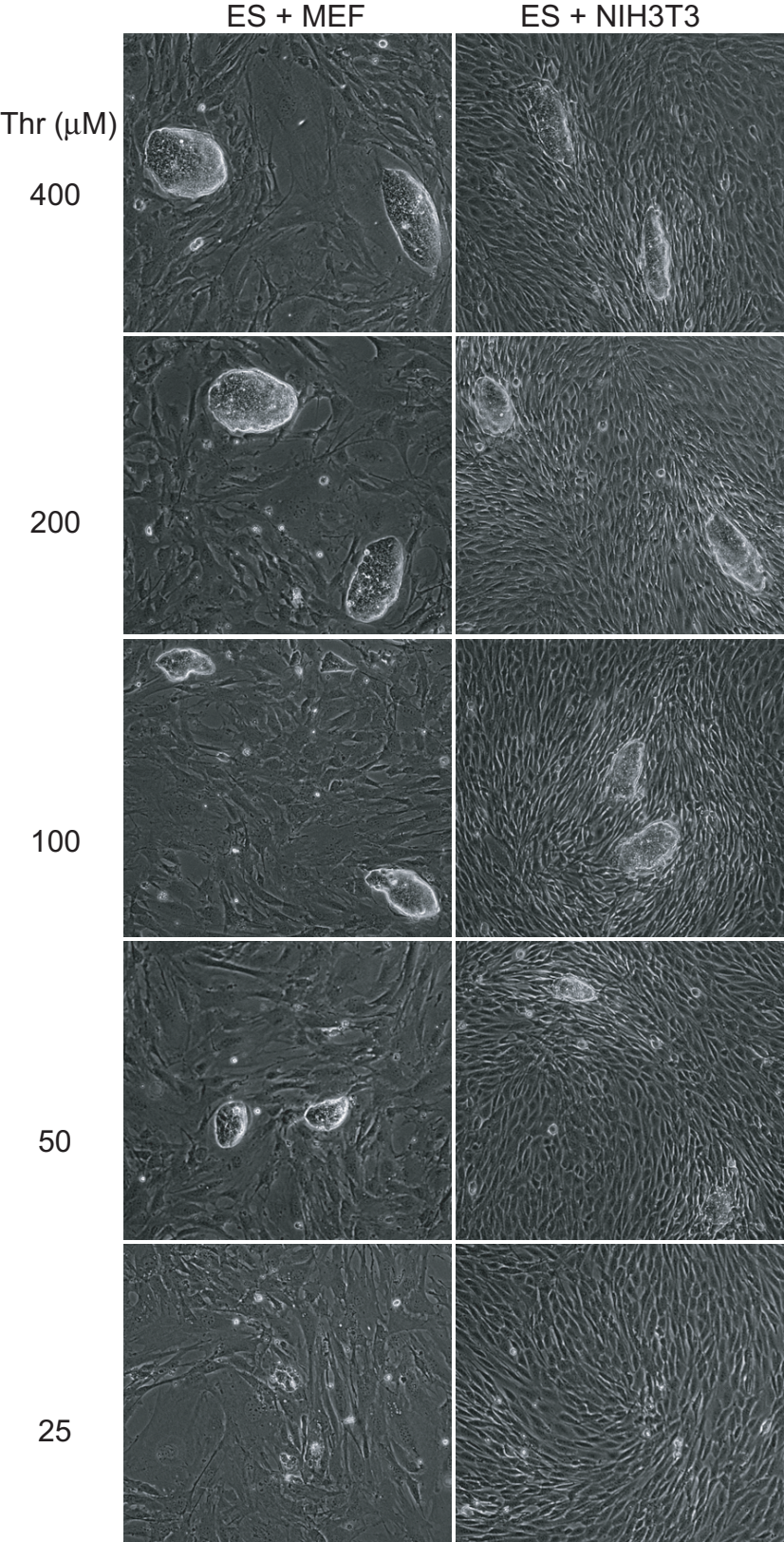
Supplemental Data
Figure S6A



Supplemental Data
Figure S6B

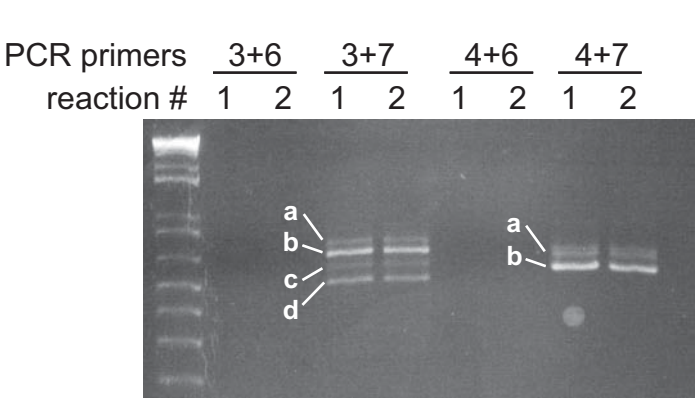


Supplemental Data
Figure S7

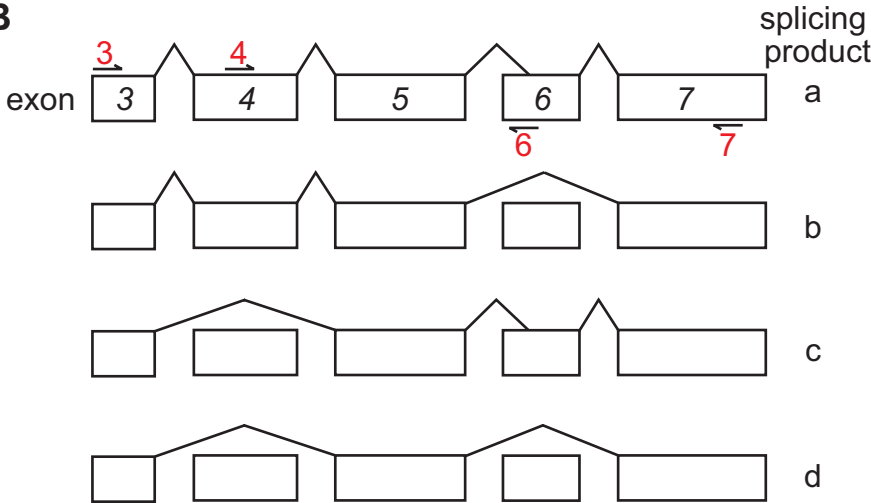


Supplemental Data
Figure S8

A



B



Supplemental Data, Figure S1. Coordinated changes in metabolite abundance during ES cell differentiation.

Bar graphs showing the fold change of indicated metabolites. White and grey bars denote quantifications from the measurements of two daughter ions of each metabolite. Experiments were performed in triplicate, with error bars indicating $^{+/-}$SD.

Supplemental Data, Figure S2. Robust expression of TDH mRNA in ES cells.

qPCR analyses of mRNA abundance of the numbered enzymes (Figure 1B) in ES cells as compared with seven tissues of the adult mouse. Two of the metabolic steps displayed in (Figure 1B), use of glycine to charge tetrahydrofolate via the glycine cleavage system and formyl-tetrahydrofolate carbon donation for purine synthesis, involve more than one polypeptide. In these cases, the mRNA abundance for each relevant enzyme subunit was quantified by qPCR. For each qPCR comparison, the tissue sample showing the lowest signal was arbitrarily set at a numerical value of 1. Experiments were performed in triplicate, with error bars indicating $^{+/-}$SD.

Supplemental Data, Figures S3. Growth dependence of the ES cell line E14.Tg2A, CCE and iPS cell line AOK-5P on threonine supplementation to culture medium.

(A and B) Following plating at single cell density and growth on gelatinized dishes for 6h, cells of the E14Tg2A (A) or CCE (B) line of mouse ES cells were exposed for 36h to complete culture medium or that prepared to be missing a single amino acid. Colonies were stained with an alkaline phosphatase detection kit (Chemicon), and photographed under a Zeiss AxioObserver microscope using bright field optics.

(C) Following plating at single cell density and growth on irradiated MEF feeder cells for 6h, cells of the AOK-5P line of mouse iPS cells were exposed for 36h to complete culture medium or that prepared to be missing a single amino acid. Colonies were visualized on a Zeiss AxioObserver microscope using phase contrast optics.

Supplemental Data, Figure S4. Immunohistochemical staining of ES and iPS cells with antibodies specific to TDH.

Colonies of the E14Tg2A line of mouse ES cells (top), CCE line of mouse ES cells (middle) and AOK-5P line of mouse iPS cells (bottom) were grown on MEF feeder layers in chamber slides. After fixation cells were stained with TDH-specific antibodies, followed by Alexa488-labeled goat anti-rabbit secondary antibodies (green). Prior to fixation cells were exposed to Mitotracker dye as a

means of localization of mitochondria (red). Merged images show that TDH immunoreactivity co-localized with mitochondria.

Supplemental Data, Figure S5. Effects of amino acid dropout on the growth of HeLa, MEF and 3T3 cells.

Hela (A), MEF (B) and 3T3 (C) cells were grown in the indicated media for either one week (HeLa cells) or three days (MEF and 3T3 cells). Cells were visualized by phase contrast microscopy and photographed by use of a Zeiss AxioObserver microscope.

Supplemental Data, Figure S6. Selective inhibition of ES cell proliferation by 3-hydroxynorvaline.

(A) ES cells co-cultured with MEF cells (left panels) or 3T3 cells (right panels) were treated for two days in complete culture medium supplemented with increasing concentrations of 3-hydroxynorvaline (3-HNV). ES colony size decreased as a function of 3-HNV concentration. Neither MEF nor 3T3 cell growth was affected by 3-HNV supplementation.

(B) Tolerance of HeLa, MEF and 3T3 cells to 3-hydroxynorvaline. 3T3 (left panels), MEF (middle panels) or Hela (right panels) cells were grown for two days in complete culture media supplemented with indicated concentrations of 3-hydroxynorvaline. Cells were visualized by phase contrast microscopy and photographed by use of a Zeiss AxioObserver microscope.

Supplemental Data, Figure S7. Effects of threonine deprivation on the growth of ES, MEF and 3T3 cells.

Co-cultures of either ES/MEF, or ES/3T3 cells were subjected for two days to media containing varying amounts of supplemented threonine. Threonine deprivation reduced ES cell colony size at the 100uM and 50uM levels, and fully eliminated ES colony formation at the 25uM level. Threonine deprivation did not affect the growth of MEF or 3T3 cells even at the lowest level tested (see also Supplemental Data, Figure S5).

Supplemental Data, Figure S8. Aberrant splicing of the human TDH mRNA in embryonic liver tissue.

mRNA isolated from two samples of 13 week gestational human liver tissue was converted to cDNA and subjected to PCR amplification using oligonucleotide primers specific to exons 3 and 4 (forward primers), and 6 and 7 (reverse primers) of the human TDH gene. No PCR product was observed in reactions supplemented with the reverse primer prepared from sequences proximal to the 5′ end of exon 6 (A). DNA sequence analysis of PCR reaction products derived

from the “3 X 7” and “4 X 7” primer combinations revealed aberrant splice variants a, b, c and d as schematized in panel (B).