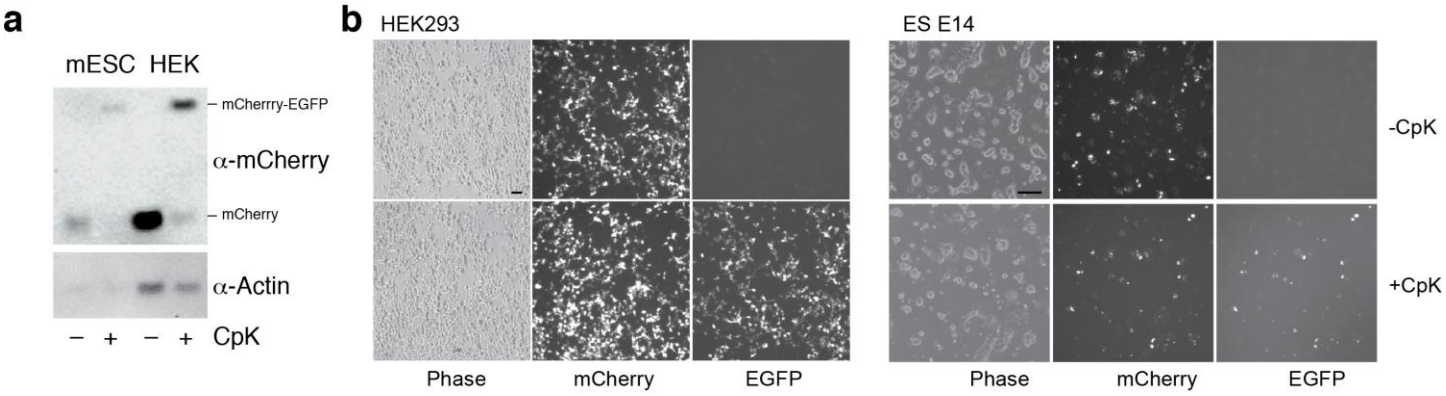
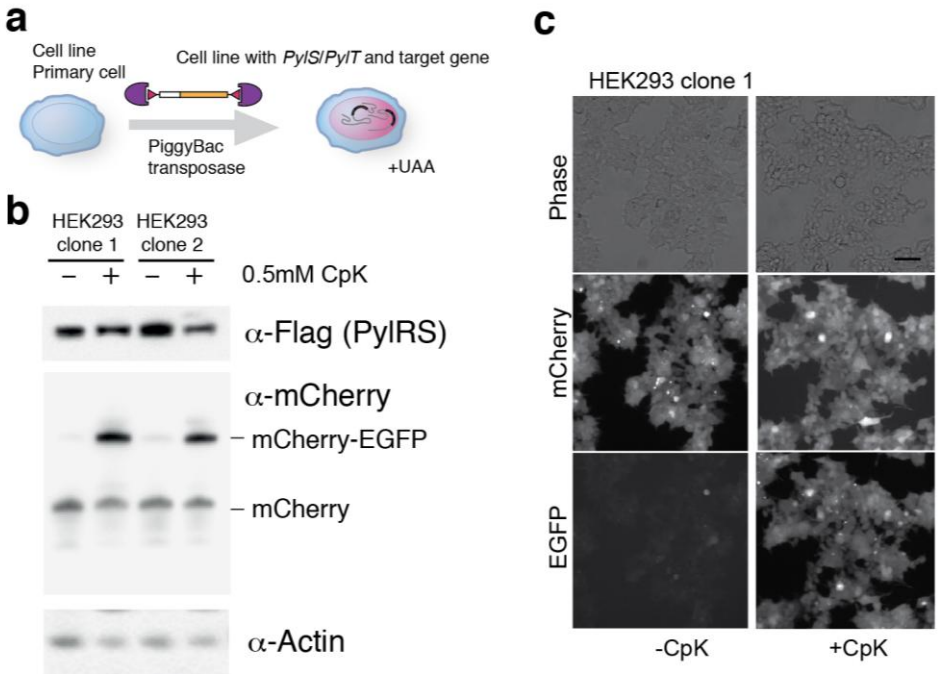


Supplementary Figure 1

Transient transfection leads to heterogeneous levels of an unnatural amino acid–containing protein, with many cells untransfected; this problem is solved by PiggyBac integration of the unnatural amino acid incorporation machinery.

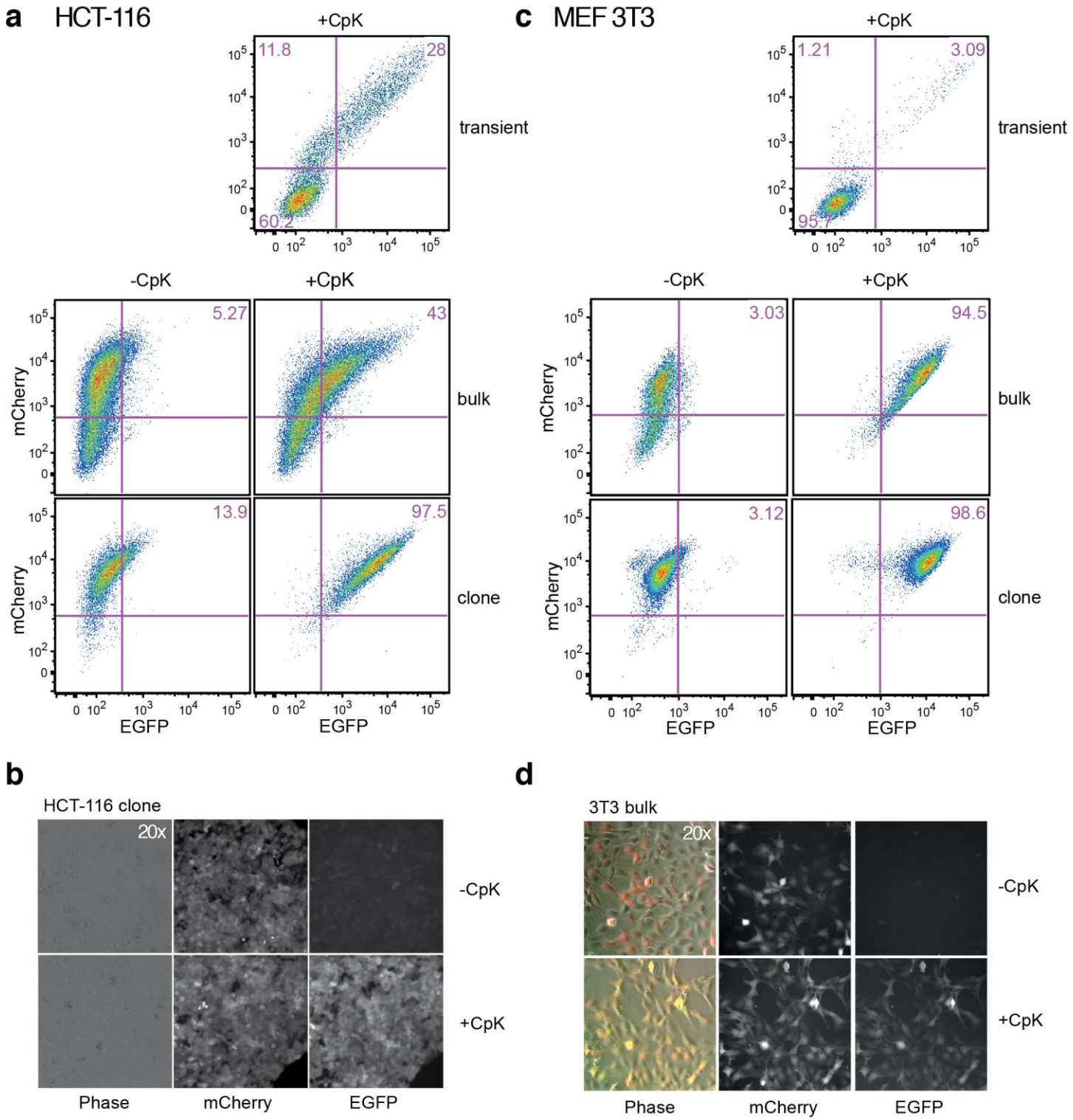
(**a**) Western blot of transiently transfected HEK293 and mESC, with 4x*PylT*/*PylS* and 4x*PylT*/*mCherry-TAG-EGFP*. (**b**) Representative images of transient transfection experiments in HEK293 and mESC, 48 h after transient transfection with 4x*PylT*/*PylS* and 4x*PylT*/*mCherry-TAG-EGFP*, and addition of amino acid (0.5 mM CpK). Scale bars, 100 µm.



Supplementary Figure 2

PiggyBac-mediated generation of HEK293 cell lines for unnatural amino acid mutagenesis.

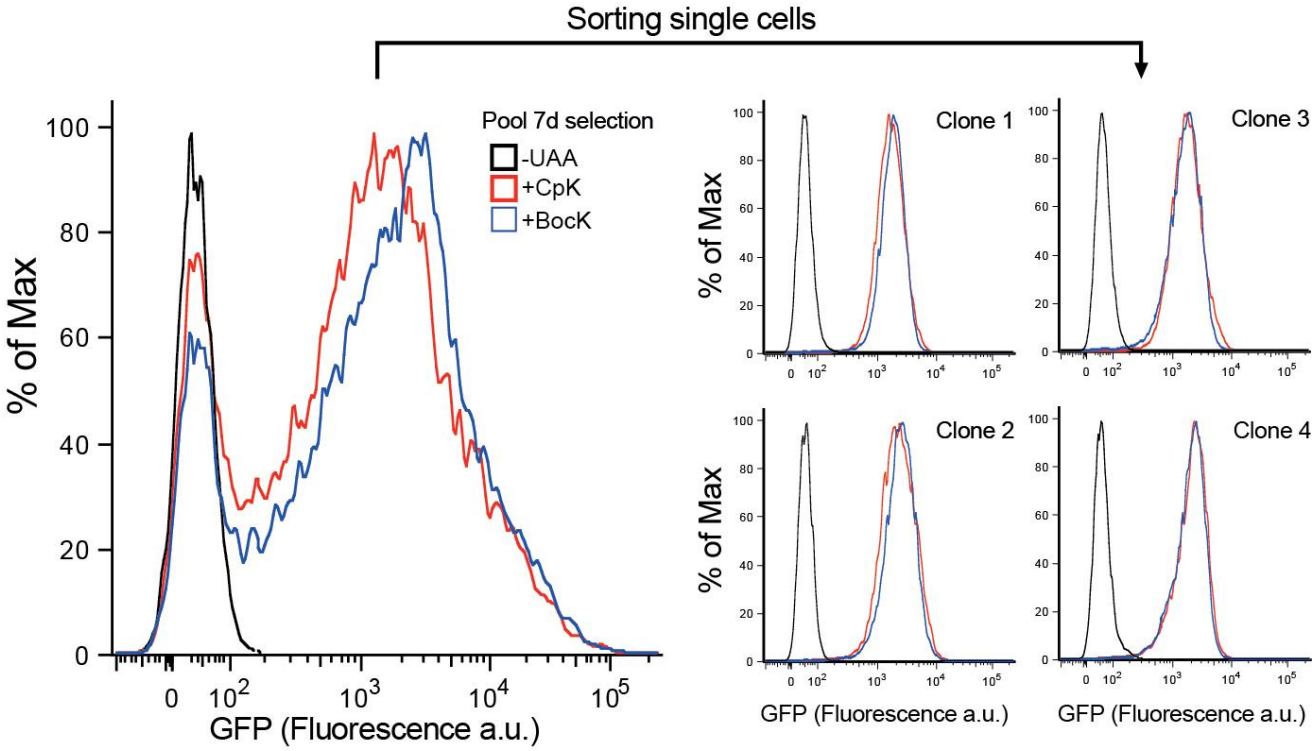
(**a**) Scheme for PiggyBac-mediated generation of stable cell lines bearing pyrrolysyl-tRNA synthetase/tRNA (*PylS*/*PylT*) pair for unnatural amino acid (UAA) incorporation. (**b**) Western blot of two HEK293 cell line stably expressing 4x*PylT*/*PylS* and 4x*PylT* /*mCherry-TAG-EGFP* cassettes, cultured 48 hours with or without addition of 0.5 mM CpK. α-FLAG detects N-terminally tagged PylRS. (**c**) Representative fluorescent microscopy images of a HEK293 cell line, cultured 48 hours with or without addition of 0.5 mM CpK.



Supplementary Figure 3

PiggyBac-mediated generation of HCT-116 human colorectal cancer and 3T3 mouse embryonic fibroblast cell lines for unnatural amino acid mutagenesis.

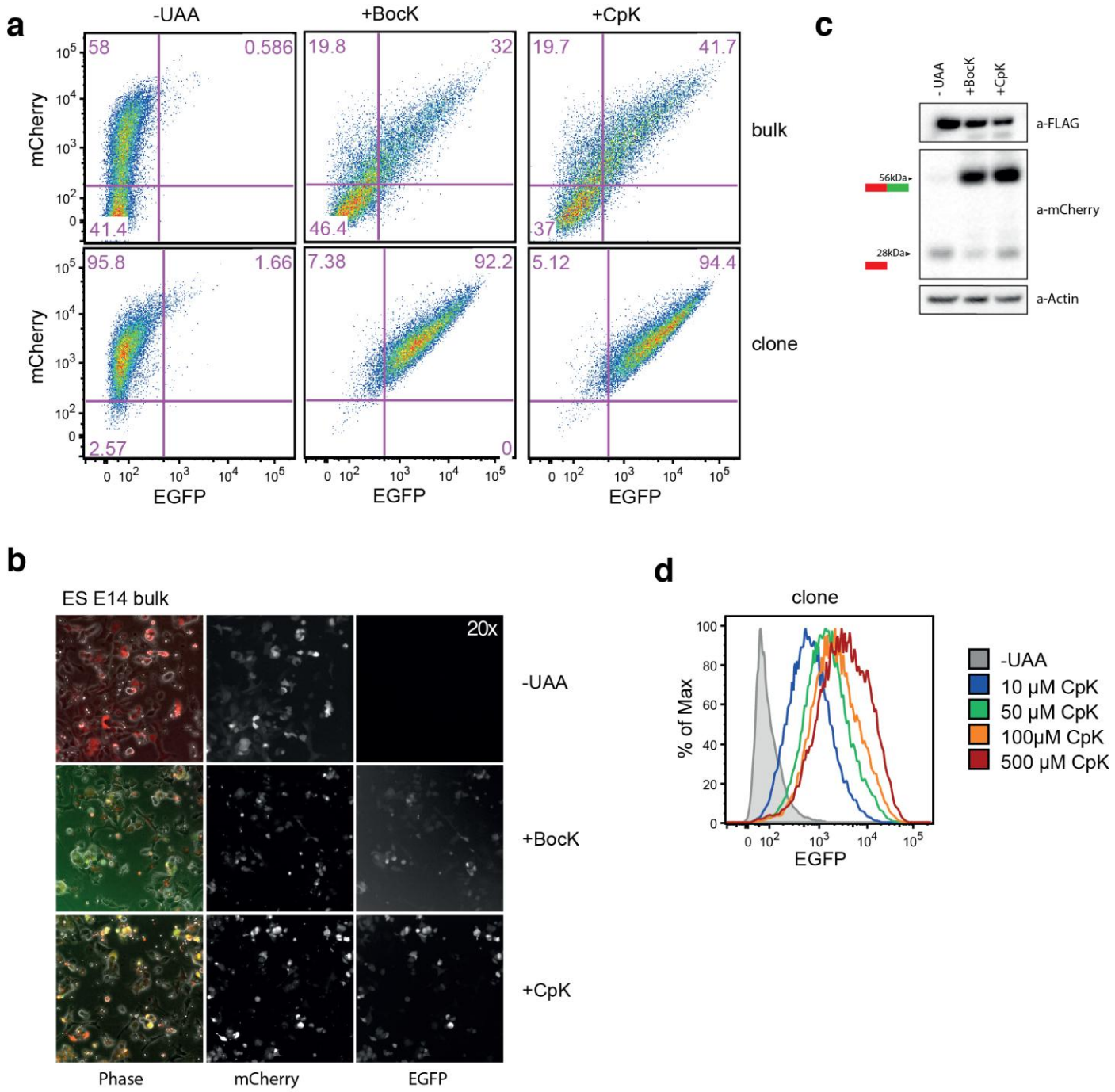
(**a**) FACS analysis of HCT-116 transient (top) and stable (bottom) amber suppression using the 4x*PylT*/*PylS* and 4x*PylT /mCherry-TAG-EGFP* cassettes targeting vectors shown in **Figure 1a**. A drug selected bulk population and a derived single clone is shown. Cells were grown for 48 hours with or without 0.5 mM CpK. (**b**) Representative image of experiment in **a**. (**c**) FACS analysis of MEF 3T3 transient (top) and stable (bottom) amber suppression using the targeting vectors shown in **Figure 1a**. A drug selected bulk population and a derived single clone is shown. Cells were grown for 48 hours with or without 0.5 mM CpK. (**d**) Representative image of experiment in **c**.



Supplementary Figure 4

PiggyBac-mediated generation and differentiation of mouse embryonic stem cell lines for unnatural amino acid mutagenesis.

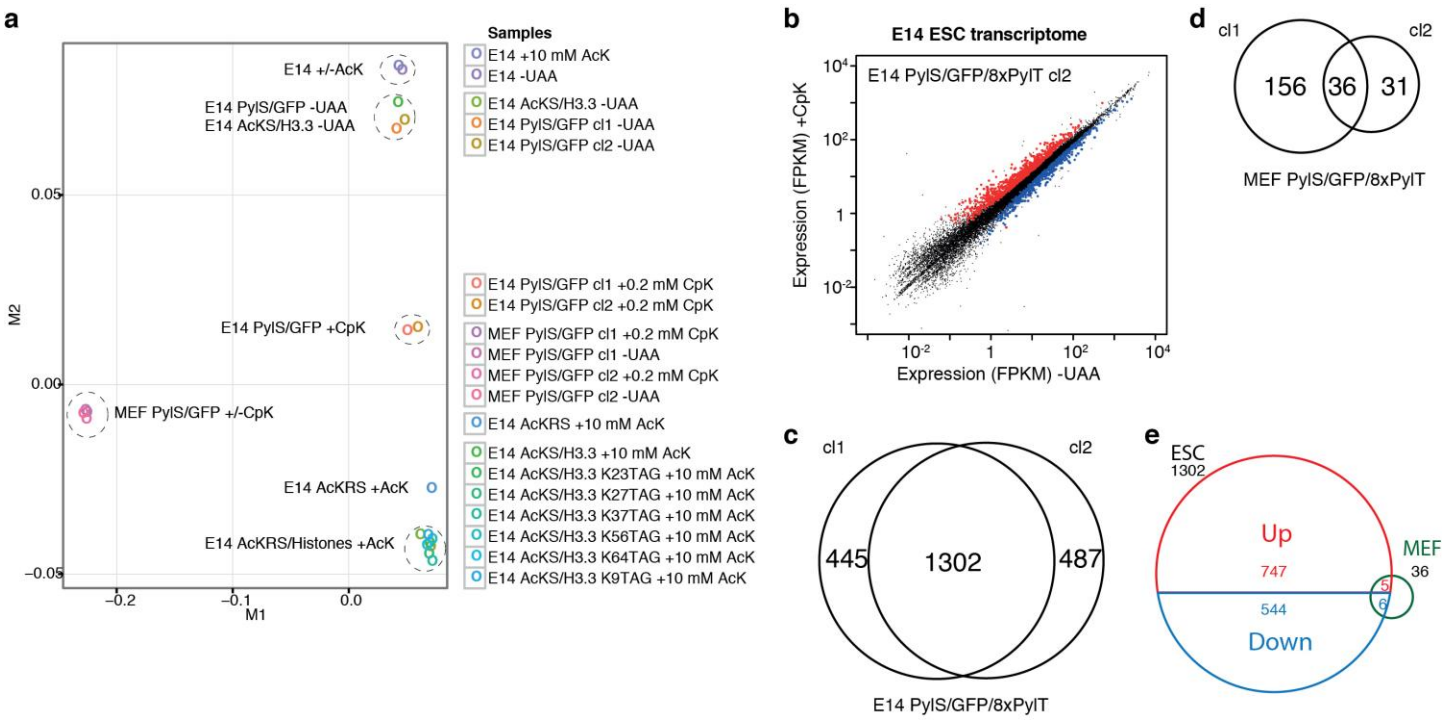
FACS analysis of bulk drug selected ESC population bearing 4x*PylT/PylS* and 4x*PylT/sfGFP*^{150TAG} and four clones derived from the bulk selection, grown in the absence (-UAA) or presence of 0.2 mM CpK or 2 mM BocK for 48 hours.



Supplementary Figure 5

PiggyBac-mediated generation of mouse embryonic stem cell lines for unnatural amino acid mutagenesis.

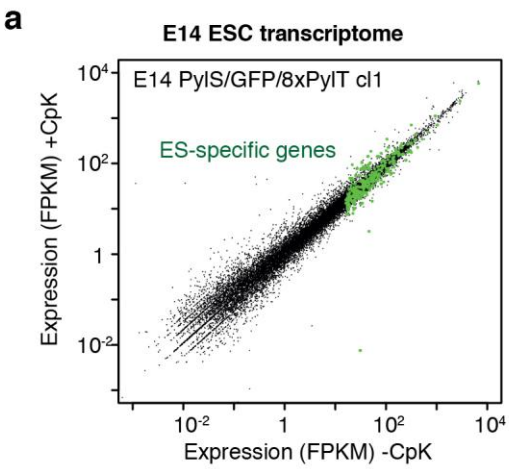
(**a**) FACS analysis of E14 mouse ESC generated with 4x*PylT*/*PylS* and 4x*PylT/mCherry-TAG-EGFP* targeting vectors shown in **Figure 1a**. A drug selected bulk population (top) and a derived single clone (bottom) is shown. Cells were grown for 48 hours without unnatural amino acid (UAA), with 2 mM BocK or 0.5 mM CpK. (**b**) Representative image of the bulk selected population from **a**. (**c**) Western blot of the bulk selected population, showing PylRS expression with α-FLAG and high read-through of the amber codon as judged by α-mCherry western blot. Actin is shown as a loading control. (**d**) Dose-dependent amber suppression (EGFP production) as measured by FACS in the clone shown in **a**. Cells were grown in the presence of indicated concentrations of CpK for 48 hours.

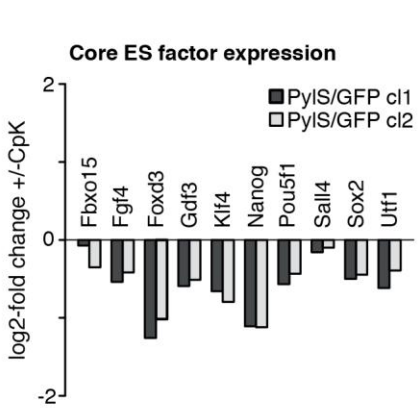


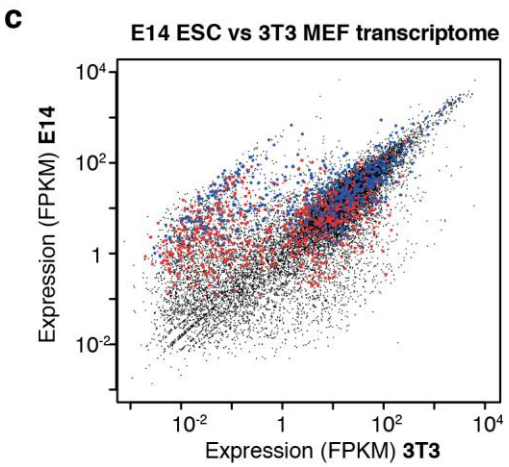
Supplementary Figure 6

RNA-seq analysis of E14 ESC and MEF cell lines incorporating CpK or AcK unnatural amino acids.

(**a**) Multidimensional scaling (MDS) plot summarizing all RNA-Seq datasets used in this study. The first two principle components are roughly corresponding to cell type specific differences (M1) and an effect of amber suppression in ESC (M2). Wild type E14 cell lines in the presence or absence of unnatural amino acid cluster closely with amber suppression cell lines in the absence of unnatural amino acid. (**b**) RNA-seq analysis of a second clone bearing 4x*PylT/PylS* and 4x*PylT/sfGFP*^{150TAG}, a biological replicate of **Figure 3a**. Whole transcriptome FPKM values in the presence versus absence of 0.2 mM CpK for 48h are plotted. Common significantly (P<0.005) up- and downregulated genes are colored in red and blue, respectively. (**c**) Venn diagram showing the overlap of significantly (P<0.005) dysregulated genes among two independent clonal cell lines shown in **Figure 3a** and **Supplementary Figure 6b**. (**d**) Venn diagram showing the overlap of significantly (P<0.005) dysregulated genes among two independent MEF cell lines. (**e**) Venn diagram of significantly deregulated genes in ESC and MEF, further divided into up- and downregulated gene sets in ESC.







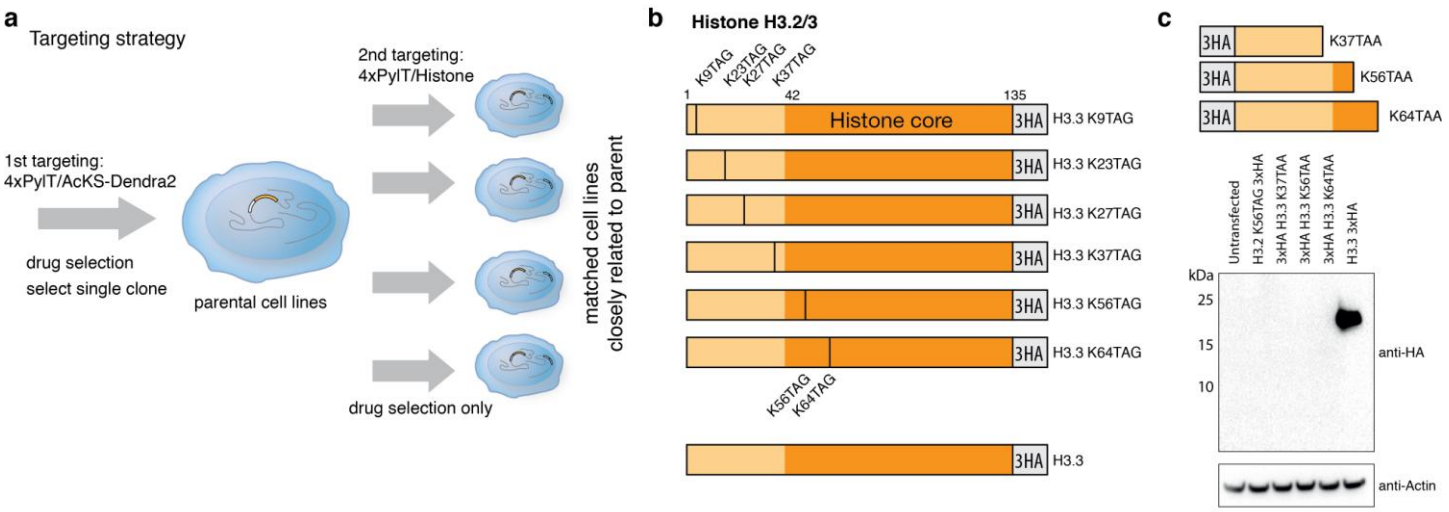
d

Top 10 GO terms upregulated					Top 10 GO terms downregulated				
GO Term	Freq	Datse	E Value	p Value	GO Term	G Fre	Datse	E Value	p Value
positive regulation of biological process (GO:0048518)	4387	232	1.42E+02	1.18E-11	metabolic process (GO:0008152)	8031	305	1.81E+02	1.09E-25
developmental process (GO:0032502)	4453	230	1.44E+02	2.29E-10	organic substance metabolic process (GO:0071704)	7455	282	1.68E+02	7.82E-22
regulation of molecular function (GO:0065009)	2082	133	6.74E+01	2.70E-10	cellular metabolic process (GO:0044237)	6983	269	1.57E+02	2.64E-21
single-organism process (GO:0044699)	11k	481	3.82E+02	4.24E-10	single-organism process (GO:0044699)	11k	376	2.66E+02	1.91E-20
single-organism developmental process (GO:0044767)	4435	228	1.44E+02	5.25E-10	primary metabolic process (GO:0044238)	7178	269	1.61E+02	2.28E-19
positive regulation of molecular function (GO:0044093)	1192	88	3.86E+01	7.51E-09	single-organism metabolic process (GO:0044710)	3648	173	8.21E+01	2.71E-19
multicellular organismal development (GO:0007275)	3901	203	1.26E+02	8.18E-09	cellular process (GO:0009987)	12k	383	2.85E+02	3.90E-16
anatomical structure development (GO:0048856)	3943	204	1.28E+02	1.21E-08	small molecule metabolic process (GO:0044281)	1744	103	3.92E+01	1.46E-15
regulation of response to stimulus (GO:0048583)	2756	156	8.92E+01	1.81E-08	biosynthetic process (GO:0009058)	3415	157	7.68E+01	1.60E-15
regulation of signal transduction (GO:0009966)	2160	129	6.99E+01	7.31E-08	organic substance biosynthetic process (GO:1901576)	3349	151	7.53E+01	6.38E-14
regulation of signaling (GO:0023051)	2445	141	7.91E+01	7.58E-08	carboxylic acid metabolic process (GO:0019752)	686	58	1.54E+01	1.01E-13

Supplementary Figure 7

Stem cell–specific gene expression changes in response to amber suppression.

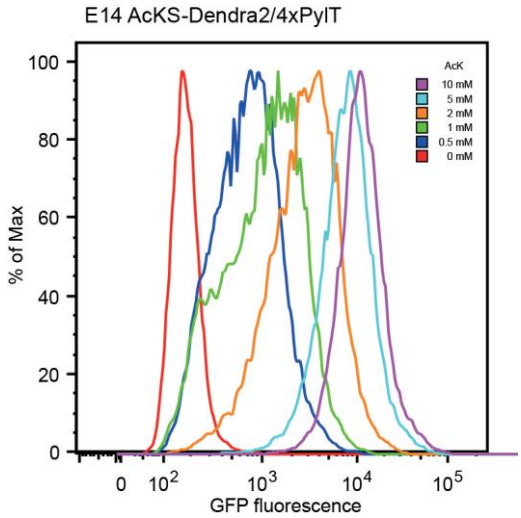
(**a**) RNA-Seq analysis of a clone bearing 4x*PylT/PylS* and 4x*PylT/sfGFP*^{150TAG} as shown in **Figure 3**. Whole transcriptome FPKM values in the presence versus absence of 0.2 mM CpK for 48h are plotted. All genes with ESC-specific expression are colored in green. ESC-specific genes were defined by an at least 10-fold higher expression in ESC over MEF cells. (**b**) Expression changes amongst a set of well-known core ESC genes, including master regulators Oct-4/Pou5f1, Sox2 and Nanog, upon addition of 0.2 mM CpK for 48 hours. (**c**) RNA-Seq comparison of E14 ESC and 3T3 MEF clones bearing 4x*PylT/PylS* and 4x*PylT/sfGFP*^{150TAG}. Significantly up- and downregulated genes in ESC as defined in **Figure 3a** are colored in red and blue, respectively. Most of the genes in these gene sets are expressed highly in both ESC and MEF cells. (**d**) Gene ontology (GO) analysis of significantly up- or downregulated gene sets in ESC. Upregulated genes fall in a variety of ontologies, whereas downregulated genes are strongly enriched for proteins involved in metabolic processes.



Supplementary Figure 8

Scheme for creating cell lines to express acetylated histones.

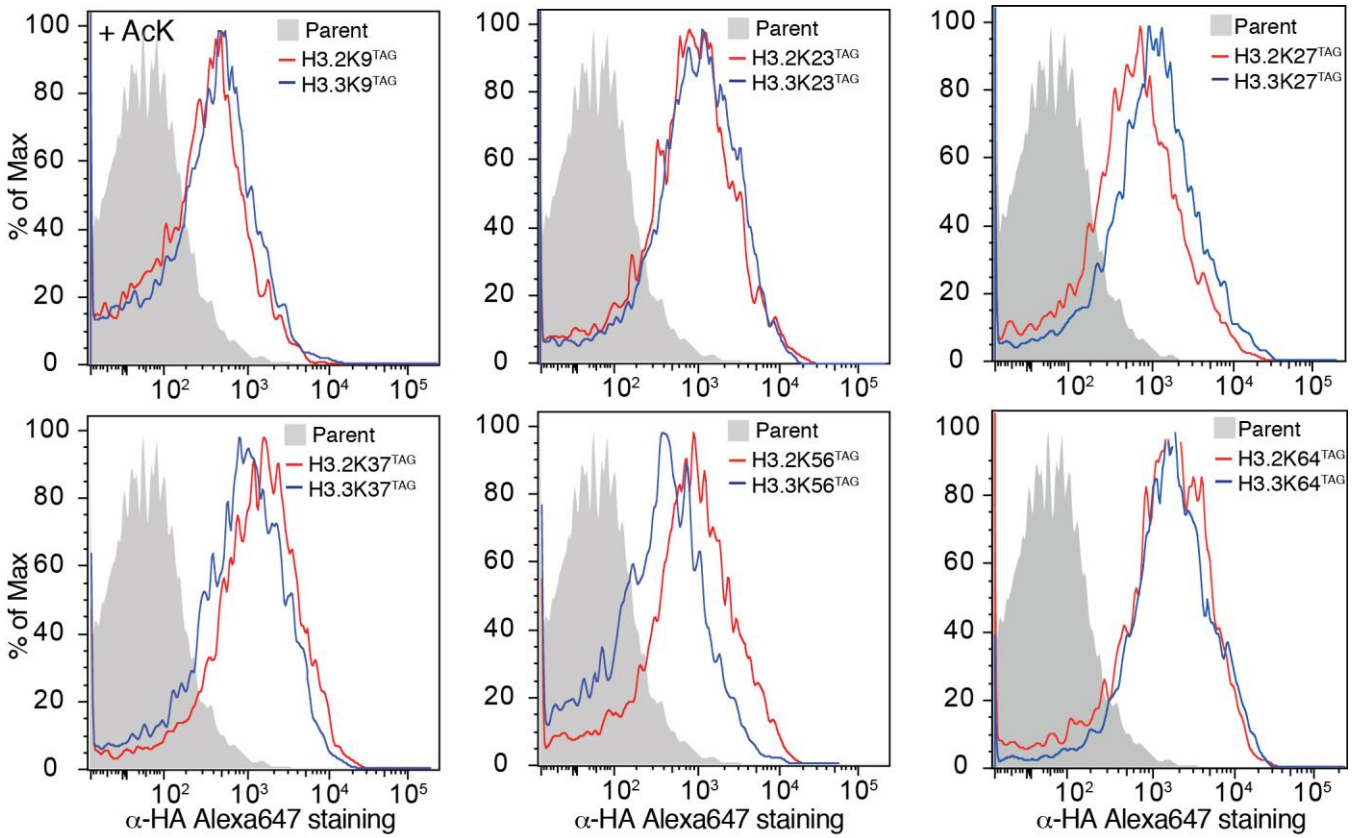
(**a**) Illustration of two-step targeting strategy to derive closely matched polyclonal pools of cells expressing the panel of acetylated histones. (**b**) Panel of acetylated histones used in the study. Location of TAG amber codons with respect to tail (light orange) and folded core (dark orange) of histone protein are indicated. (**c**) Transient expression tagged full-length histone and N-terminal histone fragments in HEK293T cells followed by western blotting corroborates the notion that truncated histones are unstable and rapidly degraded.



Supplementary Figure 9

Dose-dependent amber suppression in response to AcK.

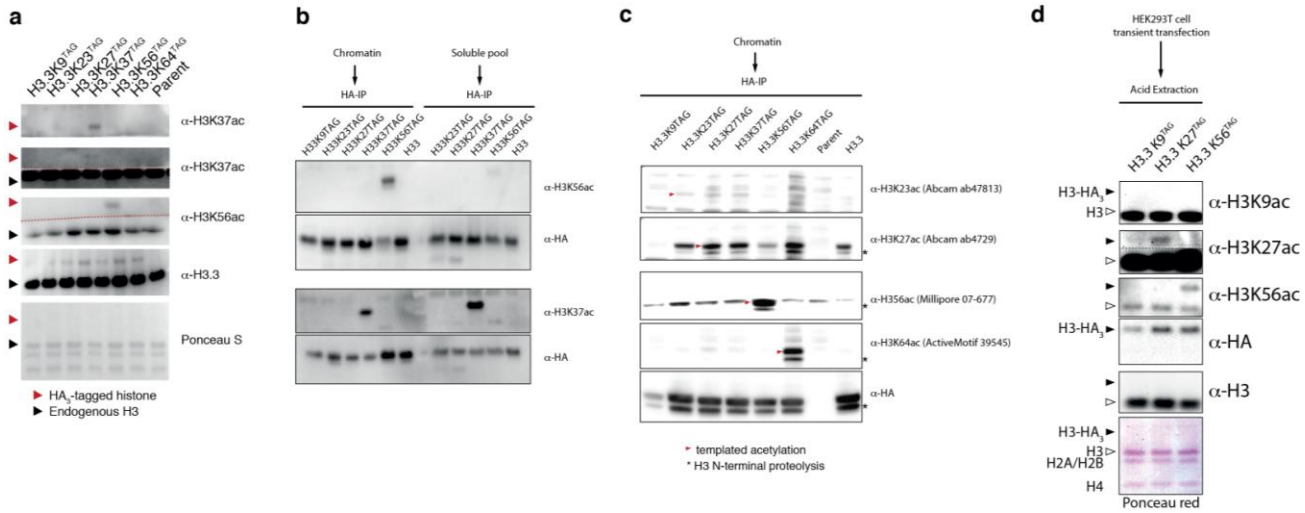
E14 ESC bearing 4x*PylT*/*AcKS*-^{TAG}*Dendra2* were incubated for 24 hours with indicated AcK concentrations.



Supplementary Figure 10

Flow cytometry analysis using immunostaining to assess expression of HA-tagged histone transgenes shown in **Figure 3b** on a single-cell level.

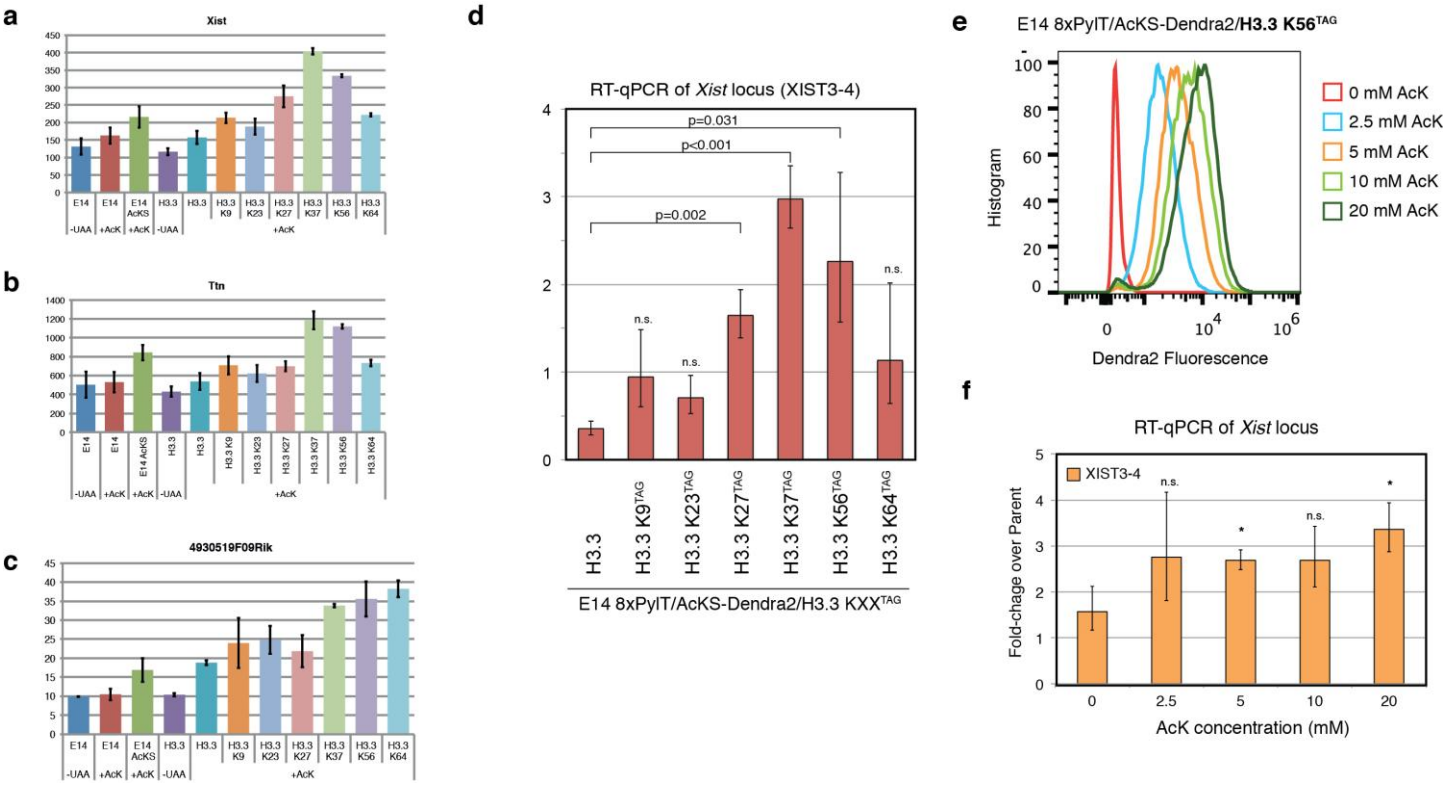
Pairs of H3.2 and H3.3 cell lines are shown which were derived independently from the same parental E14 ESC 4x*PylT/AcKS-*^{TAG}*Dendra2* clone. Expression of H3.2, H3.2(XX)TAG, H3.3, H3.3(XX)TAG bearing a C-terminal triple HA tag, as measured by fluorescent staining with anti-HA Alexa647 antibody and FACS analysis. All cells were grown for 24 hours in the presence of 10 mM AcK. The parental cell lines, which do not express any HA-tagged protein is used as a negative control for the staining procedure (gray).



Supplementary Figure 11

Genetically encoded, site-specific histone acetylation is detected on chromatin and soluble histones.

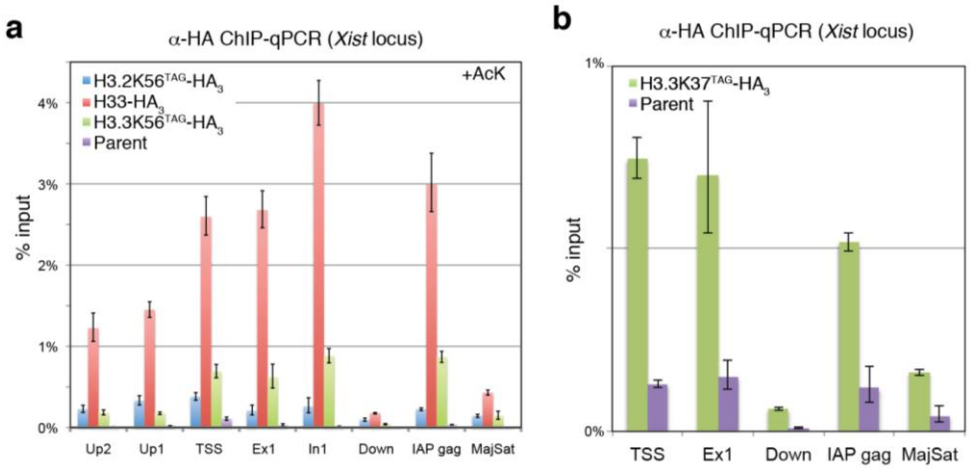
(**a**) Western blot analysis of chromatin extracts detecting genetically directed, site-specific acetylation, in comparison to endogenous levels of acetylation. Endogenous levels of H3K37ac and H3K56ac were detected on the same western blot. The dotted red line indicates where western blot was cut for imaging to spatially separate the upper and lower half during imaging, to reduce interference by the stronger endogenous signal. Loading was controlled using H3.3-specific antibody, and a membrane stain. (**b**) Western blot of histone H3.3(XX)^{TAG}-HA, immunoprecipitated from an MNase-treated chromatin fraction or from a soluble extract. Histones were probed for H3K37 and H3K56 acetylation by western blot. HA tag was blotted as a loading control. H3K37 and H3K56 are only detected when templated by genetically encoded, site-specific incorporation of acetyllysine. (**c**) Additional western blot of histone H3.3(XX)^{TAG}-HA, immunoprecipitated from an MNase treated chromatin fraction. Histones were probed for HA tag, H3K27 and H3K64 acetylation by western blot. (**d**) Histone H3.3(XX)^{TAG}-HA mutant overexpression in HEK293T cells for detection of templated histone acetylation in direct comparison to endogenous histones. H3K9 H3K27 and H3K56 acetlation was probed by western blot of acid-extracted histones. HA-tag blot shows overexpressed histone mutant. H3 antibody (ab1791) recognizes epitope at C-terminus of H3 and thus does is not able to detect HA-tagged histone species.



Supplementary Figure 12

Expression analysis of E14 ESC lines bearing AcKS-Dendra2/4×PylT and H3.3K(XX)^{TAG}/4×PylT.

(**a–c**) Absolute RNA-Seq expression (FPKM) values of representative genes from **Figure 5a** are plotted. Controls are included as follows: Wild type E14 cells with and without AcK for 24 hours (E14); parental E14 cell line bearing AcKS-Dendra2/4xPylT (E14 AcKS); E14 cell line bearing 4x*PylT*/*AcKS-*^{TAG}*Dendra2* and 4x*PylT*/*H3.3* with and without AcK for 24 hours (H3.3). (**d**) RT-qPCR validation of XIST gene expression in three biological replicates. Compare to RNA-Seq in panel **a**. Error bars indicate standard deviation of three biological replicates. Significance is given as p value based on Student's T test. (**e**,**f**) Amber suppression efficiency and XIST gene activation as a function of AcK addition. E14 ESC bearing 4x*PylT*/*AcKS-*^{TAG}*Dendra2* and 4x*PylT*/*H3.3K56*^{TAG} were incubated for 24 hours with indicated AcK concentrations. (**e**) Amber suppression as measured by the AcKS-TAG-Dendra2 reporter. (**f**) Expression of XIST gene as measured by RT-qPCR relative to the Parent cell line, as a function of AcK concentration. Error bars indicate standard deviation of three biological replicates. Significance is given as p value based on Student's T test (* p<0.05).



Supplementary Figure 13

Site-specific acetylated histone H3.3 is incorporated similarly to wild-type histone H3.3.

(**a**) ChIP assay validating variant-specific incorporation of H3.3-HA_3 and H3.3K56^{TAG}-HA_3, in the presence of 10 mM AcK for 24 h, at the *XIST* locus at primer sites tiling the *XIST* locus as indicated in **Figure 5c**. Error bars indicate 95% confidence interval of three technical replicates. (**b**) ChIP assay validating variant-specific incorporation of H3.3K37^{TAG}-HA_3, in the presence of 10 mM AcK for 24 h, at the *XIST* locus at primer sites tiling the *XIST* locus as indicated in **Figure 5c**. Error bars indicate 95% confidence interval of three technical replicates.

Supplementary Table 1

Primer Name	Sequence
IAP_Gag_F	AATCTCAGAACCGCTCCATGA
IAP_Gag_R	TTTCTTAAAATGCCCAGGCTTT
MajSat_F	GACGACTTGAAAAATGACGAAATC
MajSat_R	CATATTCCAGGTCCTTCAGTGTGC
Up2_F	AGCATTTGCAGGAGCTCTCC
Up2_R	AAGAGCAGGTCAGGATGCAC
Up1_F	CACAACTCCGGACCTGTACC
Up1_R	CGTTCCTCTGCCATCCTTCA
TSS_F	TAAGGCTTGGTGGTAGGGGA
TSS_R	TTTGCTCGTTTCCCGTGGAT
Ex1_F	ACCTCAAATACTCCTGACATCCA
Ex1_R	ACTCATCCACCGAGCTACTCT
In1_F	ATAGGAGGCAGGCTGAAAGC
In2_R	CTACCCACCCCAGTACATGC
Down2_F	TGACTCCATGGACATCAGCG
Down2_R	ACATAGGACATGTTCCCCTGC