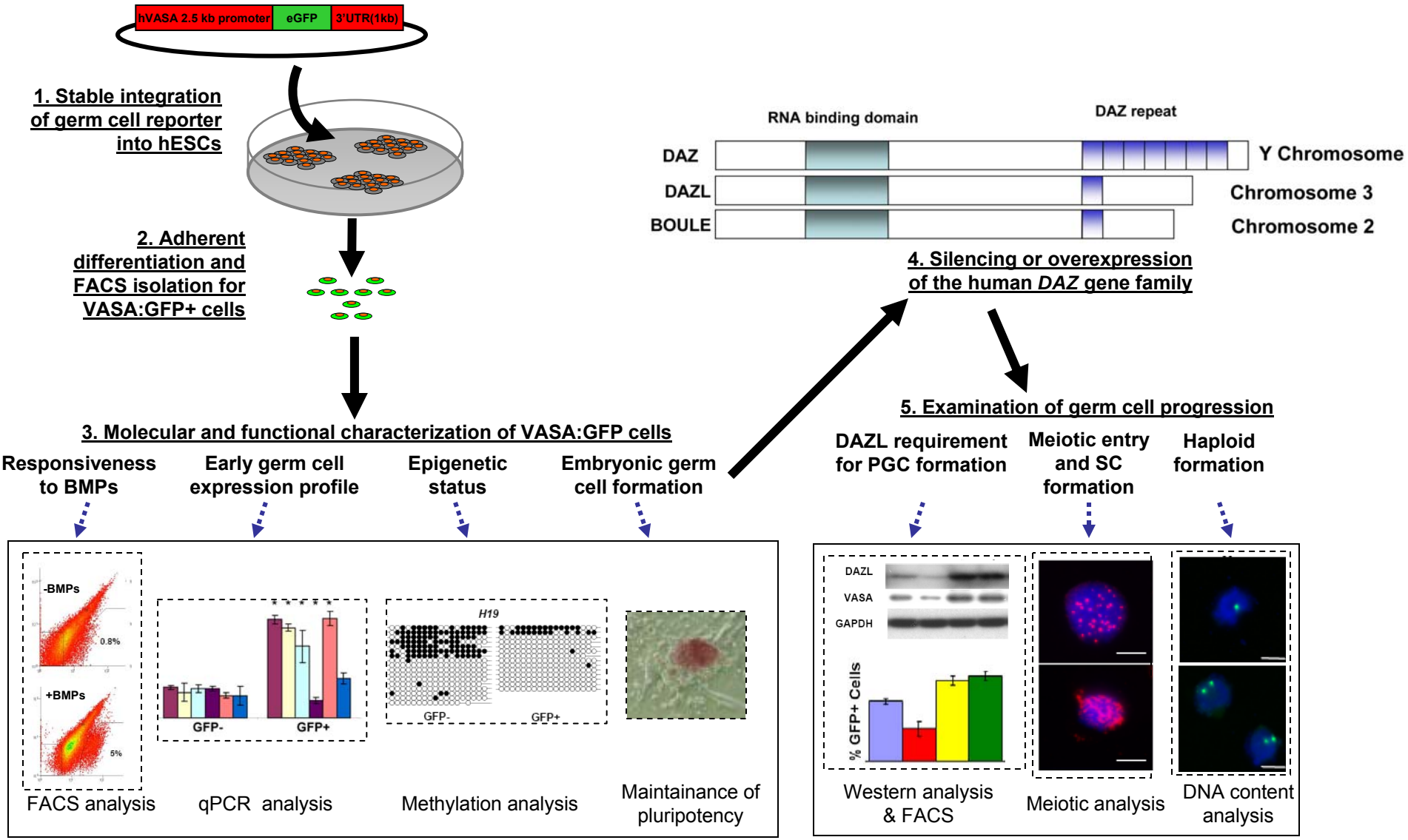
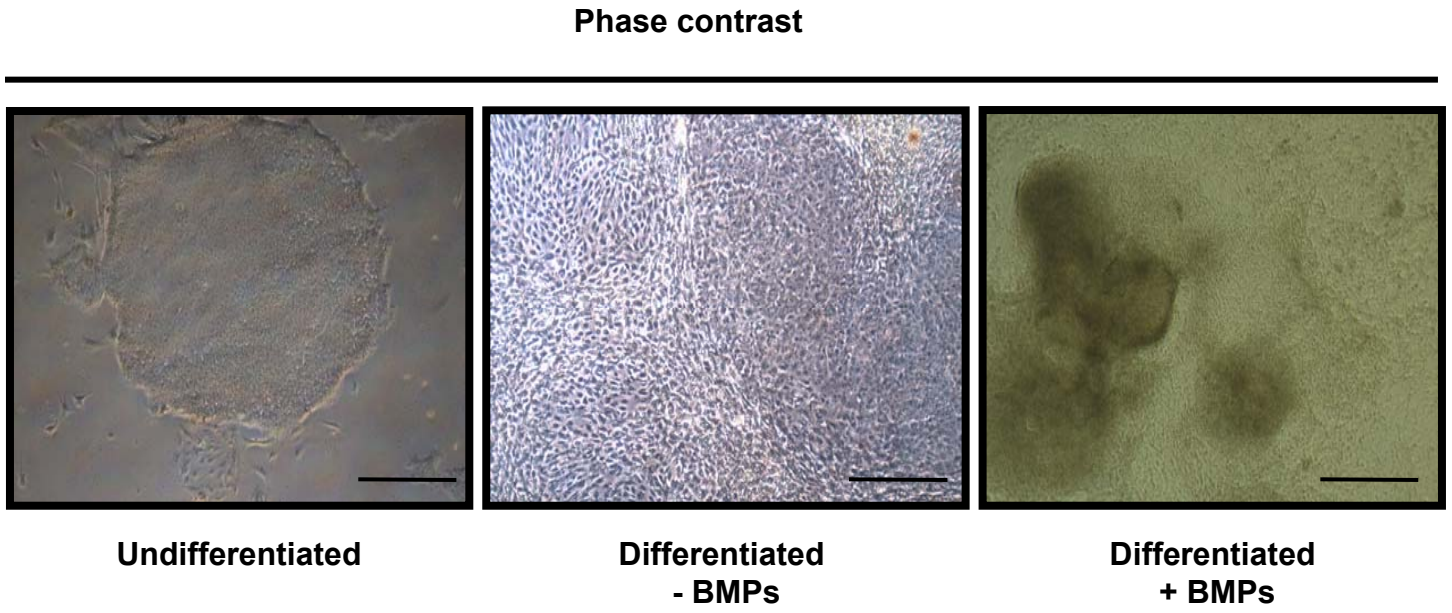
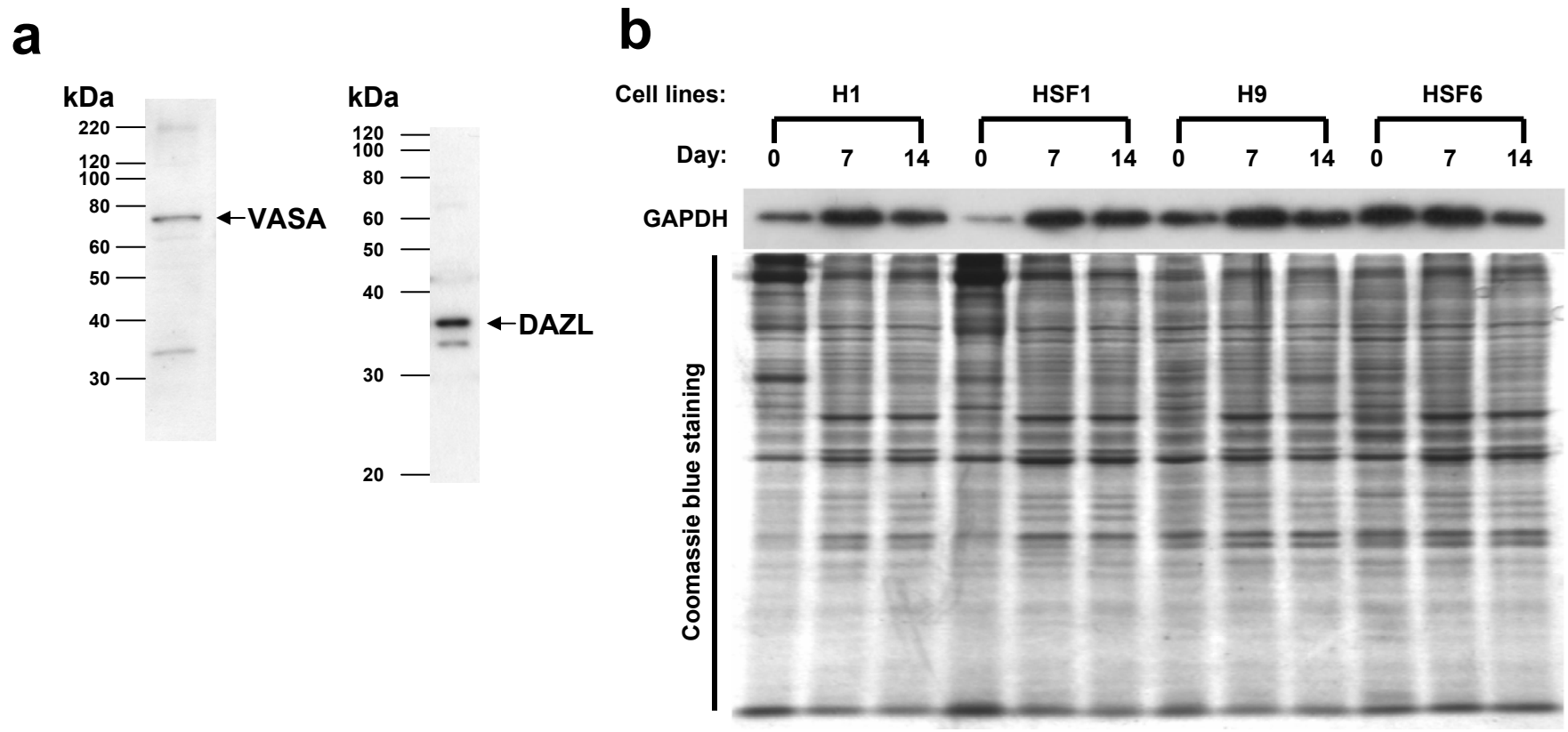


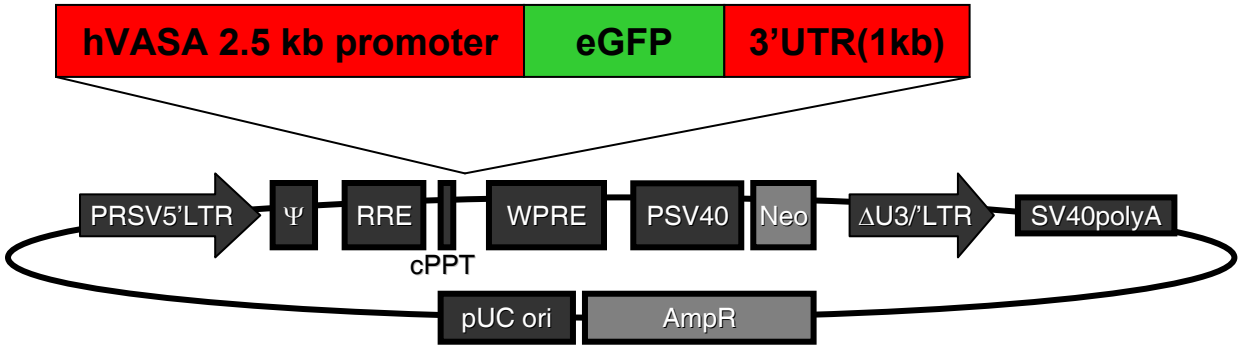
Supplementary Figure 1. Experimental overview of system to probe landmark events and genetic requirements for human germ cell formation and differentiation. A germ cell reporter (VASA:GFP) was created and stably integrated into hESCs. Adherent differentiation was developed to achieve efficient recovery of early germ cells for FACS isolation and characterization. Multiple molecular and functional assays were conducted to characterize GFP+ cells after FACS isolation, including quantitative analysis of GFP+ by FACS under various culturing condition to examine the responsiveness of hESCs to BMPs, expression analysis using more than 25 qPCR probes, methylation of genomic DNA by three independent analysis, and ability to propagate EG cells. Results confirmed identity of GFP+ cells as primordial germ cells. We further tested the utility for direct human genetic studies of germ cell formation and differentiation by silencing and overexpressing members of the germ cell specific *DAZ* gene family. Combinations of multiple functional and cellular assays demonstrated that formation of GFP+ cells was modulated by one of the *DAZ* gene family members, *DAZL*. The functional role of *DAZ* and *BOULE* in contrast was further revealed in promoting germ cell into meiosis and haploid formation.



Supplementary Figure 2. Phase contrast pictures showing an hESC colony on matrigel before and after differentiation with or without BMPs. After 7 days of differentiation with BMPs, cells on matrigel appear confluent, compact and morphologically distinct from the undifferentiated colony. In contrast, cells differentiated without BMPs appear as monolayers and more homogenous. Scale bar is 500 micron.

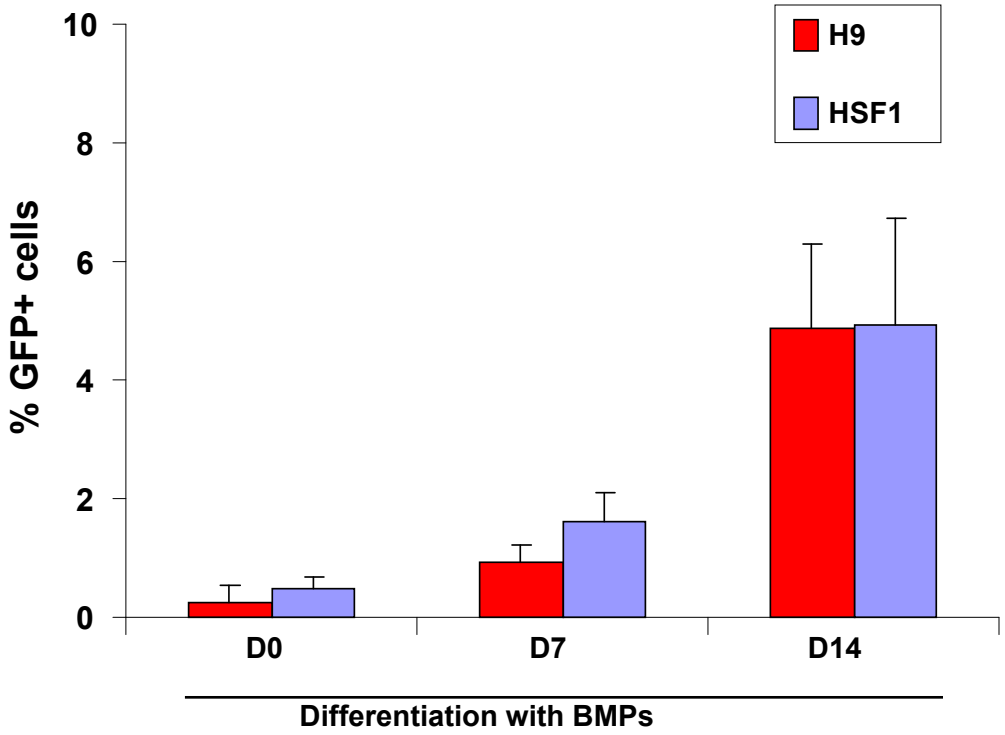


Supplementary Figure 3. Western analysis of VASA and DAZL proteins with hESC differentiation. a, Western analysis of VASA and DAZL from the cell lysates of a 14 day differentiated sample with BMPs. Polyclonal VASA and DAZL antibody detected a prominent ~79kDa band and ~35kDa band, respectively. **b,** GAPDH expression and Coomassie Blue staining of protein gel with the four hESC lines at three time points. Similar to UBC9 shown on Figure 1a, protein expression of housekeeping marker GAPDH was lower in H1 and HSF1 in undifferentiated hESCs. This may reflect previous observations that expression of these house keeping genes vary in different hESC lines or may reflect legitimate sex-specific differences. Lower panel is coomassie blue staining of the samples indicating equal amount of loading but slightly different expression patterns at various time points.

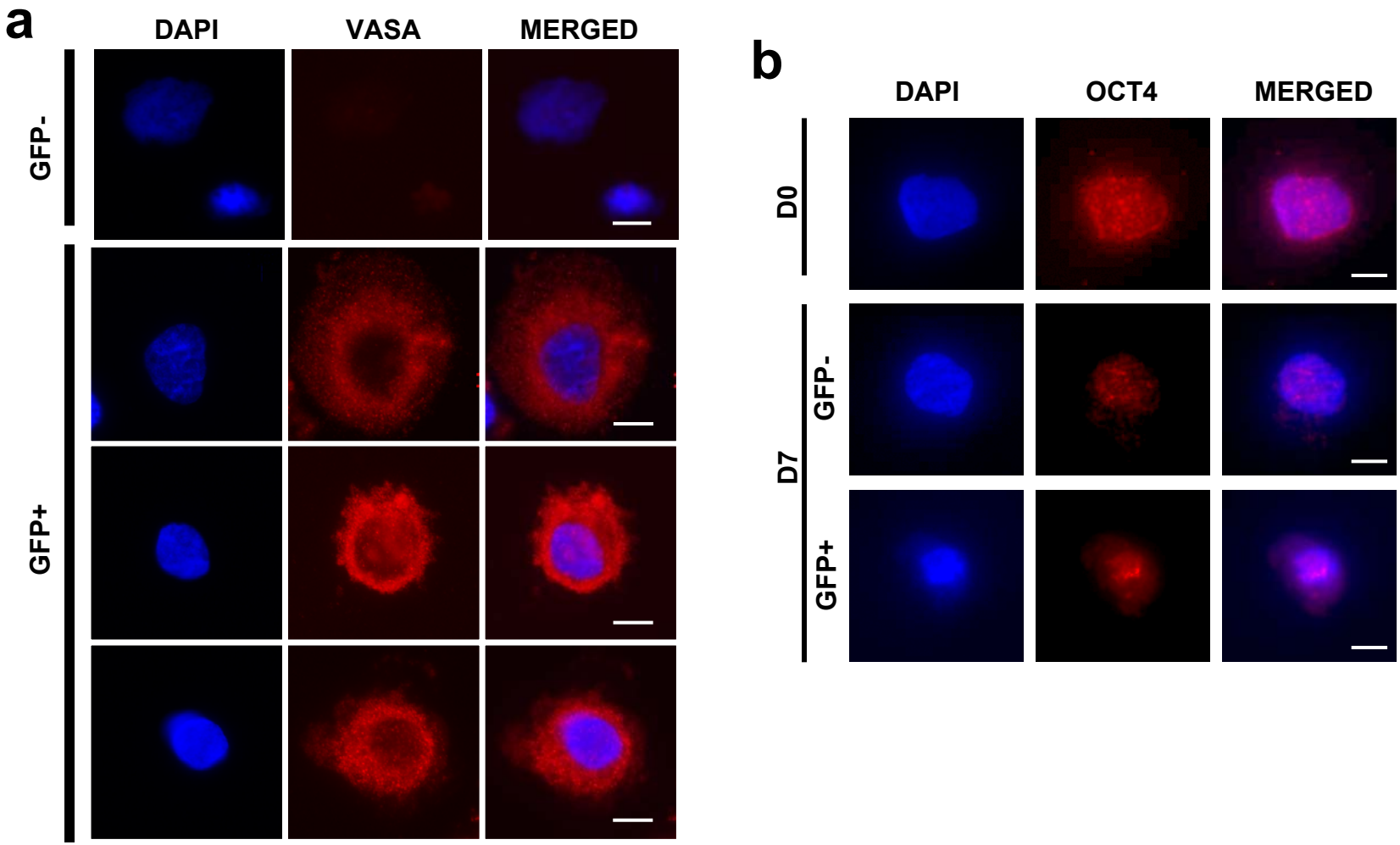


pLVGV

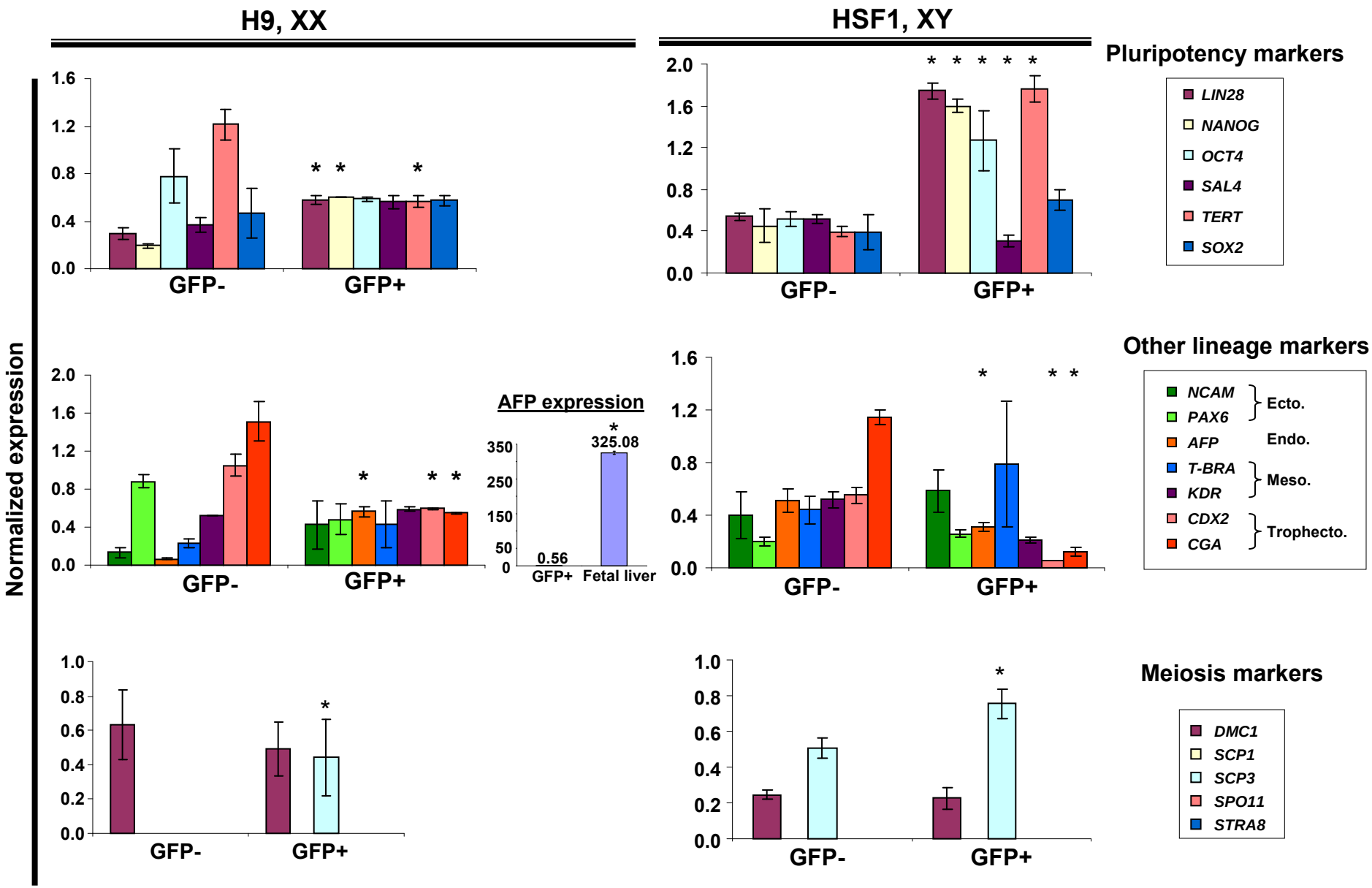
Supplementary Figure 4. Simplified diagram of the lentiviral VASA:GFP reporter construct, pLVGV. The reporter construct contains 2.5kb of promoter sequence 5-prime to the human *VASA* open reading frame (ORF) to direct expression of eGFP (enhanced Green Fluorescent Protein) expression, followed by 1kb of 3-prime untranslated region inserted into p2k7 lentiviral vector.



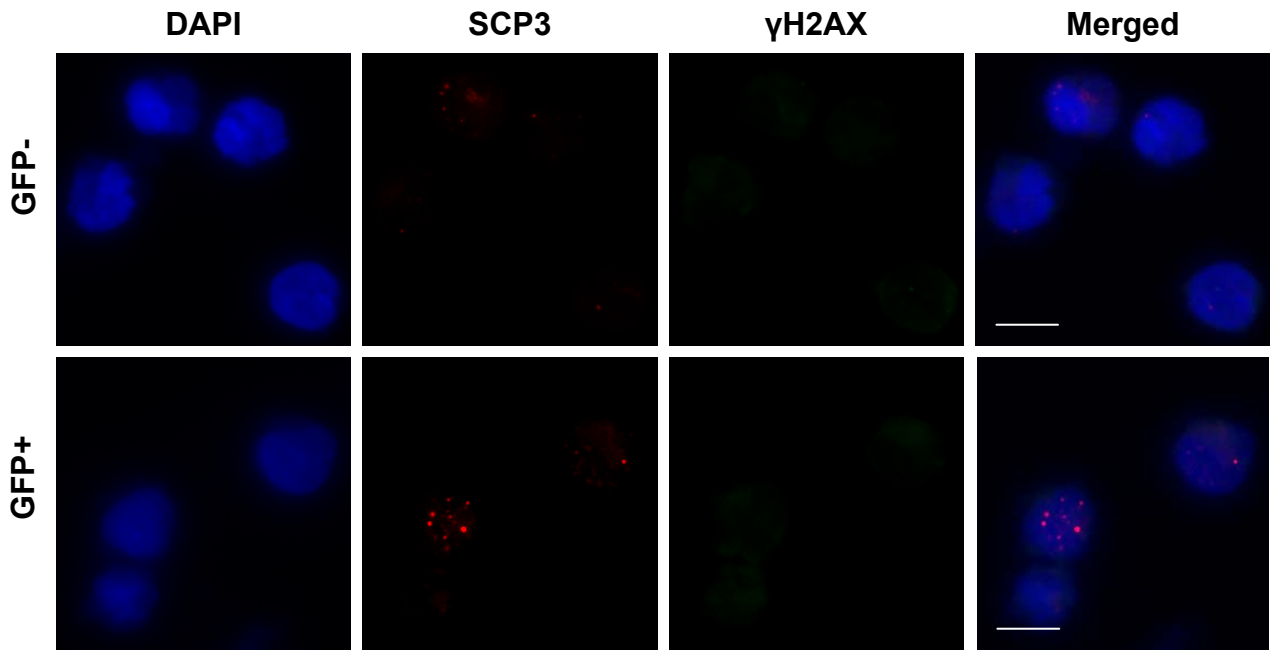
Supplementary Figure 5. Average percentages of VASA:GFP+ cells in female and male lines at three timepoints. Three independent cultures of H9 (XX) and HSF1(XY) after 0, 7 and 14 days of differentiation with BMPs show similar levels of increase in the GFP+ population in both sexes. Error bars=standard deviation, n=3.



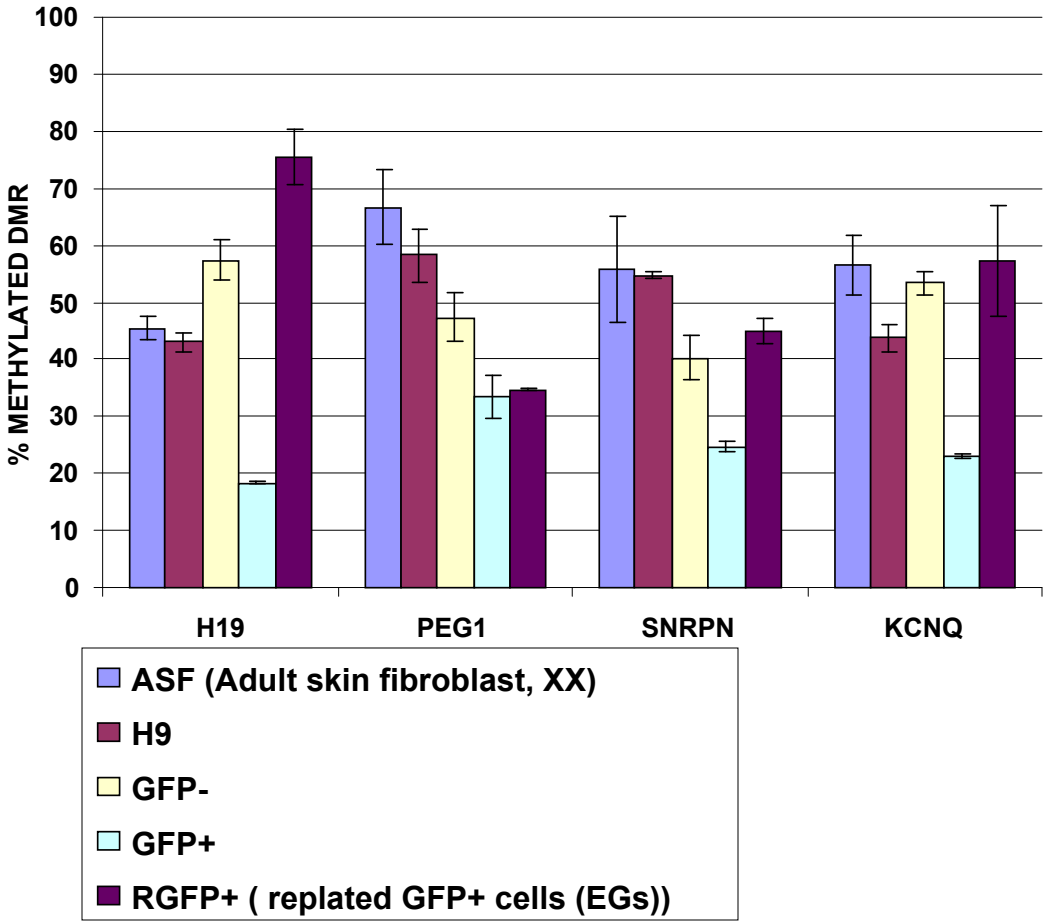
Supplementary Figure 6. VASA and OCT4 stainings of FACS-isolated cells. a, VASA immunostaining of GFP- and GFP+ cells from Day7 FACS-isolated samples. **b,** OCT4 immunostaining of Day0 undifferentiated hESCs and Day7 FACS-isolated cells. No VASA was detected in GFP- cells whereas VASA localized to cytoplasm of GFP+ cells. **b,** Punctate OCT4 staining was detected in nuclei of all three populations of cells but undifferentiated hESCs showed the strongest staining of OCT4. All cells were cytospun and fixed on slide for immunostaining. Scale bar is 10 micron.



Supplementary Figure 7. Expression profiles of GFP- versus GFP+ population from H9 and HSF1 differentiated hESCs. Expression of *LIN28*, *NANOG*, *OCT4* and *TERT* was higher in either H9 or HSF1 GFP+ cells, consistent with previous studies demonstrating the expressions of these genes was enriched in early germ cells. Among all somatic lineage markers, only *AFP* expression was found to be significantly higher in H9 GFP+ cells. However, it was much lower (<325 folds) than fetal liver. In addition, only *SCP3* expression was slightly higher in GFP+ cells but four other meiosis marker was either not detected or not significantly higher in GFP+ cells. Hence, the marker analysis depicted GFP+ cells as early germ cells. Error bar = standard deviation; asterisk = significant difference by t-test ($p<0.05$), n=2.

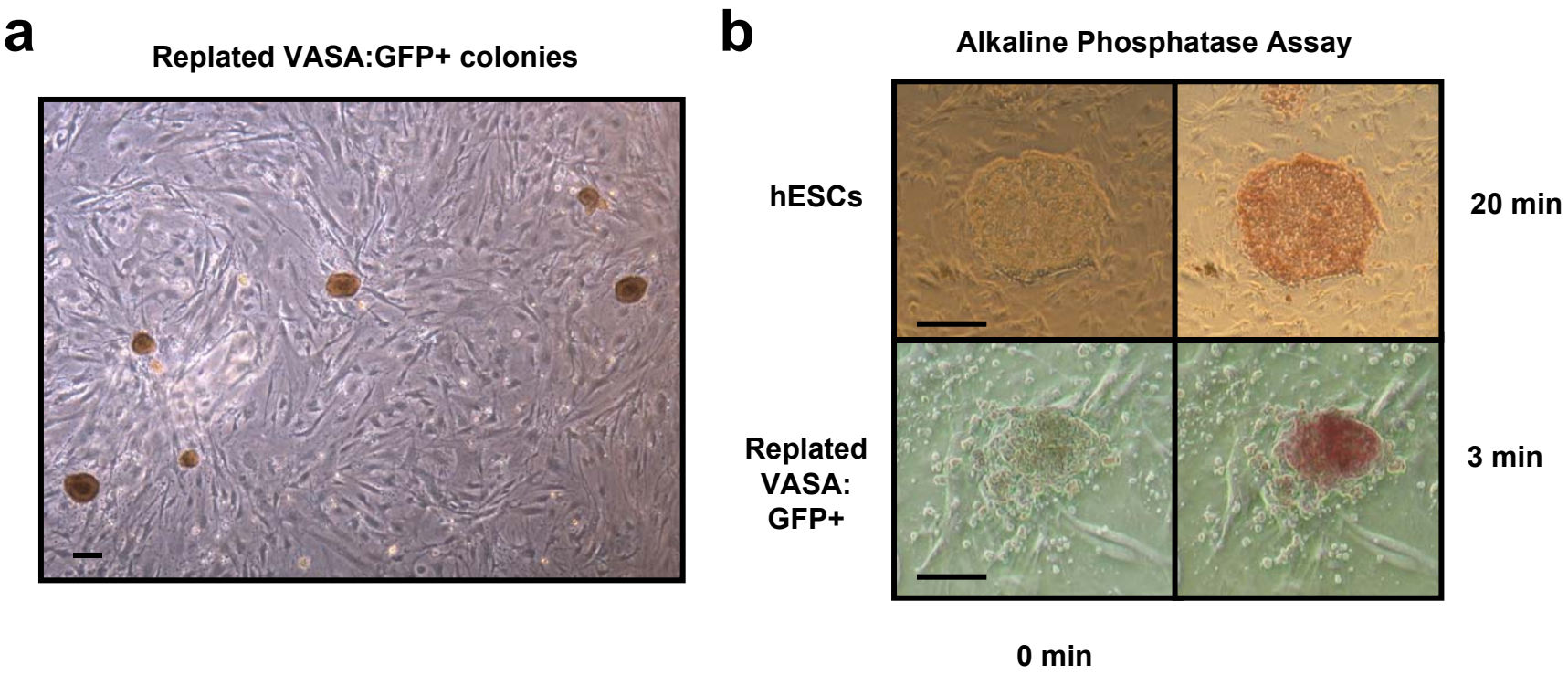


Supplementary Figure 8. Meiosis markers, SCP3 and γH2AX, was not detected in FACS-isolated cells. No SCP3 and γH2AX staining was detected in GFP- cells and a few foci of SCP3 were detected in GFP+ cells. Although slightly higher SCP3 RNA transcript was detected in GFP+ cells, but 4 other meiosis markers were no detected or not significantly higher in GFP+ cells. Taken together with the SCP3 and γH2AX immunostainings shown here, GFP+ cells most likely had not entered into meiotic prophase I. All cells were cytospun and fixed on slide for immunostaining. Scale bar is 10 micron.

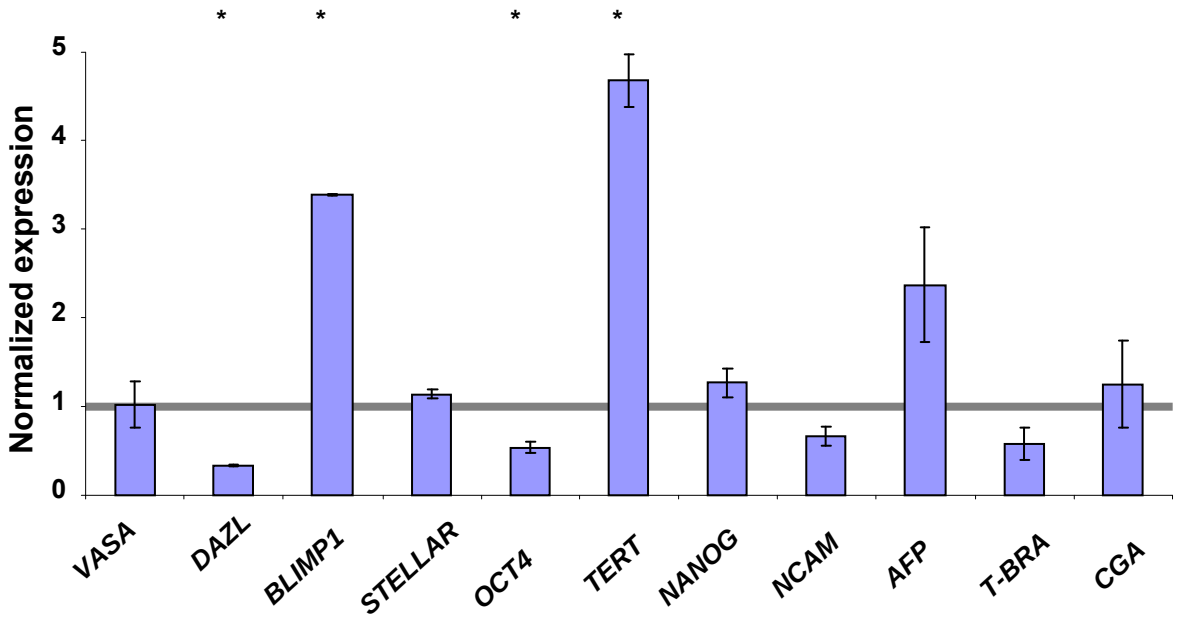


XX				
Newman-Keuls Test	Mean Diff.	q	Significant? P < 0.05?	Summary
H19 LOCUS				
GFP+ vs RGFP+	-57.18	25.36	Yes	***
GFP+ vs GFP-	-38.96	19.32	Yes	***
GFP+ vs ASF	-27.13	13.45	Yes	***
GFP+ vs H9	-24.57	12.18	Yes	***
H9 vs RGFP+	-32.61	14.46	Yes	***
H9 vs GFP-	-14.39	7.137	Yes	**
H9 vs ASF	-2.558	1.268	No	ns
ASF vs RGFP+	-30.05	13.33	Yes	***
ASF vs GFP-	-11.84	5.869	Yes	**
GFP- vs RGFP+	-18.22	8.08	Yes	***
PEG1 LOCUS				
GFP+ vs ASF	-33.13	12.3	Yes	***
GFP+ vs H9	-24.67	9.159	Yes	***
GFP+ vs GFP-	-13.81	5.129	Yes	*
GFP+ vs RGFP+	-1.078	0.358	No	ns
RGFP+ vs ASF	-32.05	10.64	Yes	***
RGFP+ vs H9	-23.59	7.834	Yes	***
RGFP+ vs GFP-	-12.73	4.229	Yes	*
GFP- vs ASF	-19.31	7.172	Yes	**
GFP- vs H9	-10.85	4.031	Yes	*
H9 vs ASF	-8.46	3.141	No	ns
SNRPN LOCUS				
GFP+ vs ASF	-34.04	6.685	Yes	**
GFP+ vs H9	-24.83	4.877	Yes	*
GFP+ vs RGFP+	-15.17	2.664	No	ns
GFP+ vs GFP-	-7.999	---	No	ns
GFP- vs ASF	-26.04	5.114	Yes	*
GFP- vs H9	-16.83	3.306	No	ns
GFP- vs RGFP+	-7.169	---	No	ns
RGFP+ vs ASF	-18.87	3.315	No	ns
RGFP+ vs H9	-9.663	---	No	ns
H9 vs ASF	-9.209	---	No	ns
KCNQ LOCUS				
GFP+ vs RGFP+	-32.86	8.47	Yes	**
GFP+ vs ASF	-32.14	9.26	Yes	***
GFP+ vs GFP-	-29.01	8.358	Yes	***
GFP+ vs H9	-24.45	7.044	Yes	***
H9 vs RGFP+	-8.417	2.169	No	ns
H9 vs ASF	-7.691	---	No	ns
H9 vs GFP-	-4.559	---	No	ns
GFP- vs RGFP+	-3.858	---	No	ns
GFP- vs ASF	-3.132	---	No	ns
ASF vs RGFP+	-0.7262	---	No	ns

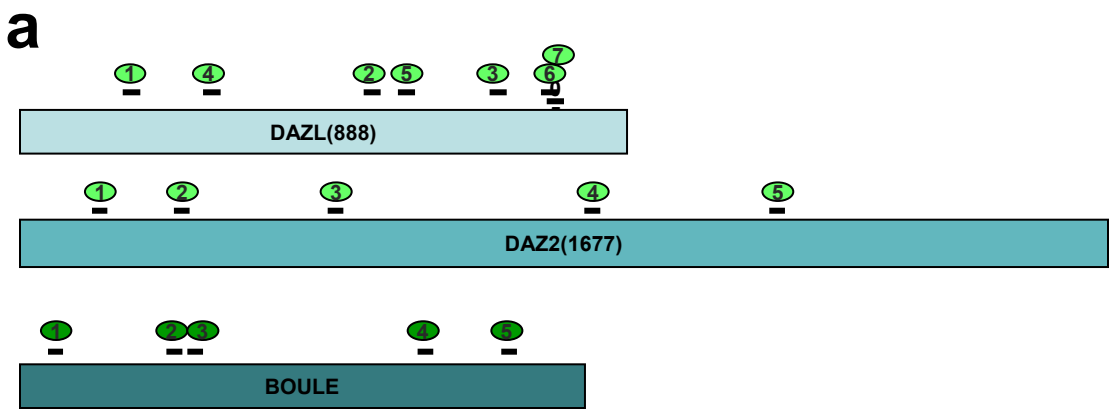
Supplementary Figure 9. Methylation status of various XX bearing cells at four imprinted DMRs. Genomic DNA of adult skin fibroblast (ASF), undifferentiated hESCs H9, GFP-, GFP+, and replated GFP+ (RGFP) cells were subjected to methylation sensitive and dependent enzyme digestions. qPCR was carried out to measure the methylation level at DMRs of H19 (paternal imprinted), PEG1, SNRPN, KCNQ (maternal imprinted) loci. Table at right shows the results of statistical tests for all samples and loci. In short, GFP+ cells were hypomethylated at all four loci, consistent with bisulfite sequencing analysis and 5MC staining shown in Figure 2. RGFP+ (EG) cells had increased methylation at DMRs of H19, SNRPN and KCNQ relative to freshly-isolated GFP+ cells, suggesting that prolonged culturing changed the methylation status of GFP+ cells. Error bar = standard deviation; asterisk = significant difference by Newman-Keuls test ($p<0.05$), n=3.

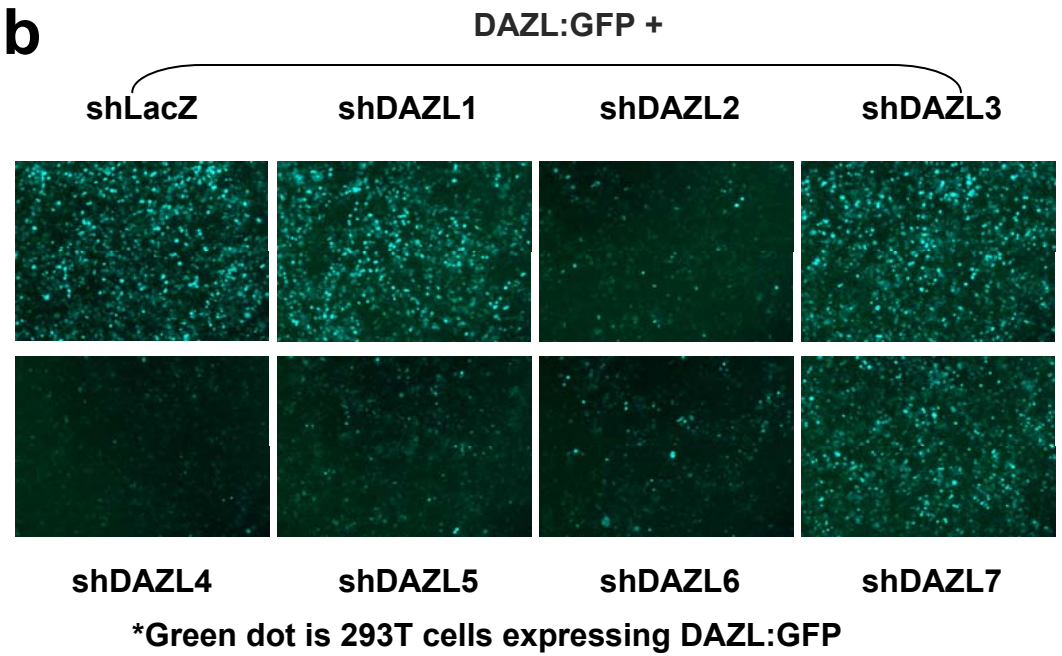


Supplementary Figure 10. Phase contrast images showing multiple colonies and alkaline phosphatase (AP) activity of the VASA:GFP+ replated colonies. a, Multiple colonies arise after 7 days of replating VASA:GFP+ cells onto inactivated MEFs. **b,** Clustered colony shows stronger AP activity after 3 min of staining than an undifferentiated hESC colony stained for 20 min. Scale bar indicates 100 micron in **a** and **b**.



Supplementary Figure 11. Expression profiles of the replated VASA:GFP+ cells . Expression was normalized first to 4 housekeeping genes and to corresponding expressions of freshly isolated VASA:GFP+ cells without replating. Asterisk indicates a significant difference in expression to freshly isolated VASA:GFP+ cells at $p < 0.05$, n=2.



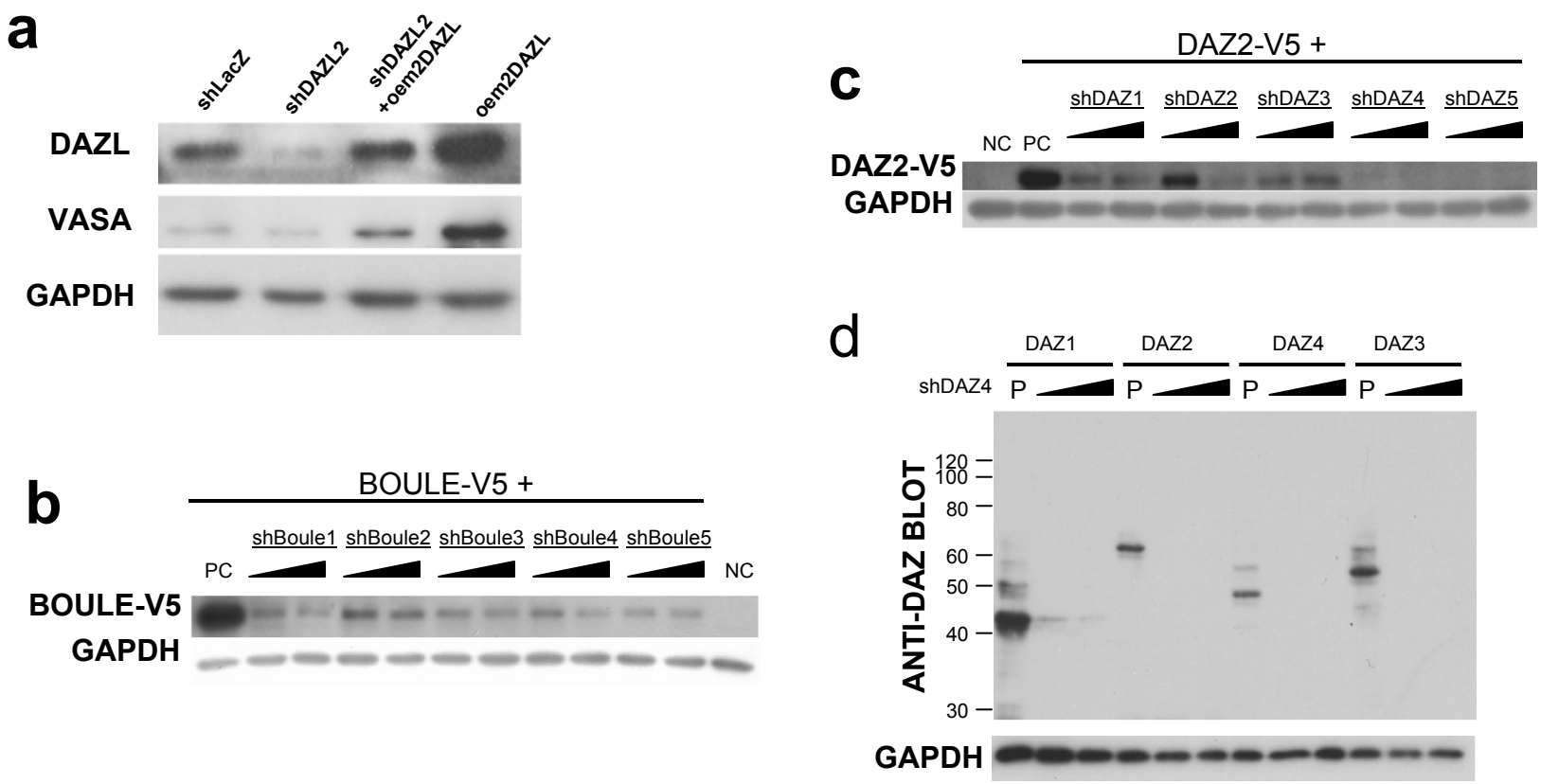


c

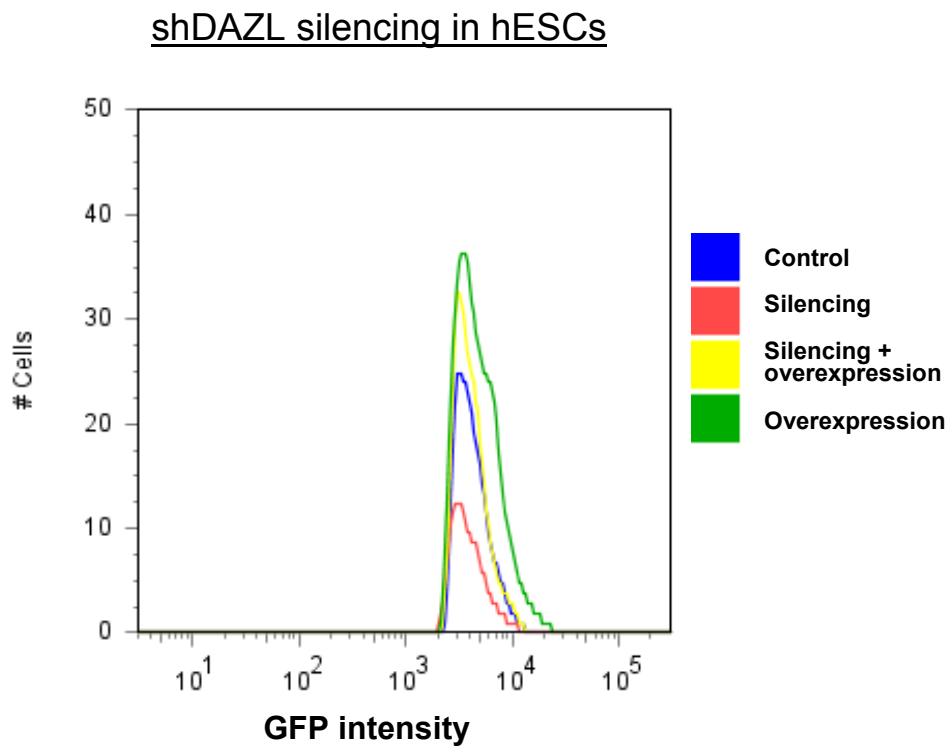
DAZL2: GCA TAT CCT ACT TAC CCA AAT
a.a. A Y P T Y P N
mut2 DAZL2: GCA TAT CCA ACA TAT CCA AAT

DAZL4: G CAG AAG ATA GTA GAA TCA CA
a.a. Q K I V E S
mutDAZL4: G CAG AAA ATC GTC GAG TCA CA

Supplementary Figure 12. Silencing of *DAZ* gene family members. a, shRNA target sites at *DAZL*, *DAZ2*, and *BOULE*. **b,** Silencing of DAZL:GFP by different shDAZLs. We identified 3 shRNAs which significantly reduced DAZL:GFP fusion protein expression when cotransfected in 293T cells. Initial screening of shDAZL1-3 showed that shDAZL2 was most effective, but additional screening of shDAZL4-7 identified shDAZL4 as the most effective silencer for DAZL. **c,** Synonymous mutations for shDAZL2 and shDAZL4. Changing 3-4 nucleotide sequences within shDAZL2 and shDAZL4 targeting regions imparted resistance to silencing, as shown (Fig. 3; Supplementary Fig. 13a).



Supplementary Figure 13. Silencing of DAZL, DAZ and BOULE. a, Silencing of DAZL and VASA by shDAZL2 in hESCs. DAZL and VASA expression was reduced when shDAZL2 was expressed and rescued by overexpression of mut2DAZL. Similar to mut4DAZL (Fig. 3b), mut2DAZL elevated VASA expression in hESCs. **b,** Western analysis of BOULE silencing vectors. BOULE-V5 was cotransfected with control or 5 unique shBOULEs in 293T cells. PC was positive control with shLacZ vectors and NC was negative control without BOULE-V5 or silencing vectors. Two levels of silencing vectors were added to show the silencing effect is dependent on the level of transfected silencing vector. **c,** Western analysis of DAZ2 by silencing vectors. Same experimental design was used as in **b**. We identified 4 shRNAs, shDAZ1-4, that were able to target all four copies of DAZ based on sequence comparisons, and another shRNA, shDAZ5, that targets *DAZ2* only. When the five shDAZs were cotranfected with DAZ2-V5, shDAZ1, 3, 4, and 5 significantly decreased protein level of DAZ2-V5. **d,** Silencing of DAZ1-4 by shDAZ4 in 293FT cells. In the case of *DAZ,* there are four copies of the *DAZ* genes on the human Y chromosomes with highly-conserved sequences. Western analysis shows that all four DAZ proteins were silenced by shDAZ4.



Supplementary Figure 14. VASA:GFP+ cells were reduced by shDAZL4 and rescued by overexpression of mut4DAZL. Histogram of FACS results with silencing of *DAZL* in H9 cells carrying the VASA:GFP reporter. >10,000 cells of each treatment were analyzed and compared.

H9, XX, Day 7

	P.	E.	N.S.
Control	0	0	100
DAZL	9	5	86
BOULE	13	9	78
DAZL+BOULE	10	10	80

HSF1, XY, Day 7

	P.	E.	N.S.
Control	0	0	100
DAZ	22	0	78
DAZL	8	9	92
BOULE	7	1	92
DAZ+DAZL	3	7	90
DAZ+BOULE	11	6	83
DAZL+BOULE	6	3	91
DAZ+DAZL+BOULE	27	6	67

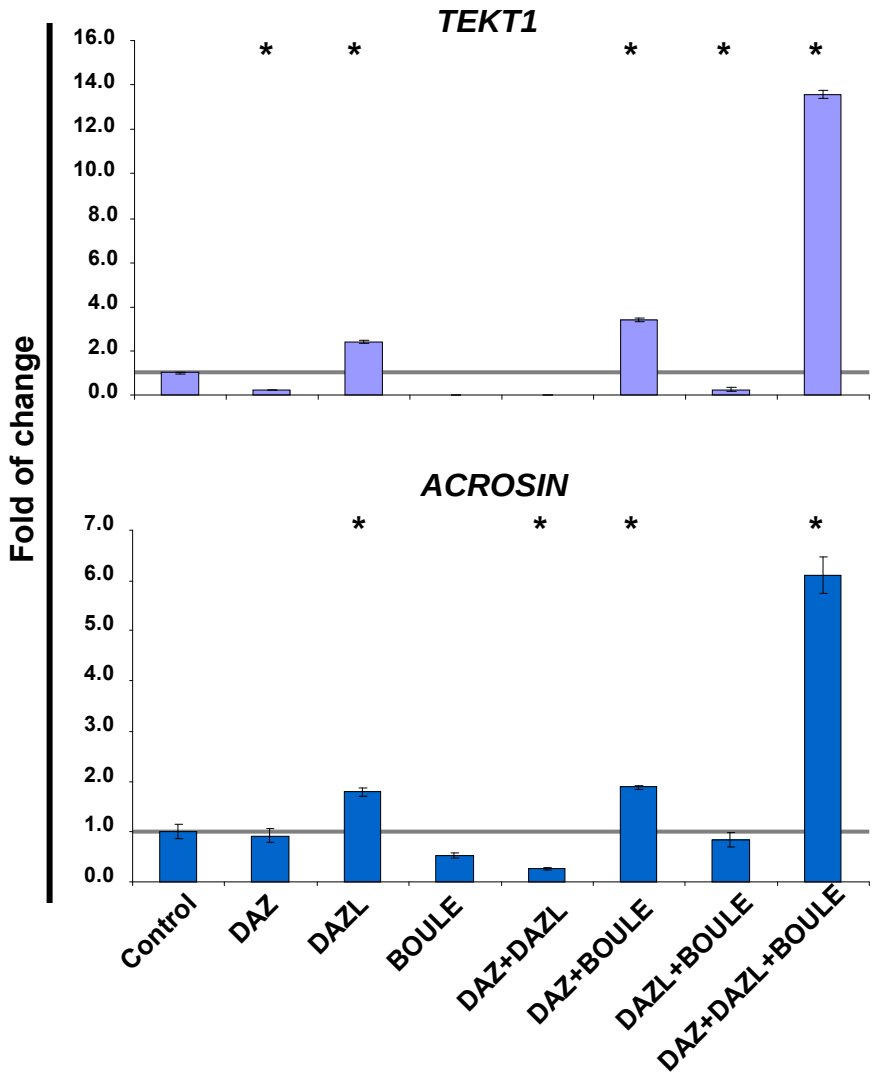
H9, XX, Day 14

	P.	E.	N.S.
Control	3	0	97
DAZL	4	1	93
BOULE	2	3	98
DAZL+BOULE	8	0	92

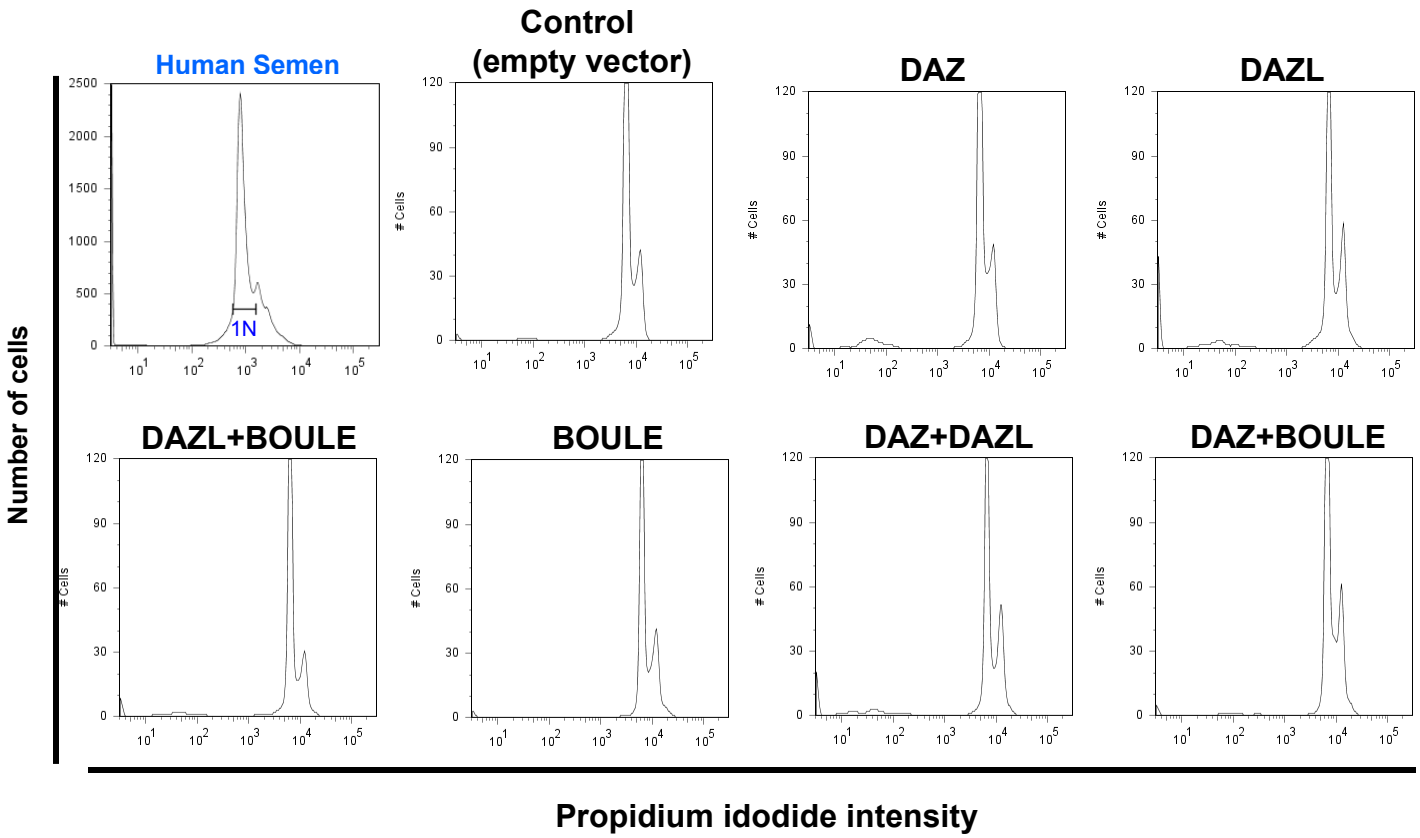
HSF1, XY, Day 14

	P.	E.	N.S.
Control	2	0	98
DAZ	29	4	67
DAZL	3	1	96
BOULE	5	1	94
DAZ+DAZL	16	6	78
DAZ+BOULE	5	3	92
DAZL+BOULE	5	10	87
DAZ+DAZL+BOULE	11	11	80

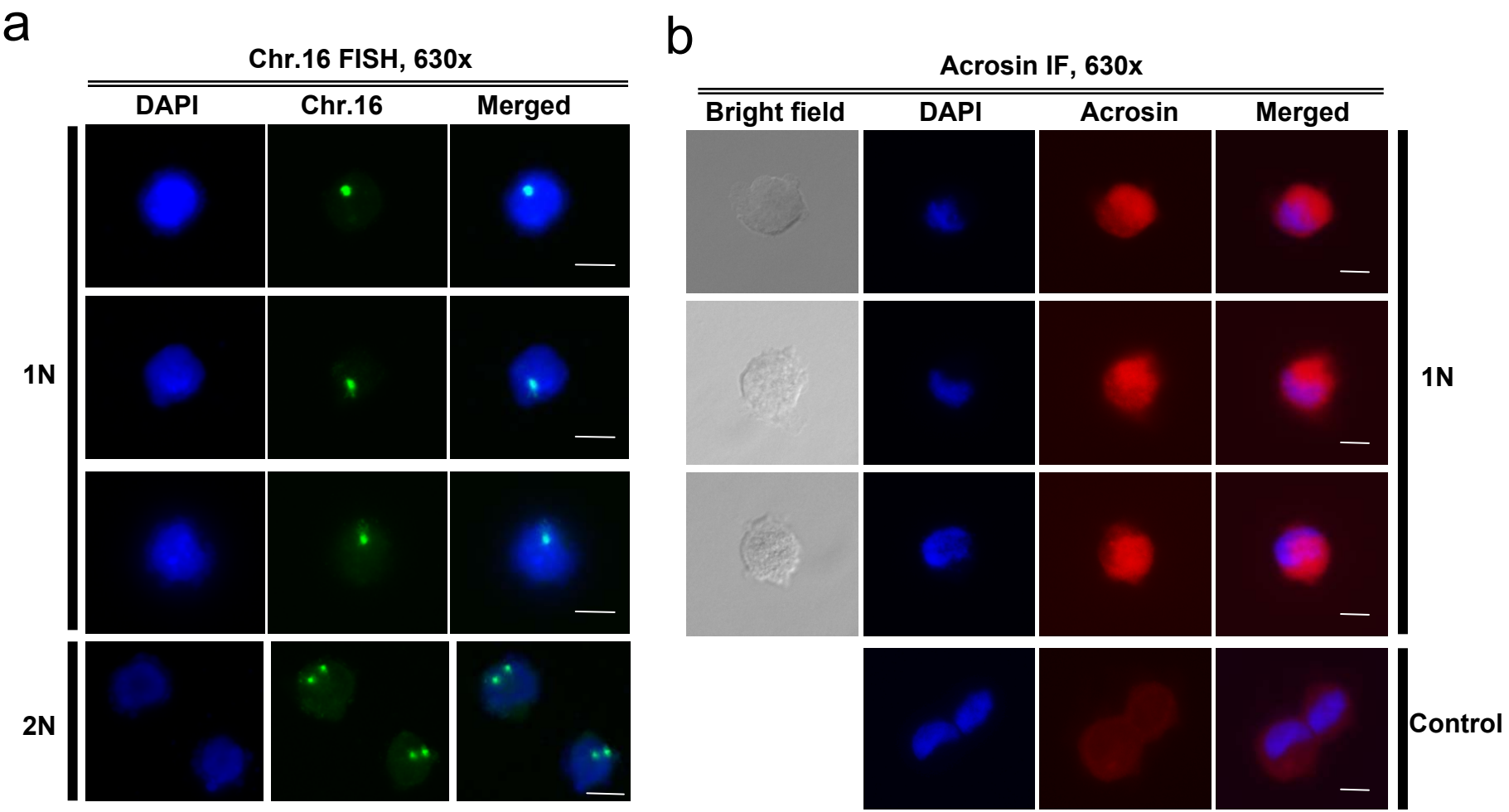
Supplementary Figure 15. Percentage of cells in each category of SCP3 staining as a function of different combinations of overexpressing vectors. 200 nuclei were counted per each sample. P.: Punctate, E.: Elongated, N.S.: no staining. Same data set were used to make the graphs in Figure 4c.



Supplementary Figure 16. Expression of *TEKT1* and *ACROSIN* in the XY line with overexpression of different factors. Differentiated hESCs (Day14) without BMPs and without FACS were subjected to RNA extraction and qPCR analysis. Expression was normalized to 4 housekeeping genes followed by normalization to control (carrying empty overexpression vector). Asterisk indicates significant difference compared to control sample at $p < 0.05$. Overexpression of DAZ, DAZL, and BOULE together induced the highest *TEKT1* and *ACROSIN* expression, consistent with the DNA content and ACROSIN immunostaining results shown on Figure 4d, f. We also note that the high percentage of meiotic cells at both Days 7 and 14 indicates clearly that meiotic progression is not synchronized. Moreover, we observed that ACROSIN and TEKT1 expression was only enriched in the cells at Day 14, but not at Day 7 (data not shown). Thus, at other days, such as Days 8 or 9, cells are unlikely to have completed meiosis to form 1N cells. Error bar = standard deviation; asterisk = significant difference from control cells by one-way ANOVA test ($p<0.05$), n=2.

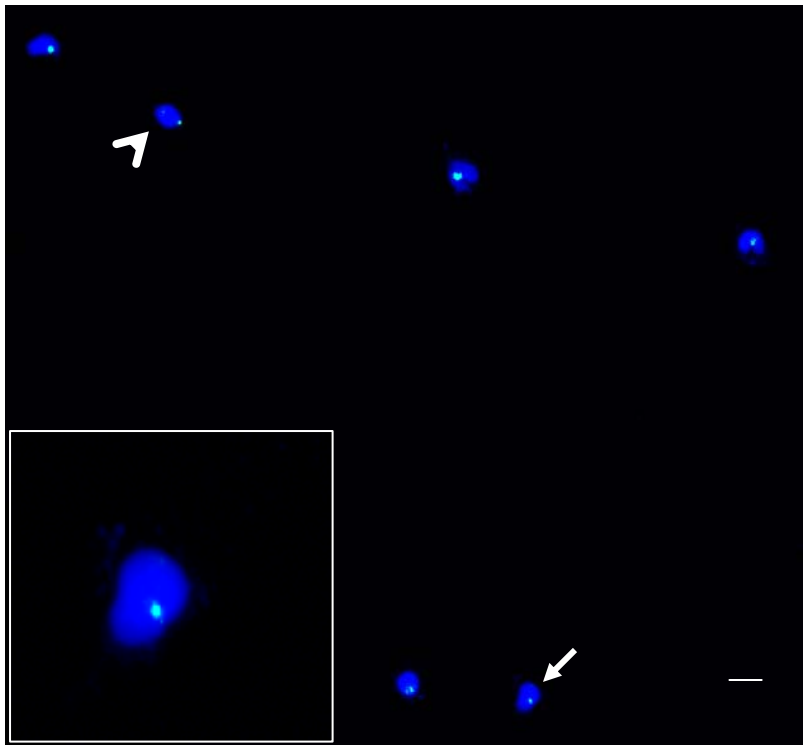


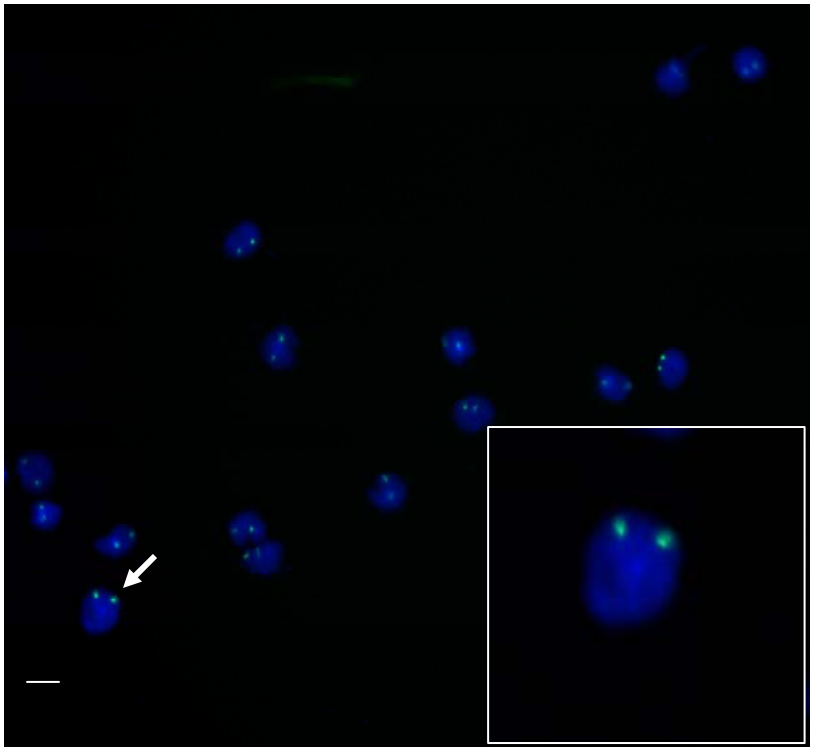
Supplementary Figure 17. DNA content (FACS) of human semen and control cells. Control hESCs carrying empty overexpression vector and different combinations of *DAZ*, *DAZL*, and *BOULE*. No specific population of 1N cells was detected at the scale setting at which the 1N sperm was detected by using human semen sample. In contrast, a small but significant peak/population was detected in cells with overexpression of DAZ, DAZL, and BOULE together (Fig. 4d).

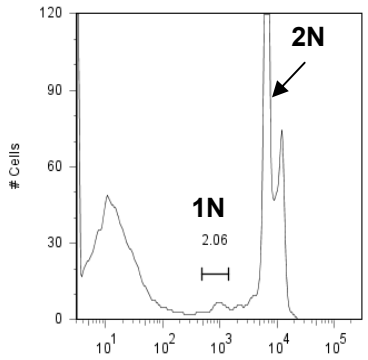


Supplementary Figure 18. Additional FISH staining and ACROSIN immunostaining at high resolution. a, Fluorescent in situ hybridization (FISH) with probe against chromosome 16 in the sorted 1N cells and 2N cells. FACS-isolated cells were cytospun onto slides and fixed for FISH analysis. Denaturation of samples at 85°C is required before specific DNA probe can bind to target chromosome sequence, in this analysis, autosomal chromosome 16. **b,** ACROSIN staining of the FACS-isolated 1N cells and control cells without any overexpression factors. Independent cell sample was used for this staining because denaturation step in FISH would denature ACROSIN making immunodetection of the antigen difficult. Scale bar is 10 micron.

Chr. 16 FISH, 200x

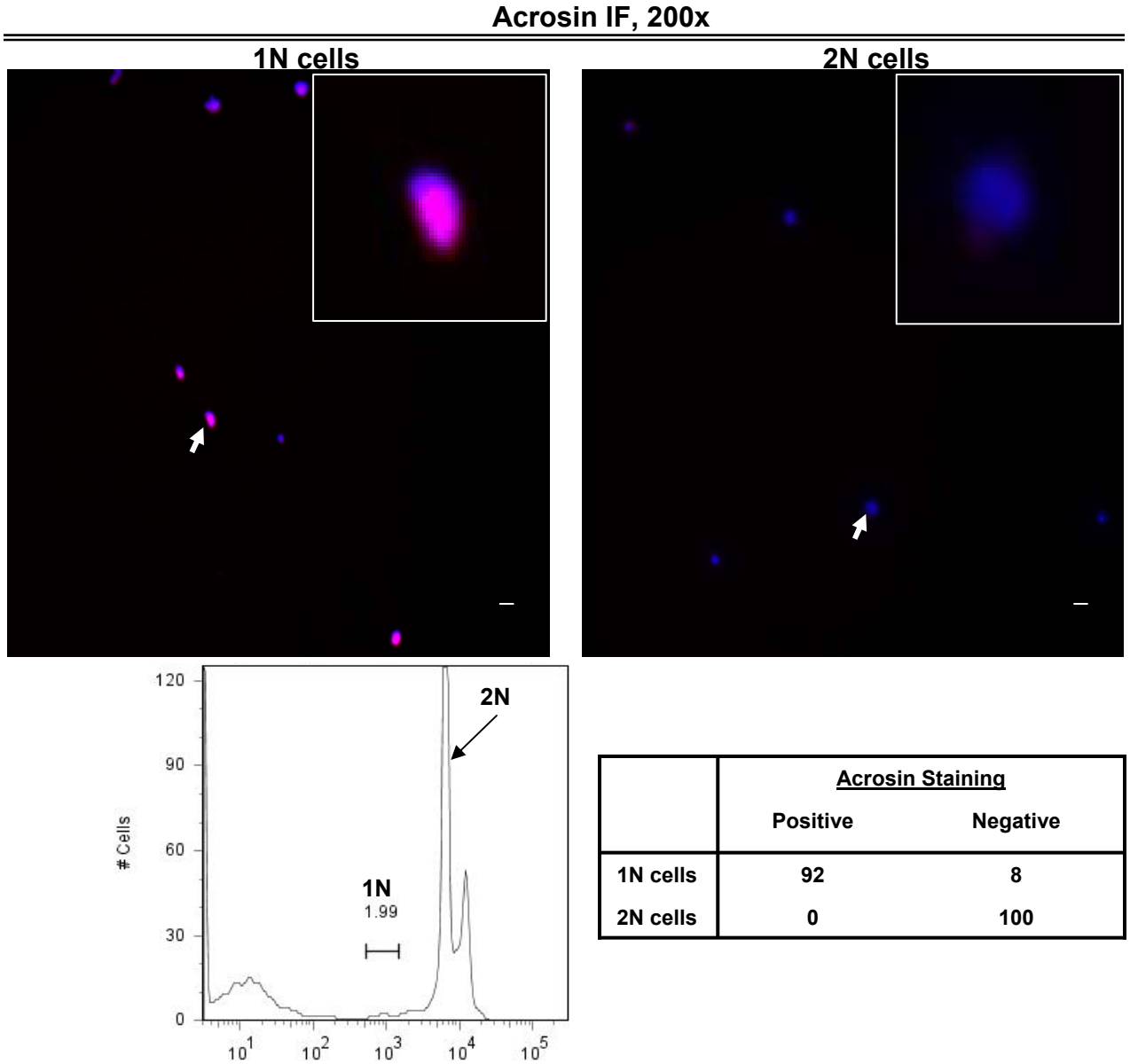






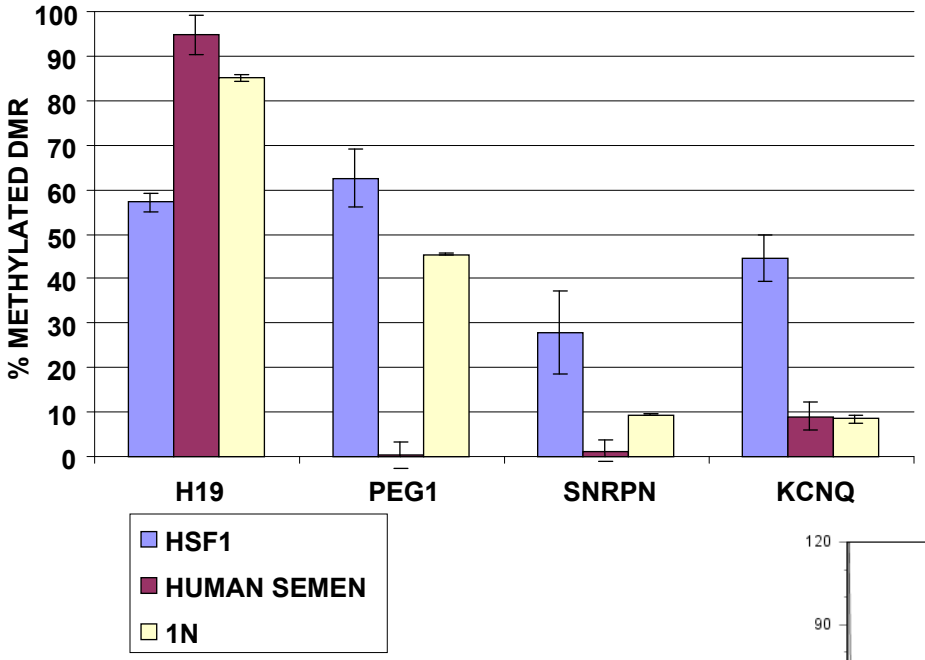
	Number of Chromosome 16		
	1	2	4
1N cells	89	11	0
2N cells	0	100	0

Supplementary Figure 19. Whole population FISH analysis of 1N and 2N cells with quantification of purity. All cells were FACS-isolated and cytospun onto slides before FISH procedures. Insets depict the cells labeled with arrows. The FACS profile is shown on the lower left and the purity quantification is shown on the lower right. 89 out of 100 cells had single focus of chromosome 16 probe, indicating single copy of chromosome 16. 11 out of 100 cells of the 1N FACS-isolated cells had two chromosome 16 (for example, the cell labeled with arrowhead at the upper left hand corner of the 1N cells image), suggesting incomplete detection of propidium iodide of 2N cells during FACS isolation or inherent chromosome aneuploidy of these cells. 2N cells had 100 out of 100 cells with 2 chromosome 16, indicating high purity of this population. Scale bar is 10 micron.

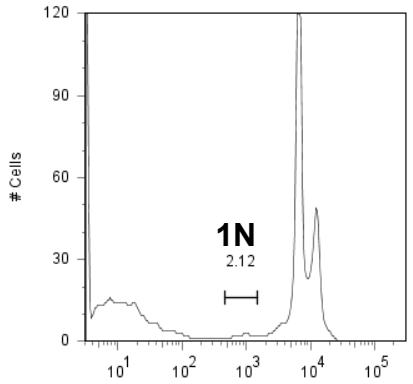


	Acrosin Staining	
	Positive	**Negative**
1N cells	**92**	**8**
2N cells	**0**	**100**

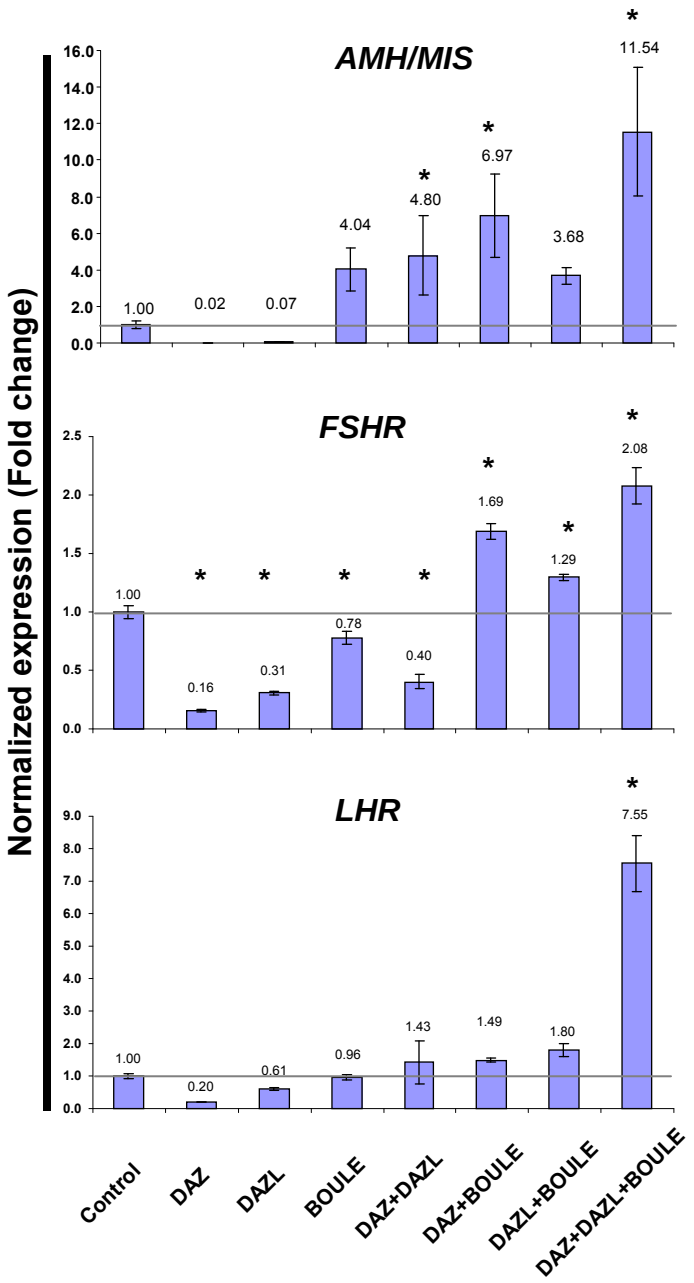
Supplementary Figure 20. Whole population ACROSIN analysis of 1N and 2N cells with quantification of purity. All cells were FACS-isolated and cytospun onto slide before immunostaining procedures. Insets depict the cells labeled with arrows. The FACS profile is shown on the lower left and the purity quantification is shown on the lower right. 92 out of 100 of 1N cells had strong ACROSIN staining and 8 cells had low or no detectable level of ACROSIN staining. In contrast, 2N cells showed no ACROSIN staining in all cells. Hence, 1N cells are strongly correlated with ACROSIN staining, supporting the FISH results that these cells are haploid cells and expressing spermatid/sperm marker, ACROSIN.

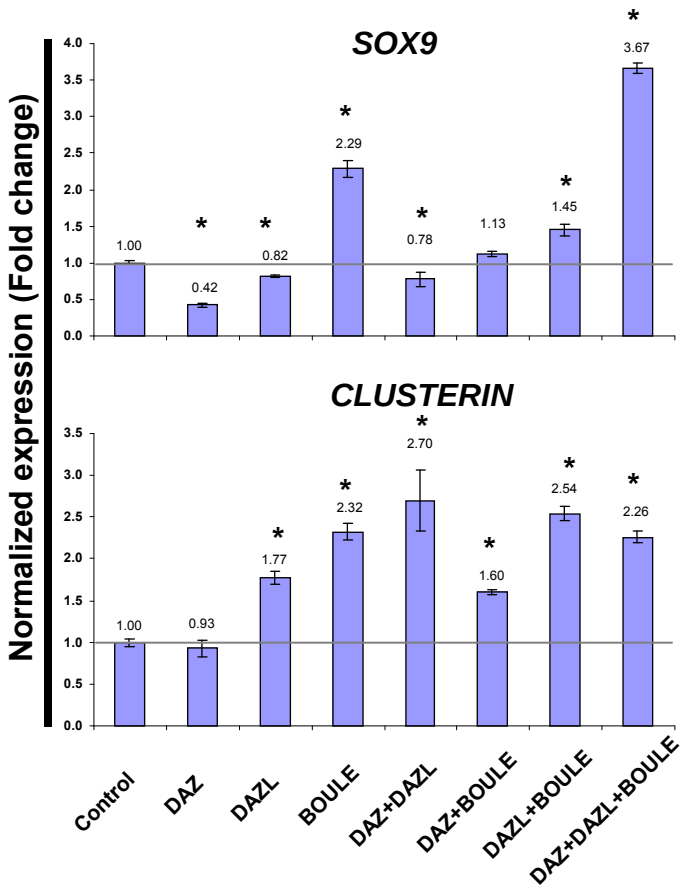


XY				
Newman-Keuls Test	Mean Diff.	q	Significant? P < 0.05?	Summary
H19 LOCUS				
HSF1 vs HS	-37.7	9.958	Yes	**
HSF1 vs 1N	-23.37	6.173	Yes	**
1N vs HS	-14.33	3.785	Yes	*
PEG1 LOCUS				
HS vs HSF1	-62.06	44.8	Yes	***
HS vs 1N	-44.97	32.46	Yes	***
1N vs HSF1	-17.09	12.34	Yes	***
SNRPN LOCUS				
HS vs HSF1	-26.52	5.179	Yes	*
HS vs 1N	-16.83	3.287	No	ns
1N vs HSF1	-9.692	1.893	No	ns
KCNQ LOCUS				
1N vs HSF1	-36.14	24.11	Yes	***
1N vs HS	-0.689	0.4596	No	ns
HS vs HSF1	-35.45	26.44	Yes	***



Supplementary Figure 21. Methylation status of various XY bearing cells at four imprinted DMRs. Genomic DNA of undifferentiated HSF1, human semen and 1N FACS-isolated cells was subjected to methylation sensitive and dependent enzyme digestions. qPCR was carried out to measure the methylation level at DMRs of H19 (paternal imprinted), PEG1, SNRPN, KCNQ (maternal imprinted) loci. Table at right shows results of statistical tests for all samples and loci. Lower panel shows DNA content profile of the 1N cells used in this analysis. In summary, 1N cells had similar profiles as human semen at H19, SNRPN and KCNQ, but not PEG1. Both 1N cells and human semen showed expected imprinted pattern at H19, SNPRN and KCNQ, i.e., hypomethylated at the maternal loci and hypermethylated at paternal locus. The difference at PEG1 suggested incomplete erasure of the 1N cells possibly caused by *in vitro* differentiation. Error bar = standard deviation; asterisk = significant difference by Newman-Keuls test ($p<0.05$), n=3.





Supplementary Figure 22. Expression of Sertoli and Leydig cell markers in the XY line with different overexpression factors. Day14 differentiated hESCs (without BMPs) were subjected to RNA extraction and qPCR analysis. Expression was normalized to 4 housekeeping genes followed by normalization to control (carrying empty overexpression vector). Asterisk indicates significant difference compared to control sample at p< 0.05. Overexpression of DAZ, DAZL, and BOULE together was associated with the highest *AMH*/*MIS*, *FSHR*, *LHR*, *SOX9* expression, suggesting the presence of Sertoli and Leydig cells in the differentiated hESC cultures. All cells are collected from whole differentiated culture without FACS for GFP+ cells. Error bar = standard deviation; asterisk = significant difference from control cells by one-way ANOVA test (p<0.05), n=3.