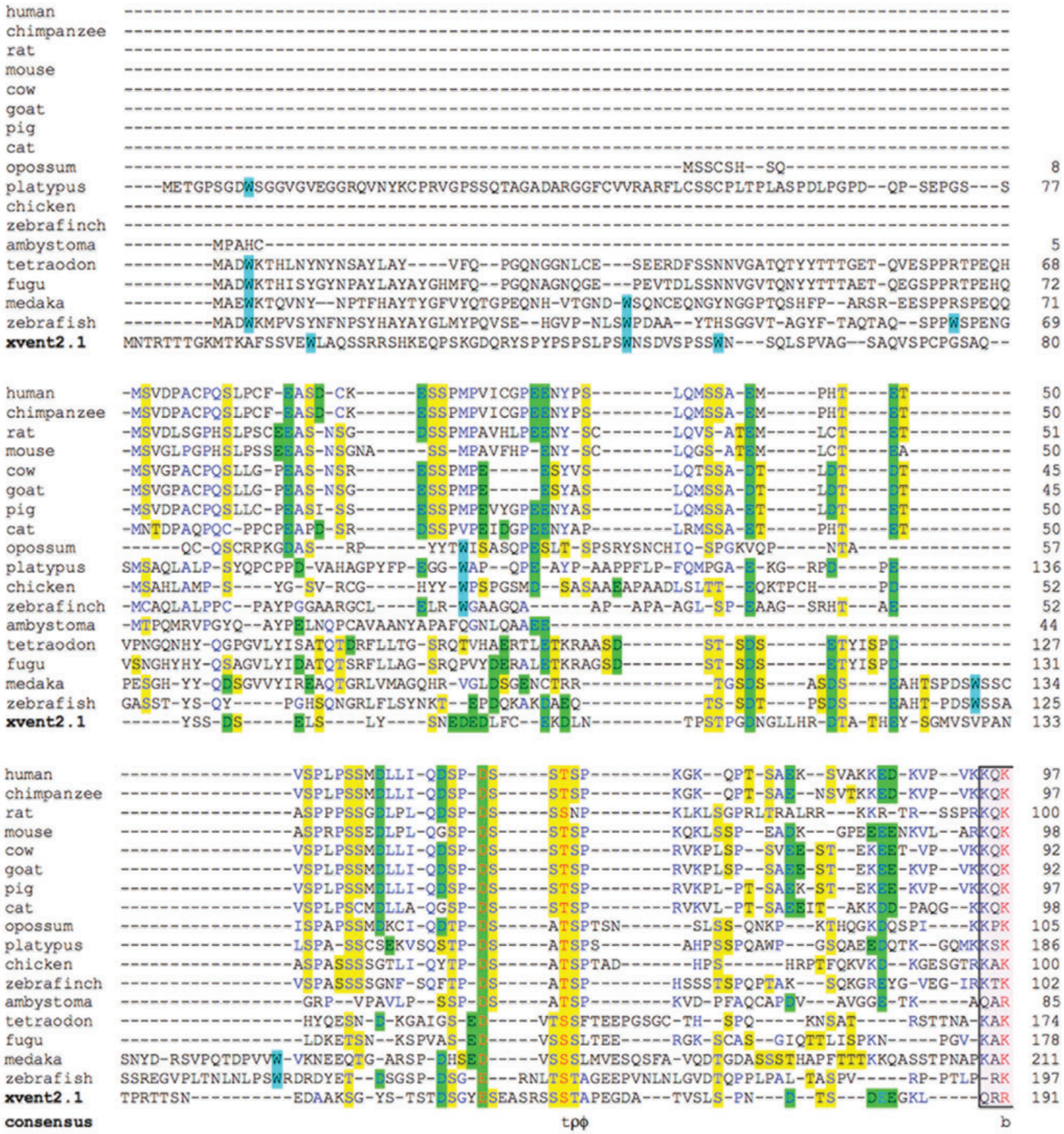
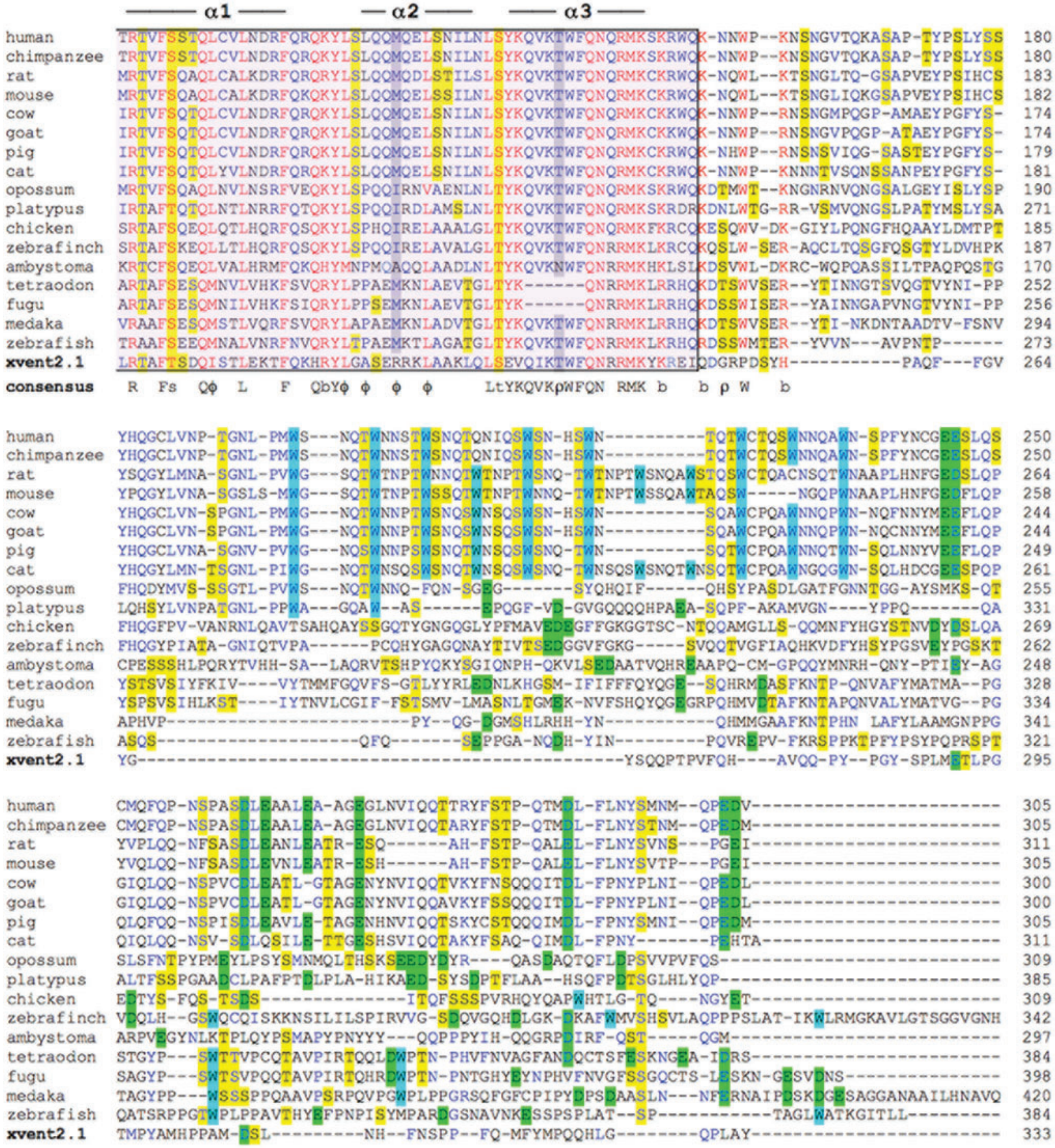
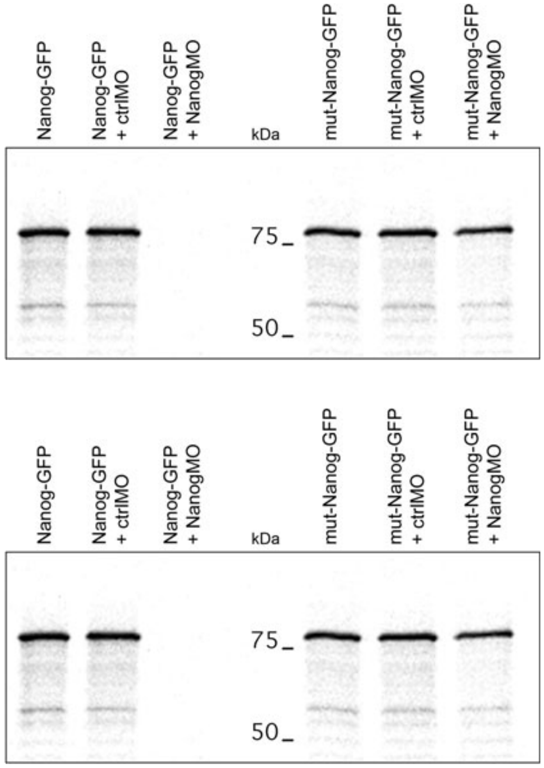
Supplementary Data



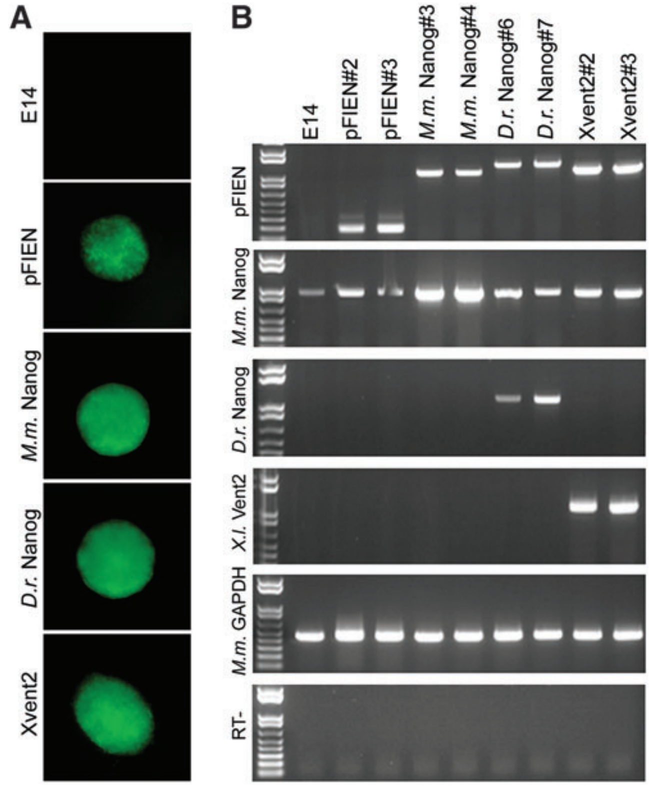
SUPPLEMENTARY FIG. S1. Alignment of Nanog proteins from human, chimpanzee, rat, mouse, cow, goat, pig, and cat (eutheria), opossum (metatheria), platypus (protheria), chicken and zebrafinch (birds), axolotl (amphibians), tetraodon, fugu, medaka, zebrafish (bony fishes), and Xvent2.1 of *Xenopus tropicalis*. Amino acids (PAM homology) are shown in blue (at least 8 of 17). Invariant amino acids in Nanog are given in red. The homeodomain (HD) is boxed, and its α-helical structure is schematically shown. Characteristic amino acid exchanges found in the Nanog HD are shaded in gray. Conserved acidic stretches are shaded in green, conserved serine and threonine residues are shaded in yellow, and tryptophan residues are shaded in bright blue, respectively. A triplet of amino acids (tρϕ), which is invariant in all Nanog proteins, is partially conserved in Xvent2.1 (S at pos. 71 in murine Nanog), but not in other BarH proteins or in the related Dll proteins (data not shown). Several S/T residues exist in the N-terminus of Xvent2.1 as described for Nanog. However, the 2 invariant amino acids flanking a polar residue adjacent to the HD domain of Nanog at the C-terminus, a basic and a tryptophan residue (b_ρ_W), are not present in Xvent2.1. A common consensus sequence for all proteins is given next. b, basic aa; ρ, polar aa; φ, hydrophobic aa; s, serine or threonine; t, threonine or serine.



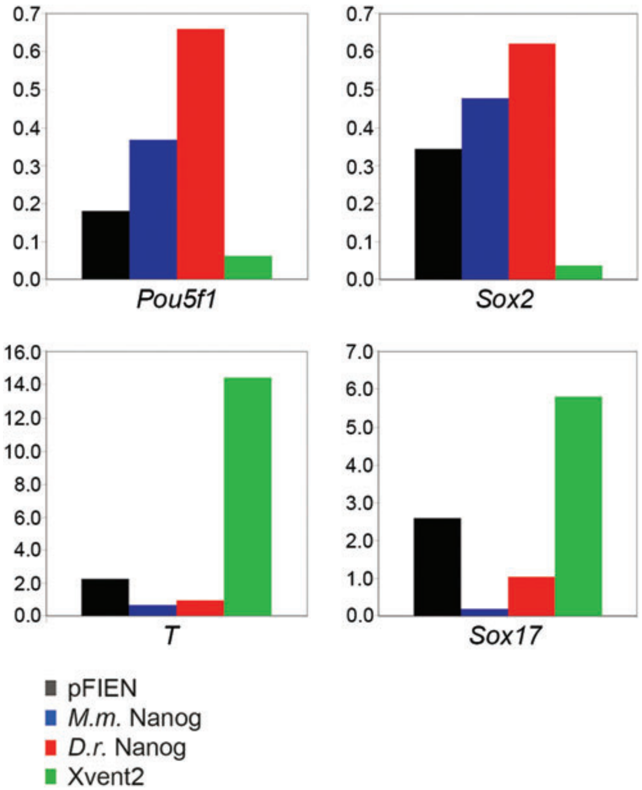
SUPPLEMENTARY FIG. S1. (Continued)



SUPPLEMENTARY FIG. S2. In vitro MO-binding test. The binding efficiency of NanogMO was tested in vitro in a coupled transcription/translation system (TNT®T7/SP6 Coupled Reticulocyte Lysate System; Promega) in the presence of ^{35}S labeled methionine. The translation of a *Nanog-GFP* fusion RNA is inhibited by NanogMO but not by ctrlMO. The inhibitory effect of NanogMO is specific, because transcription/translation of 0.5 ng *mut-Nanog-GFP* plasmid is inhibited by neither 10 ng ctrlMO nor 10 ng NanogMO. MO, morpholino oligonucleotides; ctrl MO, control MO.



SUPPLEMENTARY FIG. S3. Transfection control of mouse E14 ES cells. *M.m. Nanog*, *D.r. Nanog*, and *Xvent2* sequences were integrated into the eukaryotic expression vector pFIEN (provided by P.R. Tata, Ulm), which harbors a neomycin/kanamycin resistance cassette, a pCAGGS derived promoter, the integrated sequence, an IRES, and the EGFP/SV40 polyA part of the pIRES2-EGFP vector (Clontech). **(A)** Stably transfected E14 mouse ES cell lines with *M.m.* Nanog, *D.r.* Nanog, Xvent2, and an empty control (pFIEN) vector after 7 passages. EGFP (Enhanced Green Fluorescence Protein) signal indicates the stable expression of each construct. **(B)** Single GFP-positive ES cell clones were picked, expanded, and tested for proper stable expression of the constructs by RT-PCR with indicated primer pairs (Supplementary Table S1C). Two independent cell clones of each construct were used. RT-: cDNA synthesis without reverse transcriptase, analyzed with GAPDH primers used as an internal control. Numbers represent different cell lines. ES, embryonic stem; RT-PCR, reverse transcriptase–polymerase chain reaction.



SUPPLEMENTARY FIG. S4. Analysis of marker gene expression of embryoid bodies after 4 days of culturing in the absence of leukemia inhibitory factor. RNA was extracted and submitted to RT-PCR by using primers for *Pou5f1*, *Sox2*, *T*, and *Sox17* (see Supplementary Table S1C). Although *M.m.* Nanog and *D.r.* Nanog transfected cells show higher expression of pluripotency genes (*Pou5f1*, *Sox2*), Xvent2 and pFIEN transfected cells show higher expression of differentiation markers (*T*, *Sox17*).

Supplementary Table S1. Primers and Conditions Used for Real-Time Reverse Transcriptase-Polymerase Chain Reaction

(A) Primers Used for *Xenopus laevis*

Primer pairs	*Sequence forward primer (5′→3)*	*Sequence reverse primer (5′→3)*	*Annealing temperature (°C)/ time(s)*
BMP4	TTTATGTGGATTTCAGCGATGTG	AGCCAAGGGAAATGGACAATCT	53/5
Cdx2	TACCCCTCTGCCCCCCA	GGAAGGTGCCCCATGGTCT	53/5
Chordin	AACTGCCAGGACTGGATGGT	GGCAGGATTTAGAGTTGCTTC	52/5
Cxcr4b	CCTCACTACCGACACATTCATGAT	CCTTGTGTAGGGTGTTCTCCCA	54/5
E-cadherin	GACTCACTCCTTGTGTTCGATTACG	TGGGCGAGTTCAGAGAGCTG	54/5
Ectodermin	TGTGTAAGCTCAGCCTGCAAA	GACAGCGGCGACAGAATGA	54/5
Epidermal Keratin	GCTCATCTAGCAAAGGTGGCTT	AGCCAGAACCCACACCACTG	54/5
Fut1	GAGGGTGGGATATGGACTGTTAAA	GGACAGAGCATACAAGGTGGC	54/5
Gata4	GCTACCTCACCTGTCTATGTGCC	TTTGTTGAGATGAGCCGCTG	53/5
Gata6	ACAATCACATCAGCGCAAGGA	GACTGGAGAGCTGGCAGCG	53/5
Geminin H	GGATATGGAGAAGCCTACCATGTC	GATGCAGATGGCTGGATTACTTT	53/5
Grhl1	CATCGCTCACCCCATCAAA	CAAAGGCGACAATCCGTTCT	53/5
Gsc	GATGCCGCCAGTGCCTC	TGCAGCTCAGTTCGTGACAAA	54/5
H4	GGCAAAGGAGGAAAAGGACTG	GGTGATGCCCTGGATGTTGT	55/5
Klf4	ATGAACCGACCCGCCAC	AAGCTCGATCACATCGCTGA	53/5
Ncam	AAGTCCAACAGAGGCAACCAA	GTCCCCCCATCAATTTGAAA	54/5
Oct60	TGACGGAAATTGCCAAAGAAC	CCTTGGACATTCTGAATTTGCTC	54/5
PepCK	CCAACGTGGCAGAGACCAGT	TGACCCCAGGAGGAAGTGG	54/5
Sall4	CAGTATGCCACCGCGCTT	TACTGATAACAGGAGCACCGCC	54/5
Siamois	AAGGAACCCCACCAGGATAAAT	TGTTGTTCTGGTTCTTCAAACTTCA	54/5
Sod2	CAGGCTCAGTGTTTGTGGCA	TCATAAGGCAAGTCAGGCAGAG	52/5
Tert	CAGGTGCTTGGGATTGTGGA	CCTGAACTTCTCAGAGTCTCCCTCT	54/5
Tsg1	CAGCAAATGCCTAATACAGGAGCT	GTGCCCAGACACAGCATACACTC	55/5
Wee1b	AGCACCCCTCAGGTCAACATC	CAAAGCCCGGCAGAAGACT	54/5
Xbra	AGCTCACCAACGAGATGATCG	AACCGTATACATTGCATTGGGAT	54/5
Xema	TCCGCAGAAAGAGGAAAAGAAA	ACTTAGCGGGCTCCCTTCAG	54/5
XSox2	CCAGTCCACCTGTAGTCACCTCT	CACTTCTGCCCCAGGTAGGTAC	54/5
XSox3	GTTTATGGTTTGGTCCCGGG	AGCGCCCAATCTTTTGCTG	55/5
XSox17α	TGGCAAGTCGTGGAAGTCTCT	GTGGTCCTGCATGTGCTGC	54/5

(B) Primers Used for *Danio rerio*

Primer pairs	*Sequence forward primer (5′→3)*	*Sequence reverse primer (5′→3)*	*Annealing temperature (°C)/time(s)*
BMP4	CCCGAGGAAGGGAAGAAGAA	TCAAAGTCCCGCAGCAGC	54/5
Cxcr4b	CCTGGAACAGGGTCTGGAGA	TAGGGTTGAGACAGCAGTGGAA	54/5
Gata4	CCTCGTCCTCTCGCCATG	GGTTTTGCTGATGTTTTTGGG	54/5
Gata6	GAAGCCCGCATACCTCCAA	TATCCCTCGATGTGCCGTTAT	55/5
Gsc	CGCTGGGATGTTTAGTATCGACA	GGTGGAGCAGAACCGAGTCTT	53/5
H4	CTCCCCGCTATAAGTGTCATGTTT	GCGTAAGTGGGCTGTGAGGTT	54/5
Nanog	ACAGCCAGTACCCGGGACAC	TTGGTCGGGCTCAGTCTTGT	54/5
Pax2a	TGTTTGAGCGGCCGTCA	CCTGCTCTGGCTTGATGTGTT	54/5
Pou2	CCGCTTCATCCCCATCTCT	TCCGCTAAAAAATCCTGGGC	54/5
Shh	CACCTCCTTTTTGTCCTCGACA	TCCGGCTCTGACACTGCTG	54/5
Ved	TATCCCATCAGCCCCGC	GGATGTACAGGGATGCTCGG	54/5
Vent	GCGGATGAAGCTGAAGCG	GAAAAACAGCGGGATAGAGGAA	55/5
Vox	CCGTGGACTGGCTTGCTC	TTGTCTTTTTCTCCGGCTCCT	54/5

(C) Primers Used for Cell Culture (*M.m.* ES cell line *E14*)

Primer pairs	*Sequence forward primer (5′→3)*	*Sequence reverse primer (5′→3)*	*Annealing temperature (°C)/ time(s)*
pFIEN	TGGGCAACGTGCTGGTTGTTGT	TTCCAAGCGGCTTCGGCCAG	60/70
M.m. Nanog	GGCAATTGATGAGTGTGGGTCTTCCTG	CCGCTCGAGTCATATTTCACCTGGTGGAGTC	60/70
D.r. Nanog	GGAATTCATGGCGGACTGGAAGATGC	ACGCGTCGACTCACAGCAAAGTTATTCCT	60/70
Xvent2	GGAATTCATGACTAAAGCTTTCTCCTC	CCGCTCGAGCTAATAGGCCAGAGGTTGCCCA	60/70

(*continued*)

Supplementary Table S1. (Continued)

Primer pairs	Sequence forward primer (5′→3)	Sequence reverse primer (5′→3)	Annealing temperature (°C)/ time(s)
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	57/70
M.m. H4	AAGCGCAAGACGGTTACGG	TACAGAGTGCGGCCCTGG	54/5
M.m. T	CCGGTGCTGAAGGTAAATGTG	GGTGTCAGCCGTCACGAAGTC	55/5
M.m. Sox17	CCCTTTGTGTATAAGCCCGAGA	CGTGGCTGTCTGAGAGGTTCA	52/5
M.m. Pou5f1	GTTGGCGTGGAGACTTTGCA	GAGGTTCCCTCTGAGTTGCTTTC	54/5
M.m. Sox2	TGGCCCAGGAGAACCCC	GACAAAAGTTTCCACTCCGCG	54/5

Denaturation temperature was set to 95°C and extension was done by 72°C for 14 s.

Supplementary Table S2. Frequencies of Observed Phenotypes After Gain and Loss of Function and Changes in Marker Gene Expression

(A) In Situ Hybridization: Delocalized Expression After Nanog Gain of Function in *Danio rerio* Embryos

	Control injected % ([a])	D.r. Nanog RNA (400 pg) % ([a])
Patched-1 at tailbud stage	0 (0/17)	72 (13/18) (lower expression, expression domain quite different)
Sox17 at 80% epiboly	6 (2/31)	46 (20/44) (dorsal forerunner cells dispersed along margin)
Shh at tailbud stage	0 (0/33)	82 (32/39) (expression domain shorter and wider)

(B) Southpaw In Situ Hybridization After Nanog Gain of Function in *Danio rerio* Embryos

At 20 hpf	Wild type % ([a])	Control injected % ([a])	D.r. Nanog RNA (400 pg) % ([a])
Left side	76 (57/75)	73 (35/48)	50 (35/70)
Right side	4 (3/75)	2 (1/48)	7 (5/70)
Both sides	19 (14/75)	23 (11/48)	43 (30/70)

(C) Loss of Function, Gain of Function, and Rescue: *Danio rerio* Embryos

Analyzed at Phenotype	70%–80% epiboly Disturbed epiboly % ([a])	1 dpf Lethality % ([a])
Wild type	1 (1/85)	9 (20/212)
ctrlMO	2 (2/83)	11 (13/116)
NanogMO	99 (102/103)	86 (120/139)
NanogMO + *D.r. mut-Nanog* RNA	33 (9/27)	21 (18/86)
NanogMO + *M.m. Nanog* RNA	8 (3/31)	13 (4/31)

(D) In Situ Hybridization: Decreased Expression After Nanog Loss of Function in *Danio rerio* Embryos

At 70%–80% epiboly	controlMO % ([a])	NanogMO % ([a])
Ved	0 (0/40)	78 (28/36)
BMP4	0 (0/53)	100 (33/33)

(E) Gain of Function: *Xenopus laevis* Embryos

Analyzed at Phenotype	Stage 11.5 Failure in blastopore formation % ([a])	Stage 29 No head, no cement gland % ([a])	Stage 29 Head, but no cement gland % ([a])	Stage 29 Head, but reduced cement gland % ([a])
Wild type	5 (2/37)	2 (1/54)	0 (0/54)	4 (2/54)
D.r. Nanog RNA (600 pg)	43 (84/196)	84 (76/91)	2 (2/91)	3 (3/91)
M.m. Nanog RNA (800 pg)	42 (58/139)	12 (14/119)	7 (8/119)	26 (31/119)
Xvent2 RNA (800 pg)	42 (79/187)	77 (97/126)	9 (11/126)	3 (4/126)

[a]Observed phenotype/total number of embryos.
MO, morpholino oligonucleotides; hpf, hours postfertilization.

Supplementary Table S3. Changes in the Rate of Transcription (Numbers in Bold) as Monitored by Reverse Transcriptase-Polymerase Chain Reaction Including Standard Deviations (Regular Numbers)

(A) Nanog Loss of Function in Zebrafish Embryos

	Pou2	*Cxcr4b*	*Gsc*	*Shh*	*BMP4*	*Vent*	*Vox*	*Ved*	*Pax2a*	*Gata6*	*Gata4*
+1.5 ng NanogMO	**0.39**	**3.75**	**0.16**	**1.16**	**1.04**	**0.41**	**0.42**	**0.38**	**6.35**	**0.10**	**0.14**
	0.02	0.47	0.03	0.42	0.59	0.18	0.10	0.24	4.22	0.03	0.00

(B) Nanog Gain of Function in Zebrafish Embryos

	Pou2	*Cxcr4b*	*Gsc*	*Shh*	*BMP4*	*Vent*	*Vox*	*Ved*	*Pax2a*	*Gata6*	*Gata4*
+15 pg *Nanog RNA*	**1.65**	**0.74**	**1.56**	**0.83**	**1.37**	**1.19**	**1.12**	**1.33**	**0.88**	**1.21**	**0.99**
	0.13	0.21	0.09	0.10	0.28	0.31	0.30	0.20	0.04	0.14	0.27
+60 pg *Nanog RNA*	**2.57**	**0.70**	**2.09**	**0.53**	**1.69**	**0.79**	**0.96**	**1.89**	**0.53**	**0.91**	**0.80**
	1.29	0.05	0.53	0.01	0.18	0.04	0.28	0.31	0.19	0.12	0.03

(C) Gain of Function of *M.m.* Nanog, *D.r.* Nanog, Xvent1, and Xvent2 in *Xenopus* Embryos at Different Developmental Stages

		Sall4	*Tert*	*Fut1*	*Klf4*	*Oct60*	*Cxcr4b*	*Wee1b*	*Cdx2*	*Xbra*	*Gsc*	*Siamois*	*Chordin*	*BMP4*	*Tsg1*	*Xema*	*Ecto-dermin*	*XSox2*	*Soxx3*	*Geminin*	*Ncam*	*Grhl1*	*Epidermal Keratin*	*Xsox17α*	*Gata6*	*Gata4*	*Sod2*	*PepCK*
Stage 10	*M.m. Nanog*	**0.84**	**0.88**	**0.73**	**1.83**	**0.52**	**1.04**	**1.99**	**1.70**	**0.42**	**2.13**	**0.73**	**1.78**	**0.48**	**2.35**	**2.76**	**0.90**	**7.25**	**0.77**	**0.89**	**0.86**	**0.87**	**0.44**	**1.25**	**0.90**	**0.93**	**2.71**	**0.97**
		0.04	0.30	0.29	0.33	0.54	0.18	0.56	0.42	0.14	0.25	0.05	0.16	0.26	1.89	0.36	0.04	4.27	0.05	0.12	0.01	0.09	0.45	0.03	0.07	0.16	0.41	0.07
	D.r. Nanog	**0.75**	**1.62**	**0.33**	**2.44**	**0.29**	**0.44**	**2.90**	**8.91**	**0.46**	**1.30**	**0.41**	**0.41**	**0.33**	**4.25**	**1.02**	**1.06**	**3.88**	**0.54**	**0.94**	**1.65**	**1.02**	**0.10**	**0.65**	**0.38**	**0.27**	**4.94**	**0.48**
		0.05	0.28	0.02	0.86	0.03	0.01	2.18	6.05	0.13	0.16	0.02	0.04	0.12	0.76	0.03	0.09	0.21	0.13	0.05	0.94	0.15	0.01	0.08	0.08	0.01	0.53	0.37
	Xvent1	**0.74**	**0.85**	**0.82**	**1.74**	**0.51**	**0.58**	**3.62**	**0.93**	**0.44**	**0.11**	**0.73**	**0.32**	**0.68**	**5.16**	**0.22**	**1.00**	**0.71**	**0.41**	**0.72**	**1.64**	**0.98**	**0.71**	**0.87**	**0.53**	**0.89**	**2.78**	**2.46**
		0.18	0.13	0.06	0.22	0.25	0.20	2.35	0.40	0.13	0.08	0.13	0.16	0.52	1.59	0.08	0.02	0.24	0.02	0.03	1.26	0.11	0.26	0.06	0.06	0.09	0.57	1.48
	Xvent2	**0.79**	**0.87**	**0.65**	**0.91**	**0.44**	**1.16**	**1.47**	**5.24**	**2.44**	**0.19**	**0.81**	**0.09**	**0.95**	**1.19**	**0.57**	**1.11**	**0.25**	**0.46**	**0.46**	**0.63**	**1.02**	**1.44**	**1.27**	**0.95**	**1.10**	**2.22**	**0.48**
		0.04	0.09	0.06	0.26	0.28	0.16	1.24	1.97	1.18	0.15	0.06	0.01	0.45	0.03	0.42	0.02	0.07	0.04	0.04	0.12	0.15	0.59	0.00	0.09	0.08	1.99	0.10
Stage 11	*M.m. Nanog*	**0.86**	**0.41**	**0.73**	**1.79**	**0.20**	**1.03**	**2.57**	**0.96**	**0.69**	**1.48**	**0.85**	**2.03**	**2.66**	**2.97**	**1.68**	**1.63**	**1.00**	**0.84**	**0.90**	**1.47**	**1.26**	**0.27**	**0.32**	**0.71**	**1.18**	**4.72**	**2.25**
		0.01	0.36	0.10	0.34	0.06	0.01	1.12	0.11	0.02	0.54	0.18	1.52	0.25	0.60	0.18	0.71	0.36	0.09	0.35	0.44	0.29	0.04	0.24	0.02	0.21	0.60	0.46
	D.r. Nanog	**0.70**	**0.54**	**0.34**	**1.69**	**0.61**	**0.57**	**1.69**	**1.26**	**0.53**	**0.99**	**0.44**	**0.39**	**0.51**	**0.76**	**0.56**	**1.32**	**0.99**	**0.57**	**1.07**	**1.16**	**1.01**	**0.18**	**0.31**	**0.70**	**0.33**	**2.40**	**1.53**
		0.01	0.18	0.05	0.51	0.32	0.06	0.88	0.28	0.21	0.15	0.15	0.03	0.05	0.21	0.13	0.06	0.68	0.4	0.04	0.37	0.08	0.04	0.11	0.09	0.11	1.11	0.65
	Xvent1	**2.26**	**1.26**	**1.09**	**1.69**	**1.30**	**0.99**	**1.74**	**0.80**	**1.16**	**0.09**	**0.93**	**0.46**	**1.36**	**1.72**	**0.22**	**3.35**	**1.25**	**0.41**	**1.41**	**4.10**	**1.64**	**1.31**	**0.49**	**0.16**	**0.70**	**2.63**	**2.14**
		0.87	0.25	0.33	1.32	0.62	0.18	0.81	0.06	0.50	0.04	0.21	0.10	0.35	1.22	0.01	0.84	0.48	0.10	0.05	2.62	0.18	0.45	0.12	0.05	0.22	1.85	0.81
	Xvent2	**0.67**	**0.54**	**0.97**	**1.72**	**0.74**	**0.69**	**0.24**	**1.03**	**1.59**	**0.12**	**0.65**	**0.10**	**1.33**	**1.05**	**0.31**	**0.93**	**0.45**	**0.56**	**0.64**	**0.42**	**1.10**	**1.93**	**1.09**	**1.17**	**0.76**	**3.92**	**0.50**
		0.15	0.21	0.06	0.63	0.11	0.08	0.08	0.06	0.01	0.01	0.06	0.04	0.03	1.16	0.05	0.16	0.23	0.08	0.12	0.05	0.19	0.21	0.54	0.20	0.10	0.48	0.16
Stage 12	*M.m. Nanog*	**1.18**	**2.55**	**0.49**	**3.58**	**2.35**	**1.34**	**2.73**	**1.43**	**0.88**	**2.39**	**0.90**	**2.14**	**3.61**	**0.12**	**3.64**	**1.71**	**0.79**	**0.98**	**1.31**	**1.79**	**1.02**	**0.44**	**0.74**	**0.23**	**0.97**	**2.45**	**1.22**
		0.42	0.61	0.27	0.11	0.21	0.43	1.24	0.22	0.03	0.41	0.53	0.62	0.11	0.07	2.32	0.23	0.18	0.32	0.11	0.30	0.07	0.13	0.31	0.08	0.52	0.36	0.17
	D.r. Nanog	**1.14**	**3.79**	**0.44**	**4.35**	**3.13**	**1.20**	**2.56**	**0.94**	**0.71**	**2.40**	**0.46**	**3.72**	**6.49**	**1.31**	**1.00**	**2.03**	**0.81**	**0.64**	**1.62**	**1.18**	**0.90**	**0.33**	**0.59**	**0.35**	**0.47**	**3.30**	**2.39**
		0.11	1.85	0.13	2.42	1.85	0.19	2.40	0.08	0.12	0.50	0.17	1.56	6.17	0.86	0.37	0.14	0.17	0.18	0.08	0.12	0.29	0.21	0.13	0.04	0.04	1.79	1.37
	Xvent1	**0.62**	**0.79**	**0.55**	**1.10**	**1.75**	**0.69**	**0.71**	**0.31**	**0.42**	**0.05**	**0.28**	**0.08**	**0.28**	**0.93**	**0.85**	**1.07**	**0.28**	**0.26**	**0.55**	**0.63**	**1.69**	**0.63**	**0.39**	**0.35**	**0.45**	**1.87**	**1.35**
		0.01	0.30	0.06	0.68	1.04	0.16	0.08	0.26	0.34	0.04	0.13	0.07	0.09	0.48	0.10	0.08	0.13	0.08	0.01	0.11	0.53	0.01	0.19	0.35	0.15	0.14	0.15
	Xvent2	**0.92**	**3.28**	**1.21**	**1.33**	**1.68**	**0.86**	**2.62**	**1.64**	**1.73**	**0.21**	**0.96**	**0.11**	**2.81**	**2.07**	**3.35**	**1.18**	**0.93**	**0.65**	**0.66**	**0.73**	**1.26**	**2.72**	**1.71**	**1.44**	**1.00**	**1.42**	**0.92**
		0.25	3.77	0.08	0.13	0.06	0.25	0.68	0.15	0.32	0.19	0.11	0.01	1.61	0.73	1.41	0.04	0.06	0.11	0.00	0.01	0.02	0.04	0.70	1.12	0.21	0.57	0.57