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Supplemental Information

NANOG Is Required for the Long-Term Establishment of Avian Somatic Reprogrammed Cells

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Figure S1: Primary CEF were infected by pMX retroviral vector expressing GFP to evaluate the titre of the pMX reprogramming vectors, used for reprogramming (Figure 1).

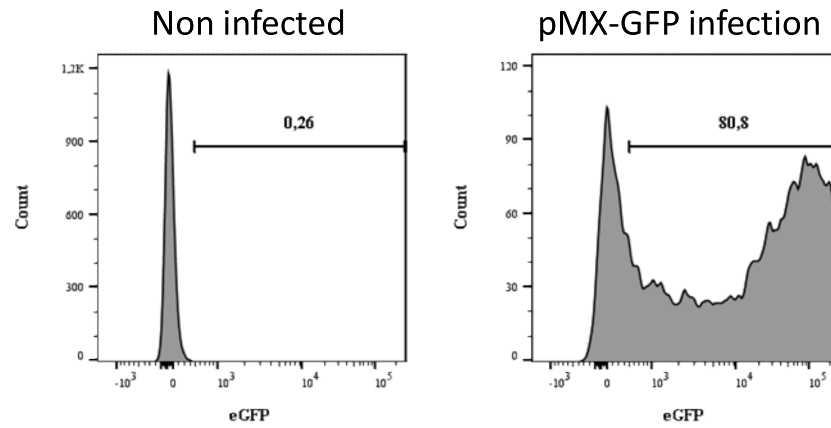
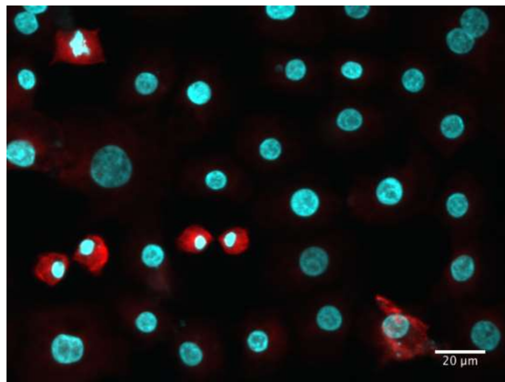
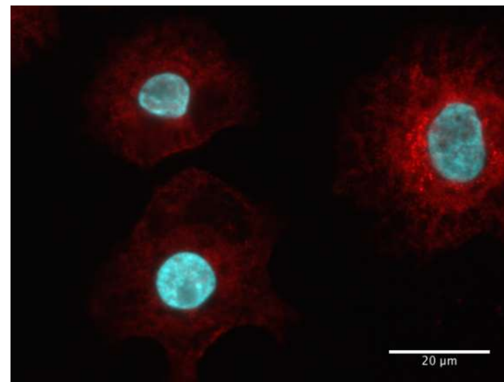


Figure S2: The reprogrammed cells differentiate into VIM-positive cells related to Figure 4 .

CEF were reprogrammed using OSKMN gene combination delivered by pMX retroviral vesctors. At passage 6 they were induced to form embryoid bodies-like structures for 4 days and then plated as described previously (Aubel and Pain, 2013) onto gelatinized coverslips. After 5 days the cells were fixed with PFA and immunofluorescence was performed using the AMF-17b antibody (DSHB) against Vimentin at 2.5µg/mL. CEF were used as a control. Epifluorescence images are shown with antibody staining in red and DAPI counterstaining in cyan; scale bar 20µm.

Reprogrammed cells





CEF

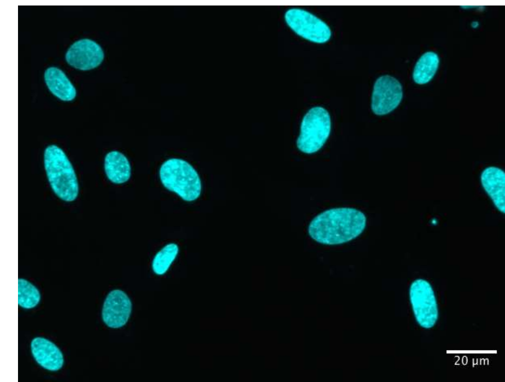


Figure S3A, related to Figure 6: A protein network of 76 genes commonly expressed between the reprogrammed clones and the chicken stem cells as established by String software. Two main interaction cores were observed, one centered on NANOG/POU5 pluripotency-associated genes and one centered on CDH1 comprising several adhesion molecules.

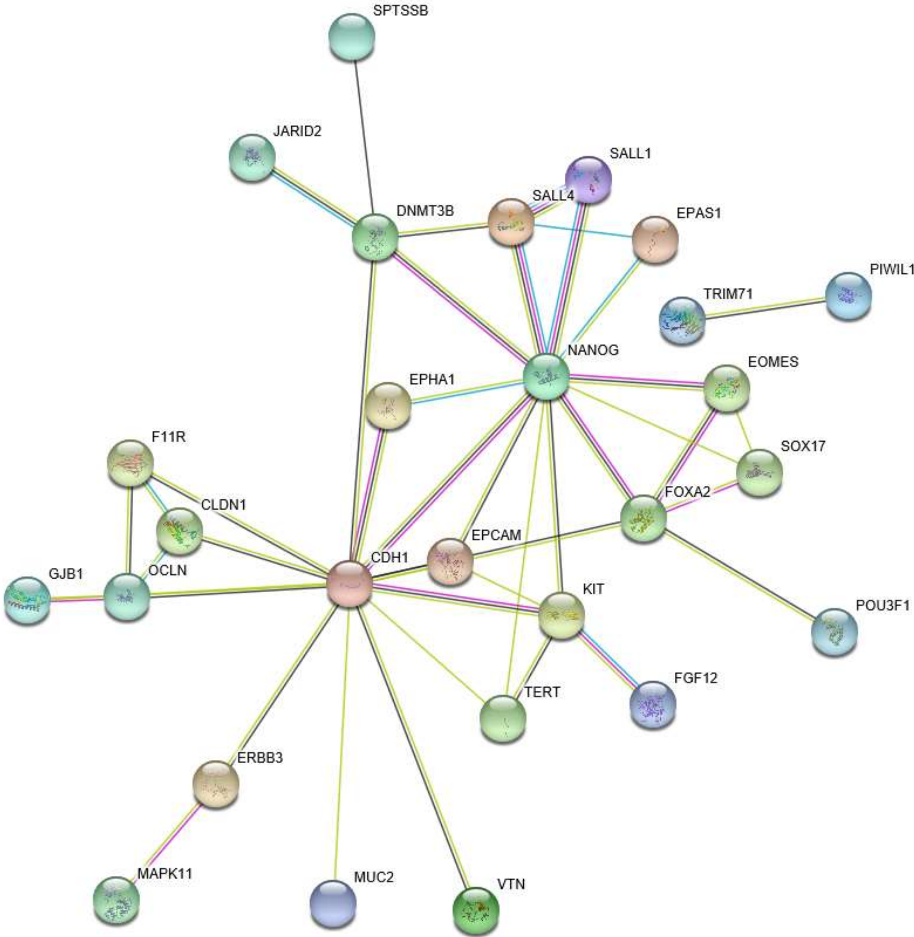


Figure S3B, related to Figure 6:: A protein network of 148 genes commonly expressed between the reprogrammed clones and the cESCs as established by String software. The pluripotency and CDH1 nodes were reinforced with additional linked genes, and two additional nodes emerged, one centered on the cKIT proto-oncogene and one on WNT3

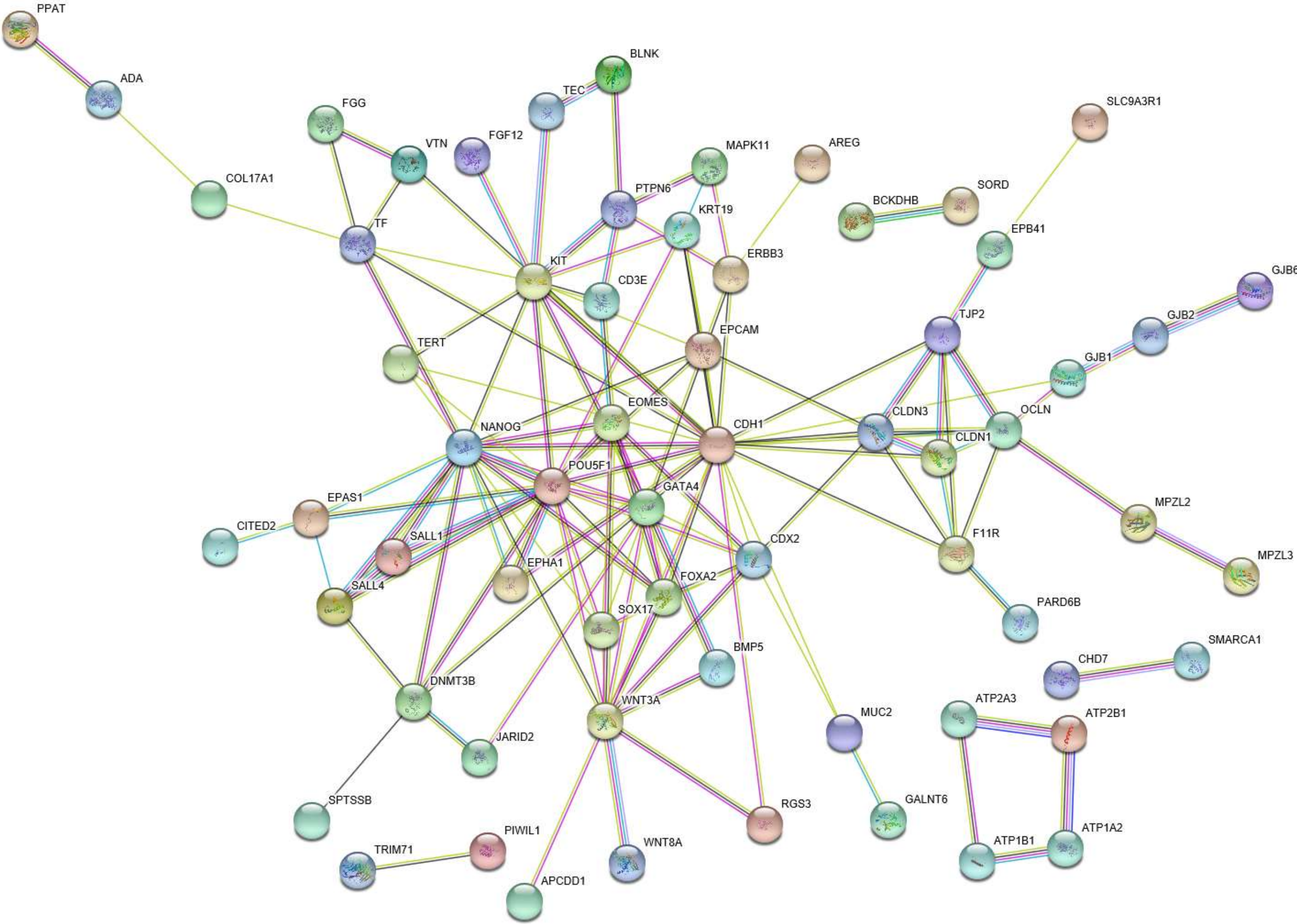


Figure S3C, related to Figure 6: A protein network of 150 genes commonly absent or expressed at low levels in the reprogrammed clones compared with their expression in chicken stem cells as established by String software. Five main nodes centered on SOX2, EFNB2, MYL3, GNAI1, and AGTR1 were identified, and each included candidate genes to improve the reprogramming process

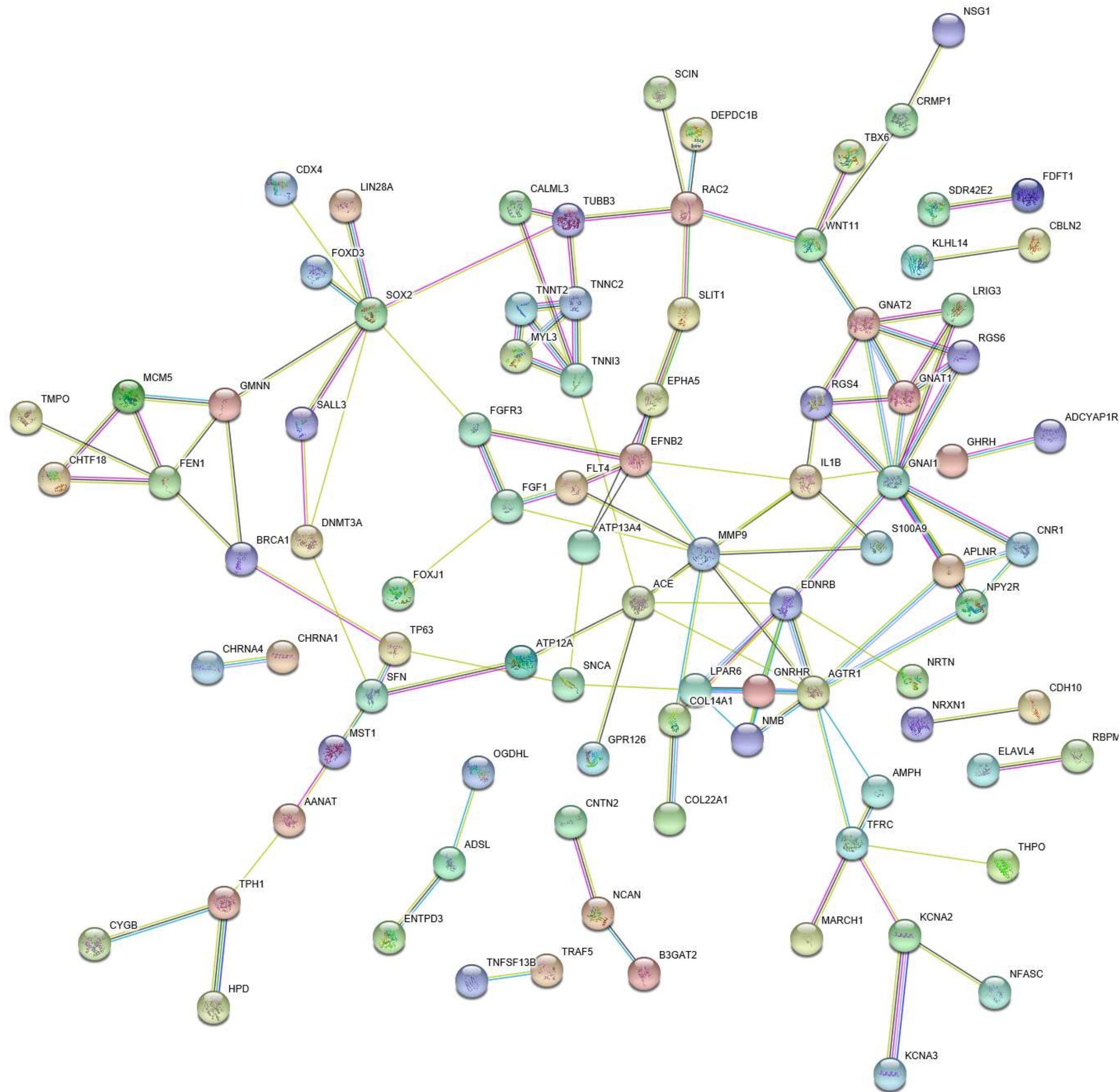


Figure S4: Expression level of endogenous and exogenous genes used for reprogramming, directly taken from the RNASeq data and complementary to the qrt-PCR illustrated on Figure 6. Expression levels of the POUV/POU5F3 (A), KLF4 (B), c-MYC (C), and NANOG (D) genes as detected by RNA-seq analysis following the total read alignment on the defined Galgal5 genome version.The expression levels of the POUV and KLF4 genes reflected a mixture of exogenous and endogenous expression, whereas the c-MYC and NANOG genes were strictly dependent on exogenous genes in the reprogrammed clones, as indicated by the lack of the 3'-UTR in the detected messenger (red arrow in C and D).

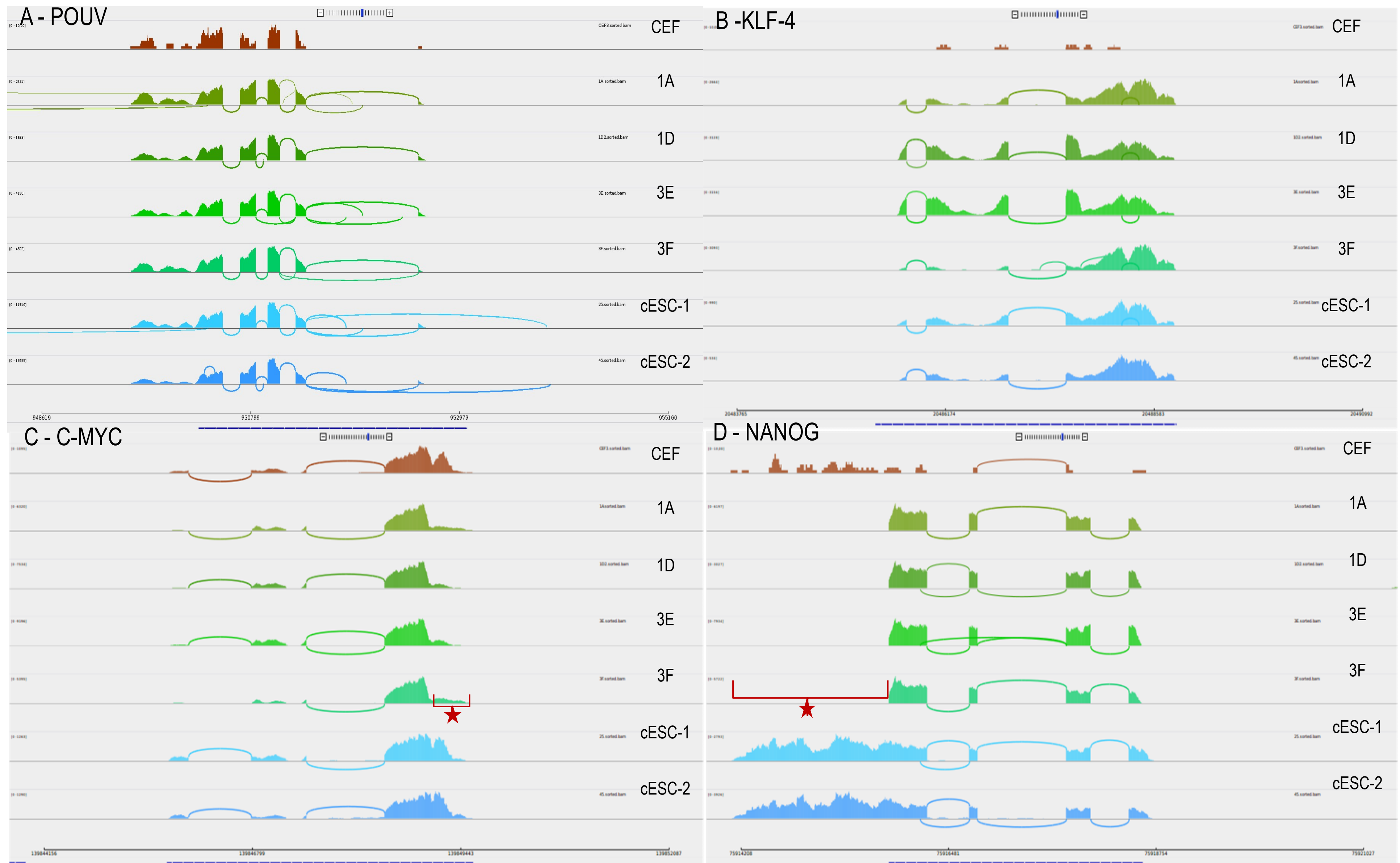


Figure S5, related to Figure 7: Developmental properties of the reprogrammed clones.

The 1D reprogrammed clone was GFP-labeled and injected into stage X-XII recipient embryos as previously described (Aubel and Pain, 2013). The green fluorescent structures observed in embryos (A) are composed of GFP-labelled cells, and not of autofluorescent cells as shown by the absence of red fluorescent signal (B) when using confocal microscope detection settings for red-labelled cells. Scale bar: 20µm.

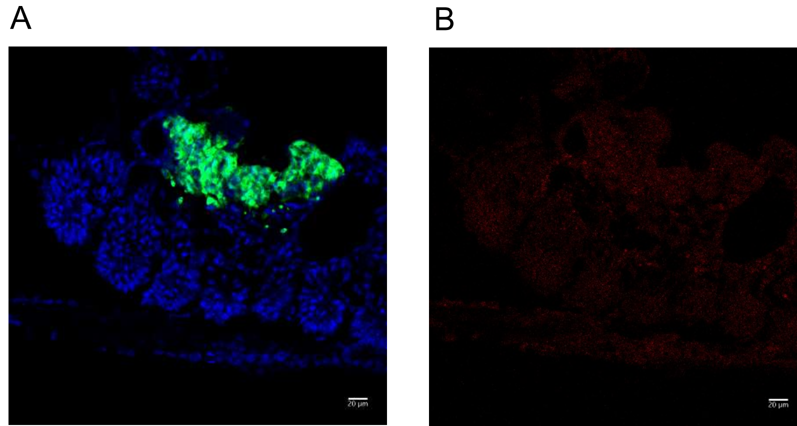


Figure S6, related to Figure 7: Developmental properties of the reprogrammed clones. The 1D and 3E reprogrammed clones were GFP-labeled and injected into stage X-XII recipient embryos as previously described (Aubel and Pain, 2013) to evaluate their developmental potential. Some injected embryos show a contribution of the injected cells as mass-like structures located in various part of the body and annexes. GFP and Hoechst detection (left panel) are done by confocal microscopy on embryo cryosection, scale bar: 100 µm.

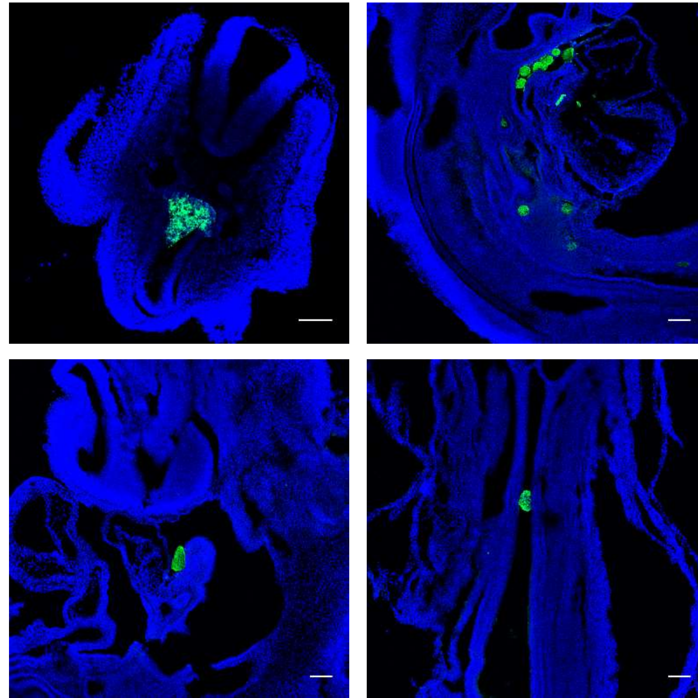


Figure S7: Duck reprogrammed cells exhibit stem cells features

(A) Reprogrammed duck cells (68-4) growing in small aggregates exhibit a typical stem cell morphology in sharp contrast with the DEFs and grow for long term culture (B). The reprogrammed representative 68-4 clone was positive for SSEA-1 and EMA-1 as detected by both FACS analysis (C) and immunofluorescence (D). Scale bar: 5 µm. (E) Endogenous telomerase activity was measured in 68-4 and cESC as positive control and in DEFs as negative control cells used as the substrates for reprogramming. (F) Analysis of cell cycle phases reveals a stem cell profile with a short G2/M phase and a long S phase for the duck reprogrammed clones (68-4) and for the established cESCs as control for avian embryonic stem cells, and DEFs as somatic negative control cells. (G) Reprogrammed cells (68-4) were probed for the presence of large nuclear foci of trimethylated histone H3 on lysine 27, which is typical for cESCs and not observed in DEFs. These foci colocalize with heterochromatin foci containing trimethylated histone H3 on lysine 9. Scale bar: 5 µm. (G) The karyotype analysis of the reprogrammed clones (68-4) reveals a normal duck karyotype with macrochromosomes and minichromosomes.

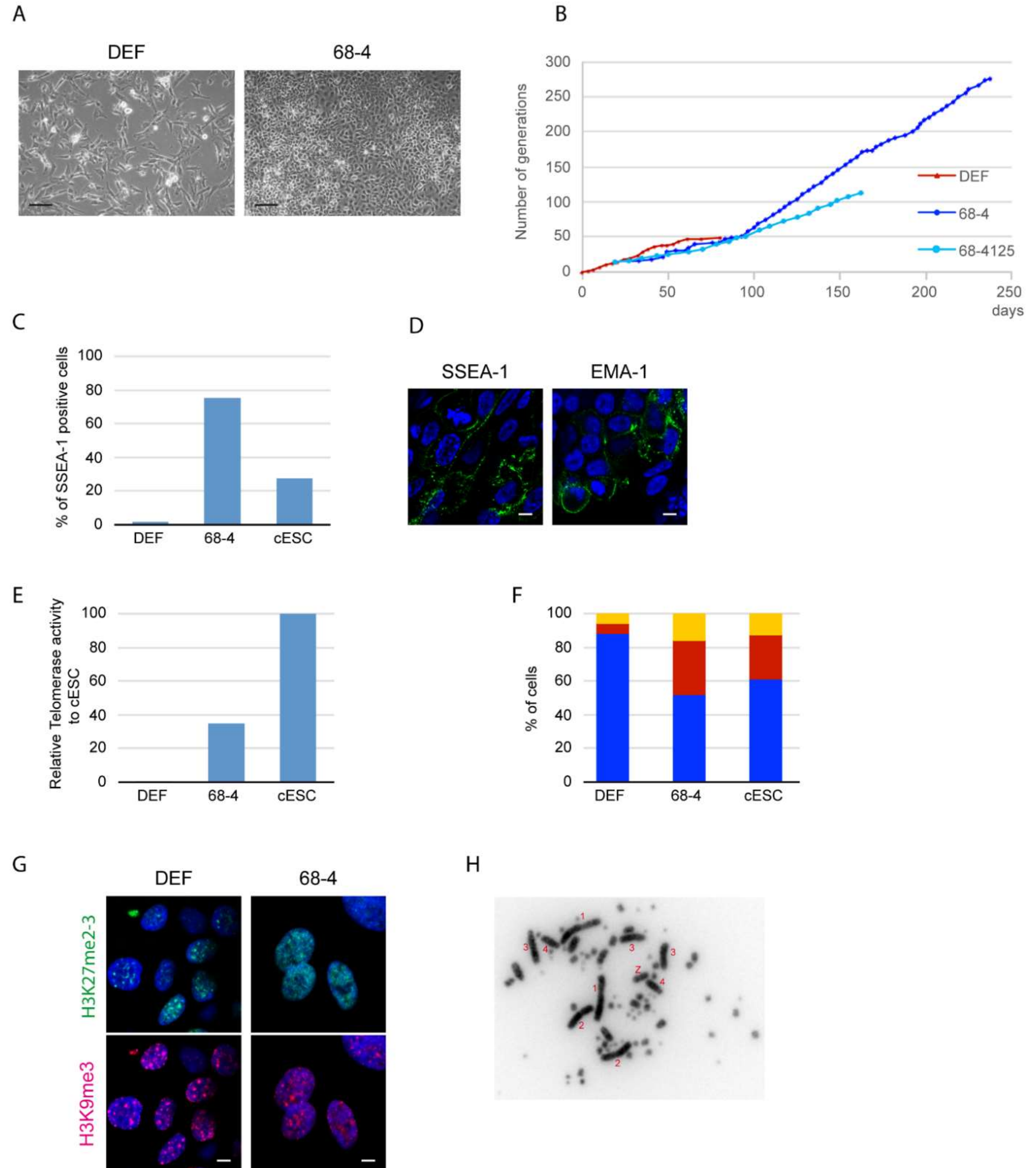


Table S4: CAM assay results

For each clone, 3×10^6 cells were placed on the surface of the chorioallantoic membrane (CAM) of 6-day-old chicken embryos. The eggs were then incubated for 10 days, and the embryonic membranes were removed and washed for further observation. The presence or absence of morphological alterations was assessed by comparison with non-injected embryos. The established cESCs did not generate a cellular mass. Some reprogrammed clones gave rise to cellular masses of various sizes in different proportions.

Cells	Number of analysed embryos	Number of tumor-like structure (> 5 mm)	Percentage
cESC	24	0	0
1A	13	7	53.8
1D	10	9	90.0
3E	17	8	47.0
3F	16	7	13.8
68-4	19	6	31.6

Table S5 : Oligunucleotide sequences

Genes	Forward	Reverse
Pluripotent genes		
POU5F3	TGCAATGCAGAGCAAGTGCTGG	ACTGGGCTTCACACATTTGCGG
POU5F3_endo	AGCACAGGAGAGGGGTTG	CAACTACAGCAGGCTCAAAGG
SOX3	GTCGGGGTGGGCCAGAGGAT	GCTGTTCATGCCCGGGTGCT
SOX3_endo	ACTGTGAACGATGTTTTGACATATCAG	GGAAGAAATCCGGTAAACAAAACAA
KLF-4	ATGCACAGGATGCTGCAACACG	TGGTGTGCGCCAGGATGAAGTC
KLF-4_endo	ACTCTGGGTTCCGCTCTTC	CCCACTCTTACCCCGTACTC
cMYC	GGAGCGCCAGCGAAGGAATG	TGGGCGCCTTCTCGTTGTTG
cMYC_endo	GATACTTTGGGCATAAGGGATGATG	CTGGGGACAACTCTATTTGGAATTC
NANOG	TGCACACCAGGCTTACAGCAGTG	TGCTGGGTGTTGCAGCTTGTTC
NANOG_endo	CTCCGCTGCCTCTTTTGC	CTGGGCTACAAATAGGGTATTTCTTG
Differentiation genes		
T (Brachyury)	AGGTCAAGCTCACCAACAAG	AGGAATGGCTGGTGATCATC
EOMES	CACTGGCGCTTCCAAGGGGG	GCTCCGGTGTTGGGCGACTC
CDX2	TGTTAGGTGTAAGGGGAGCGTGG	AACAGCATCGCTCAGACCTTCGC
GATA4	TTCGACAGCCCCATGCTGCAC	AAATTCGATGTTGGCATGCCGGG
GATA6	AAAAACGCCTCCCCAAGCACG	AAGGTTGCTGCTTTCGGGACTGG
GSC (Goosecoid)	AGACGGGAAAAGCGATTTGG	GGCAGAGCTTGTGCAAGATAG
OTX2	ACGAGAACAAAACACGCTGGGC	TTTTGTCGCCGTTGTTGTCGTCG
PAX-6	AACTCCATCAGCTCCAATGG	AGGGCTTCGATTTGCTCTTG
Housekeeping gene		
RS17	ACACCCGTCTGGGCAACGAC	CCCGCTGGATGCGCTTCATC