Supplementary Materials

7 Figures

1 Table

Supplementary Figure Legends

Supplementary Figure 1 Nanog Peptides can block the Nanog staining in CRC Clinical Samples

(a-b) 5 μ formalin-fixed paraffin embedded specimens of liver metastases sections were analyzed for the expression of Nanog protein (green) and Nanog antibody was blocked with synthetic Nanog peptides. Antibodies were a final dilution of 1:100. Patient 2 is shown. When the antibody to Nanog was blocked with synthetic Nanog peptides, the fluorescence due to Nanog was removed (b). Images were captured on a Nikon 90i microscope with a DU888 EMCCD camera and analyzed with NIS-Elements software. Object magnification, 20X. White bar 10 microns.

(c-e) Polyclonal goat anti-Nanog (R&D Cat No. AF1997(c), Mouse Monoclonal Anti-Nanog (Cell Signalling Cat No. , 4893 (d) and rabbit Monoclonal Antibody (Cell Signalling Cat No. 4903 (e) were incubated with 3 synthetic peptides (2 22mers 5' to the the homeobox domain of Nanog and 1 21mer 3' to that domain) in 0.15 M PBS overnight at 4°C. These blocked antibodies were then assessed and compared to unblocked antibody for reactivity on western blots prepared as described in Materials and Methods. Final dilutions of unblocked (UB) and blocked (B) antibodies were 1:500 (c-e). Lysates of both Clone A and CX-1 were probed in Panel A and CX-1 alone in Panels B and C. Arrowheads indicate expected mass of NANOG in each blot.

Supplementary Figure 2. Expression of stem cell-related membrane antigen genes are not Upregulated in Spheroids in CRC

(a-b) Total RNA was extracted and qRT-PCR was performed for CD44, CD133 and CD166. The results were normalized to GAPDH and HCC 2998 monolayers. Mean % ± SD.

Supplementary Figure 3 Sequence Alignment of Human Nanog and Deduced NanogP8 Protein and Allele Specific shNP8-1.

(a) The deduced NanogP8 protein was aligned with Nanog with the 2 – not just the 1 – different amino acids highlighted by the: and red underline.

(b) Allele-specific silencing of NanogP8, shNP8-1 was designed with the SNP aligned with position 10 of the guide strand according to Huang *et al* (Huang *et al*., 2009) and Dyxhoorn *et al* (Dyxhoorn *et al*., 2006).

Supplementary Figure 4 shRNA to Nanog reduces expression of Oct4 and SOX2

(a-b) shRNA to Nanog decreases Oct4 and SOX2 transcripts in CRC;

(c-d) Secondary transduction of either Nanog or NanogP8 into CloneA and CX-1 transduced previously with shNanog increases Nanog or NanogP8 expression, respectively. P values by contingency table analysis with Bonferroni correction. * P<0.01 shNanog in Clone A and CX-1 vs shNanog+Nanog, shNanog+NanogP8 in Clone A and CX-1, respectively. Mean % ± SD.

Supplementary Figure 5 Nanog is Expressed in PA-1 cells and TFs expression in SP of CX-1

(a) Restriction endonuclease digestion of 260 nt length of Nanog by RT-PCR amplified from PA-1 cells which demonstrates that only Nanog is present in PA-1 cells. Nanog and NanogP8 are two controls. (b) Expression of *NANOG*, *OCT4* and *SOX2* was analyzed in CX-1, CX-1 SP and non-SP cells by qRT-PCR. P values by contingency table analysis with Bonferroni correction. * P<0.01 vs Parental CX-1.

Supplementary Figure 6 Side Population Analyses of KM-12c Transduced with shNeg and shNp8-1.

KM-12c cells were transduced with LVs containing shNeg, shNP8-1 (shNanogP8-1) or not transduced and then analyzed for the side population as described in the text.

Supplementary Figure 7 Side Population Analyses of KM-12c Transduced with RFP, NanogP8 or Nanog. Km-12c cells were transduced with Red Fluorescent Protein (RFP), *NANOG* or *NANOGP8* and then analyzed for the side population as described in the text.
KM-12c cells were transduced with RFP alone, NanogP8 or Nanog and then subjected to analysis of the side population. The results of Supplementary Figures 6-7 are graphically displayed in Figure 5.

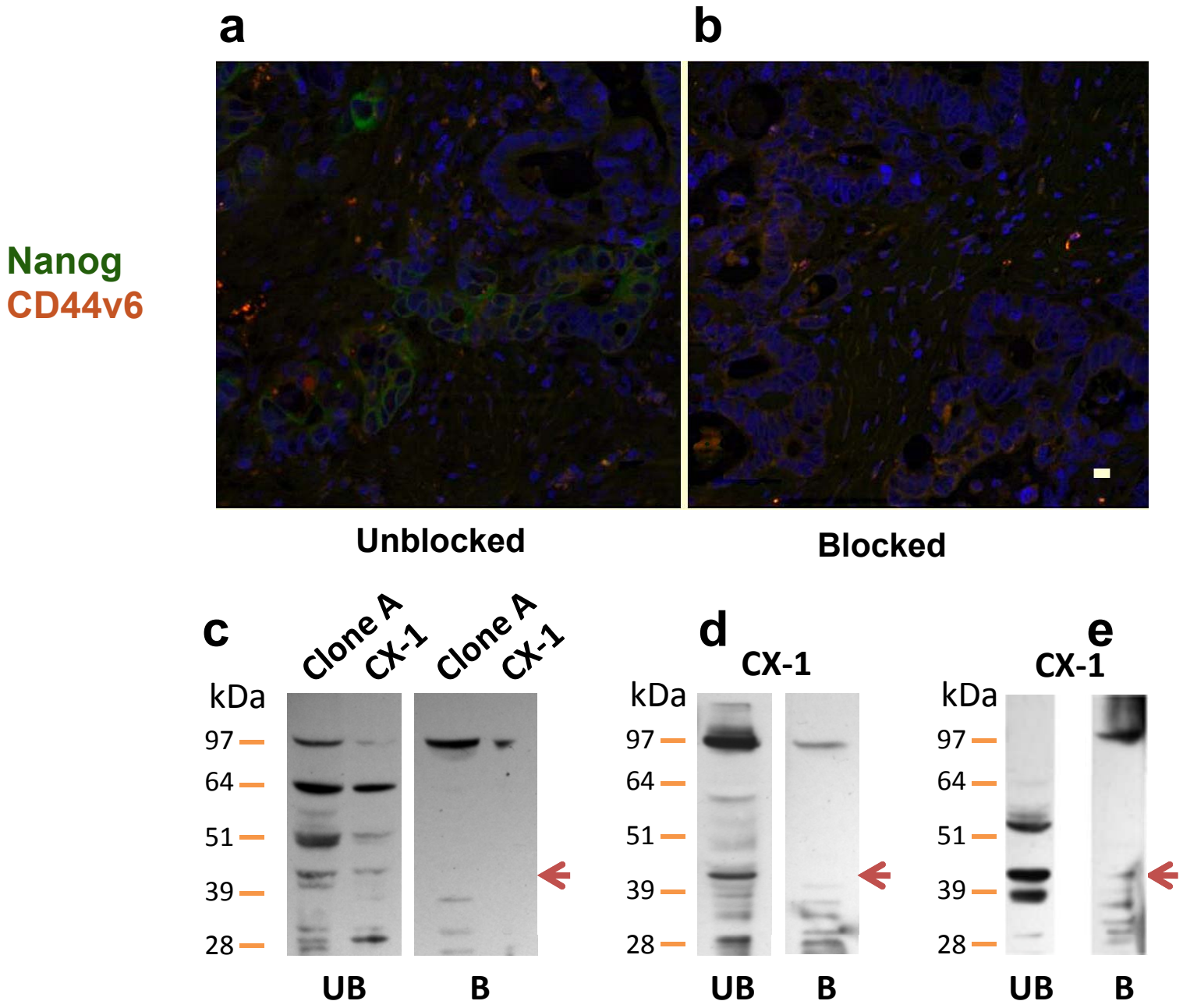
Supplementary Table

Table 1. Nanog and NanogP8 Transcripts in Deidentified Patients Whose CRC Liver Metastases Were Resected at the NIH Clinical Center

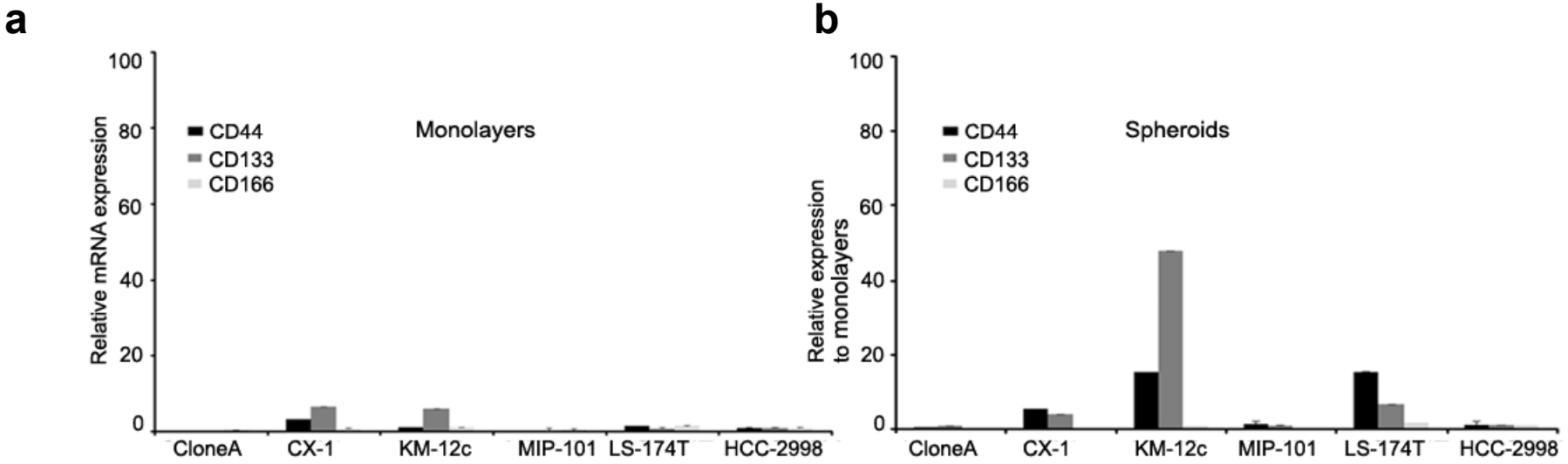
		Gene Transcript				
Patient		Normal		Tumor		
Number	Gender	Nanog	NanogP8	Nanog	NanogP8	IFA
1	F	-	-	+	-	+
2	M	-	-	-	-	+
3	M	+	-	+	+	+
4	F	-	-	+*	-	-
5	M	+	+	+	+	+
6	F	+	-	+	+	+
7	M	+	-	+	+	+
8	F	-	-	+	+	+
9	F	-	-	+	+	+
10	F	-	-	-	-	-
	6F/4M	4/10	1/10	8/10	6/10	8/10

Legend: all patients are de-identified with the only characteristic known by authors is gender (F- Female, M-Male). No other pathologic or demographic characteristics are known except that patients all have stage IV CRC with liver metastases that were resected at the NIH Clinical Center. Nanog or NanogP8 transcripts were identified as described by RT-PCR and the ALwNI endonuclease assay. Eight of 10 metastases express NanogP8, Nanog or both. IFA was performed as described in Materials and methods.

*Tumor from Patient 4 has weak expression on the total Nanog gel of a Nanog transcript (Figure 1) but was not assessable by AlwNI endonuclease digestion or Sanger sequencing so that a Nanog family member is present but it is not clear whether it is Nanog or NanogP8.

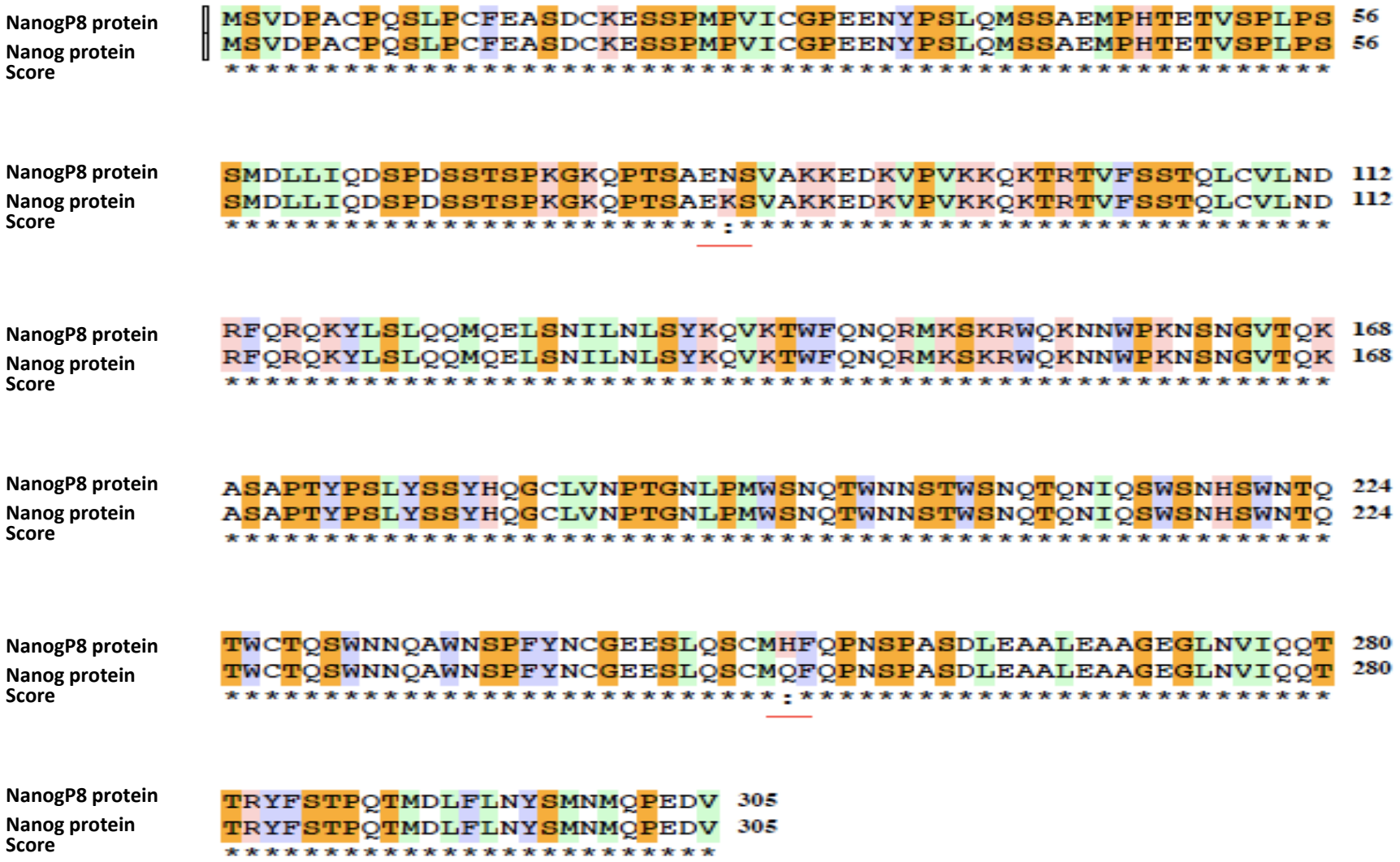


Supplementary Figure 1



Supplementary Figure 2

a



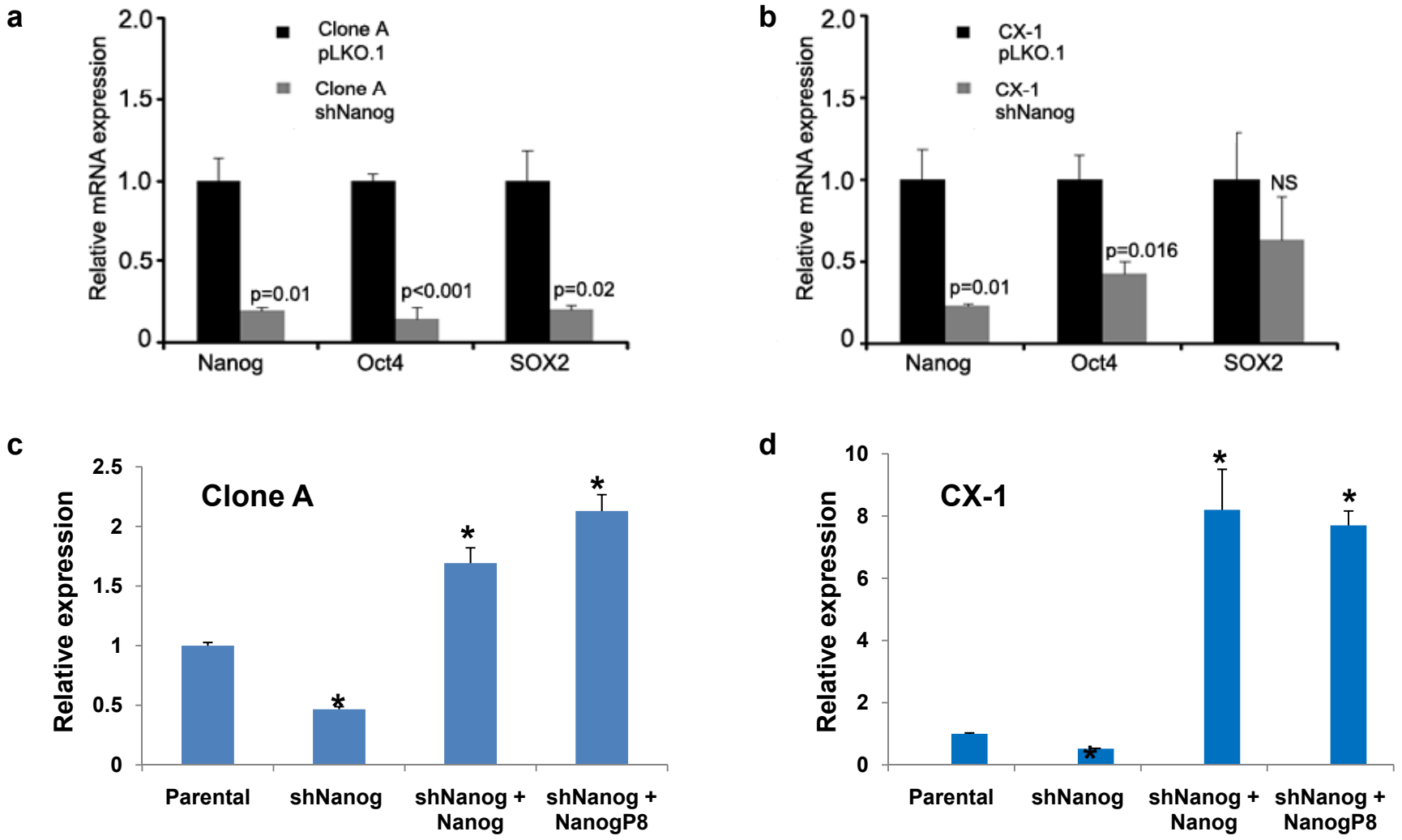
b

NANOGP8 5’—-AATCTCTGCAGTCCTGCATGCA**C**TTCCAGCCAAATTCTCCTGCC—3’

siRNA shNP8-1a 5’-CUGCAUGCA**C**UUCCAGCCA-3’

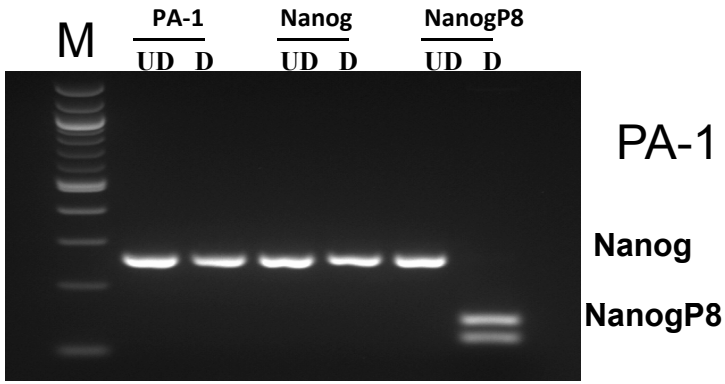
shNP8-1b 5’-UGGCUGGAAGUGCAUGCAG-3’

Supplementary Figure 3



Supplementary Figure 4

a



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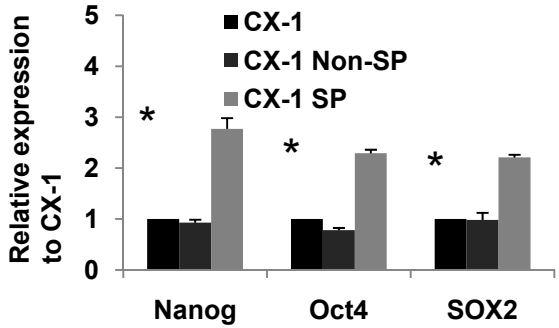
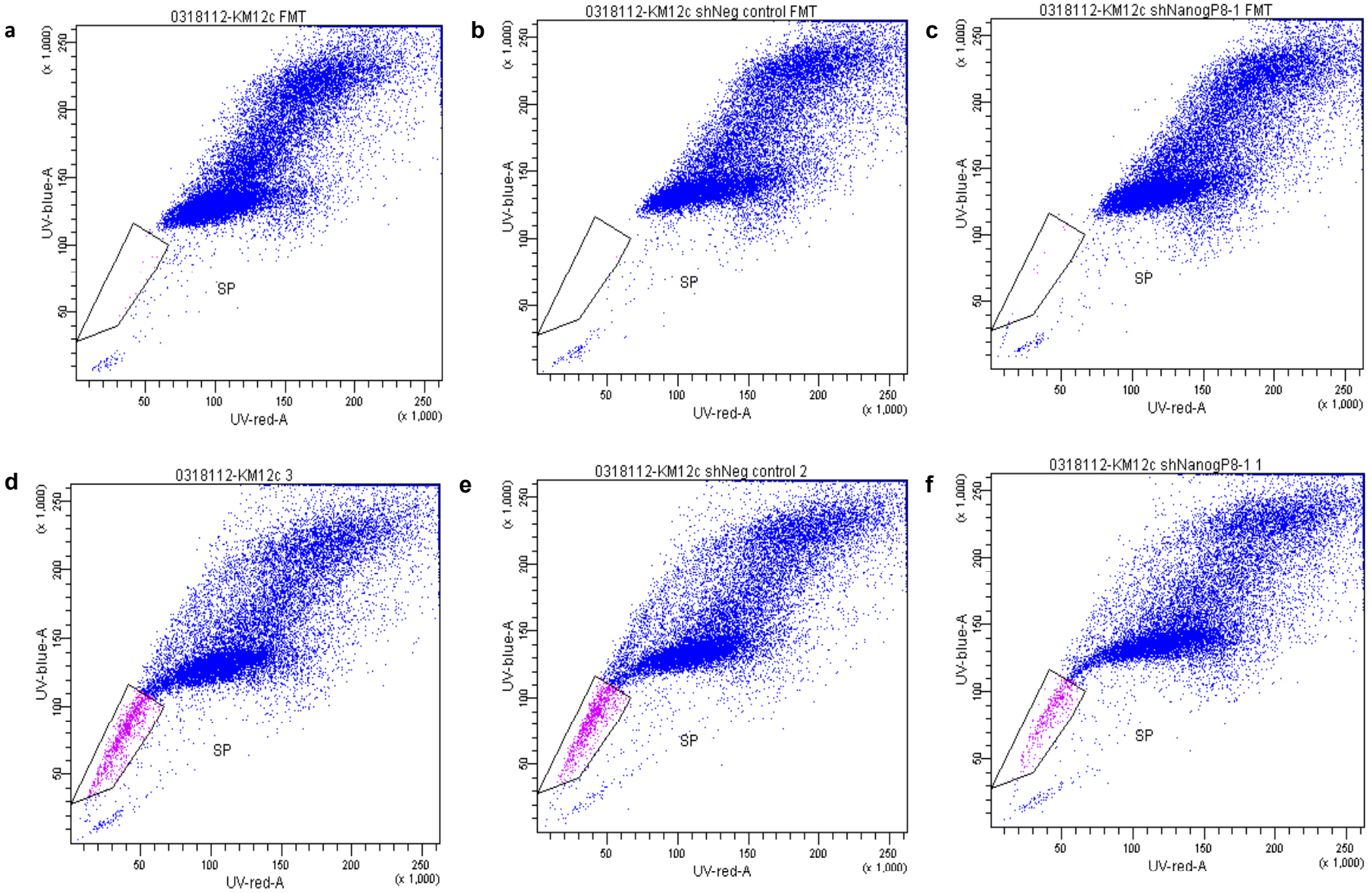
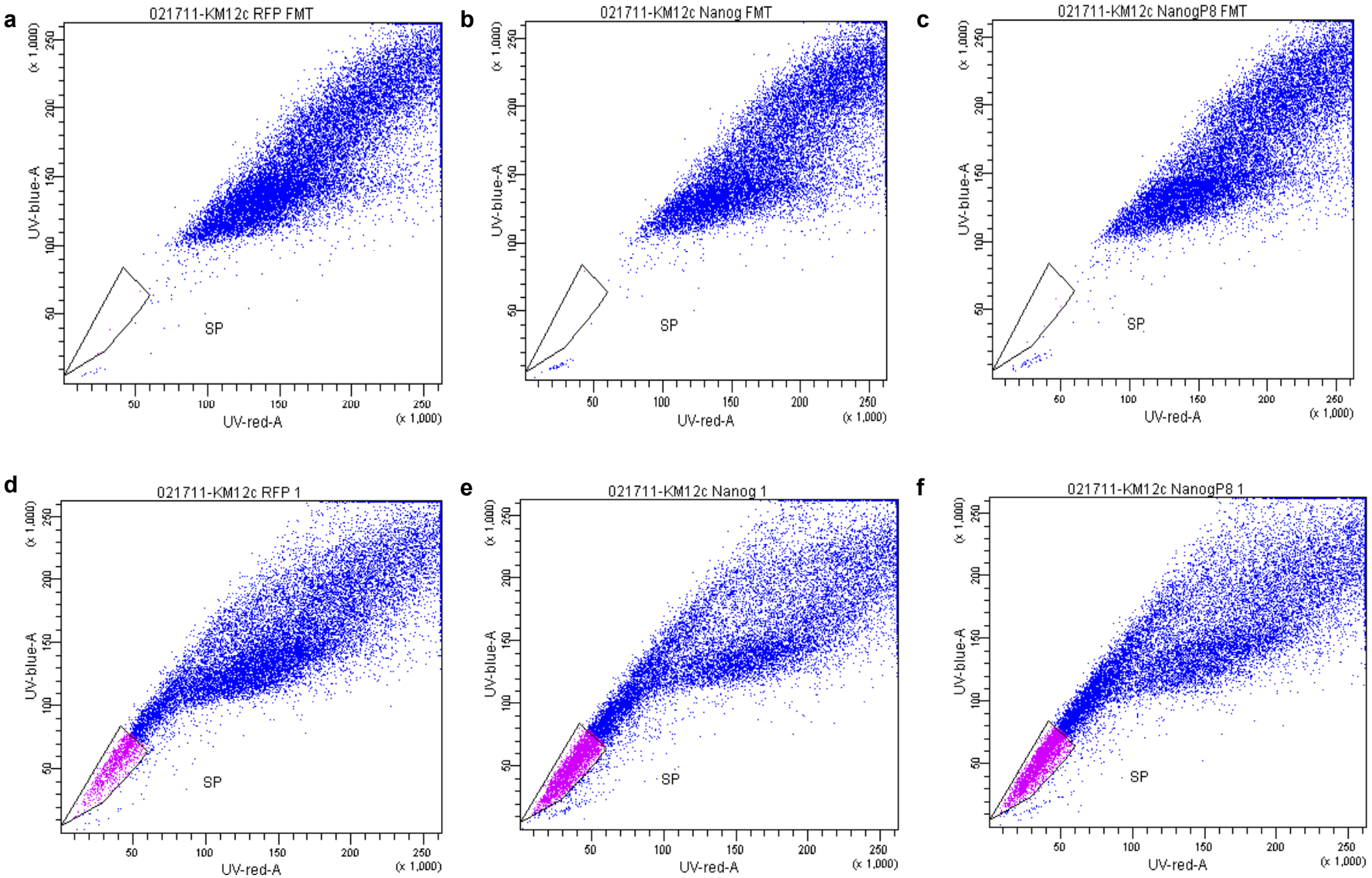


Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7