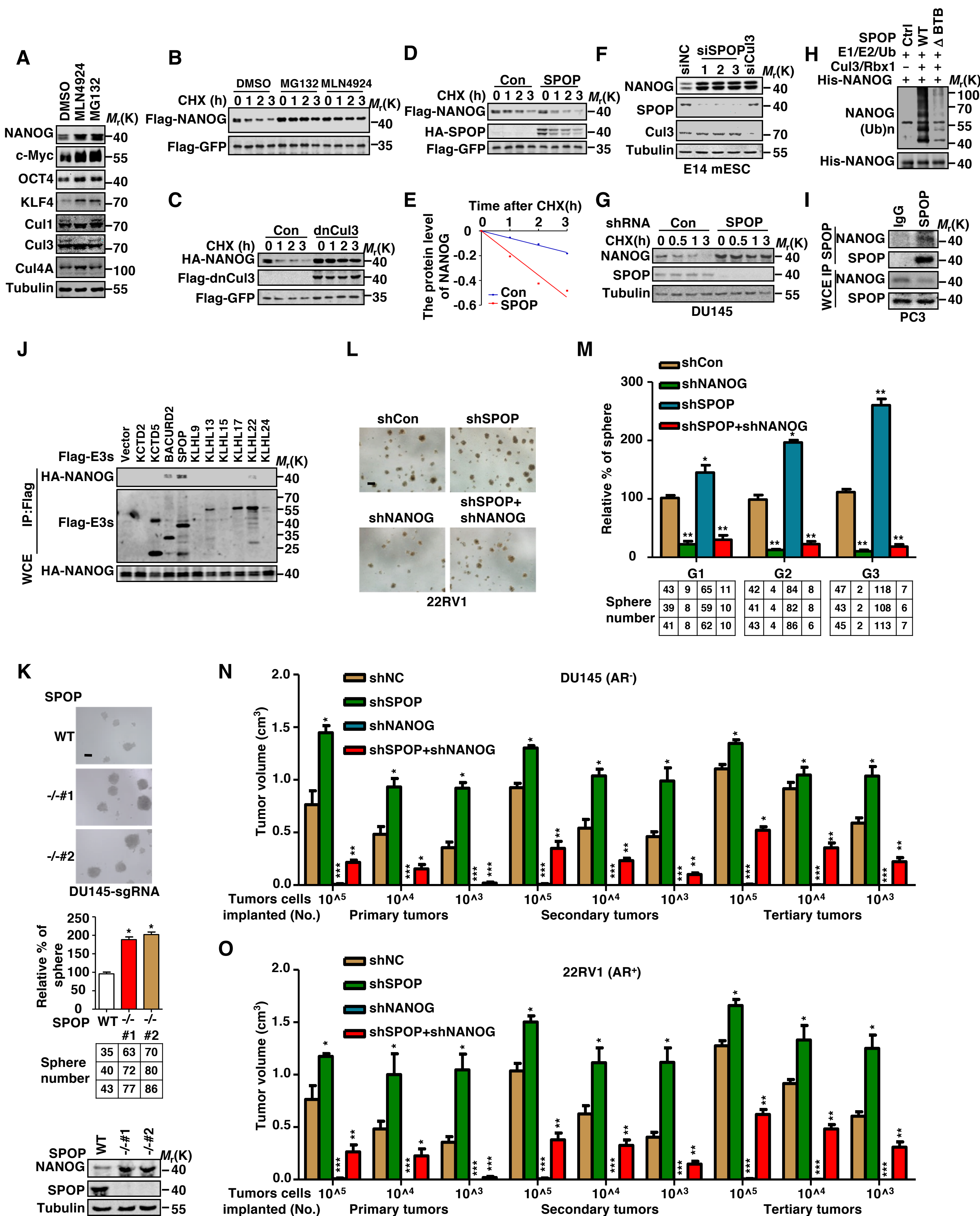
Figure S1



G1				G2				G3			
43	9	65	11	42	4	84	8	47	2	118	7
39	8	59	10	41	4	82	8	43	2	108	6
41	8	62	10	43	4	86	6	45	2	113	7

WT	-/- #1	-/- #2
35	63	70
40	72	80
43	77	86

Figure S1. Cullin3/SPOP E3 Ubiquitin Ligase Targets NANOG for Ubiquitination and Degradation (Related to Figure 1).

(A) E14 cells were treated with MLN4924 (10 μM) or MG132 (10 μM) for 6 hrs before harvesting. Expression levels of indicated proteins were analyzed by western blotting.

(B) Flag-NANOG was expressed in HEK293T cells. HEK293T cells treated with MLN4924 (10 μM) or MG132 (10 μM) for 4 hrs before performing the cycloheximide (CHX,10 μg/ml) chase analysis. Protein levels of NANOG were analyzed by western blotting.

(C) HA-NANOG was coexpressed in HEK293T cells with vector or Flag-dnCul3. After treating cells with cycloheximide (CHX,10 μg/ml) for indicated time intervals, protein levels of NANOG and dnCul3 were analyzed by western blotting.

(D) Flag-NANOG was coexpressed in HEK293T cells with vector or HA-SPOP. After treating cells with cycloheximide (CHX,10 μg/ml) for indicated time intervals, protein levels of NANOG and SPOP were analyzed by western blotting.

(E) The NANOG protein abundance in (D) was quantified by ImageJ and plotted as indicated.

(F) E14 mESCs were transfected with control siRNA or SPOP siRNA. The endogenous NANOG were analyzed by western blotting.

(G) DU145 cells were transfected with control shRNA or SPOP shRNA and then treated with CHX (10 μg/ml) for indicated times. The endogenous NANOG and SPOP were analyzed by western blotting.

(H) His-NANOG protein was purified from *E*. coli and incubated with the reconstituted E3 Ub ligase complex (Cul3-RBX1, SPOP or SPOP ∆BTB) and E1, E2, Ub. The products of *in vitro* ubiquitination assay were analyzed by western blotting.

(I) Endogenous SPOP was immunoprecipitated with an antibody against SPOP from PC3 cells, and the associated NANOG was detected by an anti-NANOG antibody.

(J) Flag-E3s and HA-NANOG were coexpressed in HEK293T cells. Flag-E3s were immunoprecipitated with anti-Flag antibody, and the associated NANOG and SPOP were analyzed by western blotting using HA antibody.

(K) DU145 SPOP WT or KO cell lines were constructed by lenti-CRISPR system. Representative sphere images from each condition of DU145 cells. Scale bar, 200 μm. Frequency of tumor spheres formed from DU145 cells Sphere counts are normalized to mock infected spheres. Data are means ± SEM (n=3). *P< 0.05 vs sgNC (Student's *t*-test). The protein level of SPOP and NANOG of DU145 SPOP WT or KO cell lines were analyzed by western blotting.

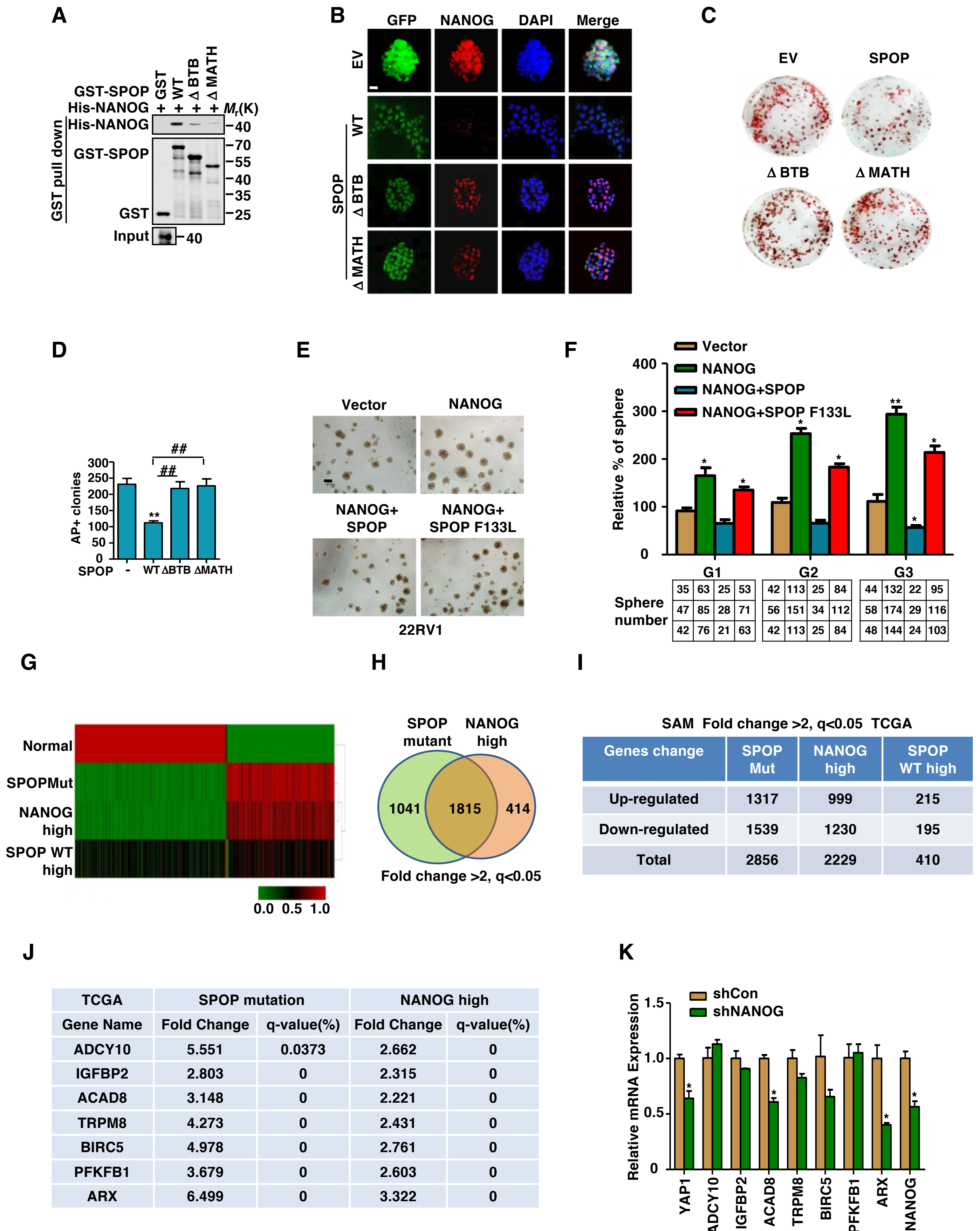
(L) Representative sphere images from each condition of 22RV1 cells. Scale bar, 100 μm.

(M) Indicated retrovirus infected 22RV1 tumor spheres were dissociated and equal numbers of cells were passaged for three generations. Spheres counts are normalized to the first generation scrambled shRNA spheres. Data are means ± SEM (n=3). **P<0.01 vs shCon (Student‘s *t*-test).

(N) Growth of tumors in nude mice derived from DU145 cells infected with retrovirus expressing indicated shRNAs of three different cell densities. Data are means ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs shNC (Student‘s *t*-test).

(O) Growth of tumors in nude mice derived from 22RV1 cells infected with retrovirus expressing indicated shRNAs of three different cell densities. Data are means ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs shNC (Student‘s *t*-test).

Figure S2



	G1				G2				G3			
Sphere number	35	63	25	53	42	113	25	84	44	132	22	95
	47	85	28	71	56	151	34	112	58	174	29	116
	42	76	21	63	42	113	25	84	48	144	24	103

Genes change	SPOP Mut	NANOG high	SPOP WT high
Up-regulated	1317	999	215
Down-regulated	1539	1230	195
Total	2856	2229	410

TCGA	SPOP mutation		NANOG high	
Gene Name	Fold Change	q-value(%)	Fold Change	q-value(%)
ADCY10	5.551	0.0373	2.662	0
IGFBP2	2.803	0	2.315	0
ACAD8	3.148	0	2.221	0
TRPM8	4.273	0	2.431	0
BIRC5	4.978	0	2.761	0
PFKFB1	3.679	0	2.603	0
ARX	6.499	0	3.322	0

Figure S2. Prostate Cancer-Associated SPOP Mutants are Defective in Promoting NANOG Ubiquitination and Degradation (Related to Figure 2).

(A) Bacterially expressed His-NANOG proteins were subjected to GST pull-down assay *in vitro* by GST-SPOP recombinant proteins and mutants purified from bacteria. The products of pull-down assay were analyzed by western blotting using an anti-His or anti-GST antibody.

(B) Immunofluorescent staining analysis for lentivirus expressed SPOP or mutants (green) and endogenous NANOG (red) in E14 mESCs. Scale bar, 100 μm.

(C) AP staining after 48 hrs SPOP or SPOP mutants overexpression in E14 mESCs.

(D) Percentage of undifferentiated colonies counted in (C). Data are means ± SEM (n=3). $^{**}P< 0.01$ vs EV (Student's *t*-test); $^{\#\#}P < 0.01$ vs WT (two-way ANOVA test).

(E) Representative sphere images from each condition of 22RV1 cells. Scale bar, 100 μm.

(F) Indicated lentivirus infected 22RV1 tumor spheres were dissociated and equal numbers of cells were passaged for three generations. Spheres counts are normalized to the first generation scrambled shRNA spheres. Data are means ± SEM (n=3). $^{*}P<0.05$, $^{**}P<0.01$ vs Vector (Student's *t*-test).

(G) Hierarchical clustering of gene expression profiles shows SPOP mutation and NANOG high tumors share a common gene signature, which are distinct from the SPOP WT high tumors.

(H) The Venn diagram shows the overlap of genes significantly differentially expressed in SPOP mutation and NANOG high samples (significance analysis of microarrays for tumor and normal samples, fold change >2, q<0.05).

(I) Table shows gene numbers differentially expressed using the significance analysis of microarrays in tumor and normal samples, fold change >2, q<0.05.

(J) List of genes that are potentially co-regulated by NANOG and SPOP.

(K) Relative gene expression of DU145 cells infected with retrovirus expressing shCon or shNANOG. *Yap1* was used as positive control. $^{*}P<0.05$ vs shCon (Student's *t*-test).

Figure S3

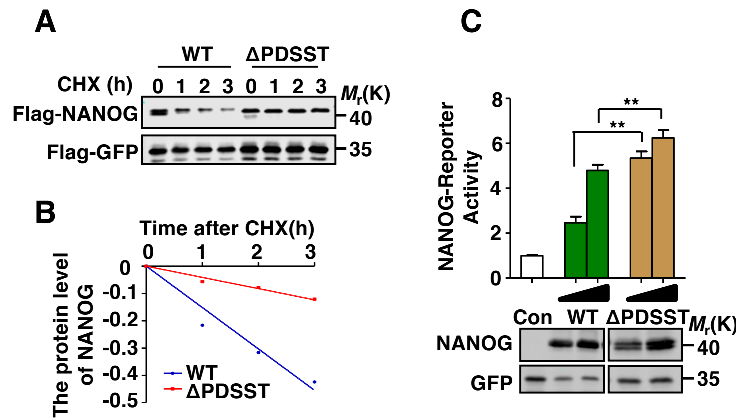


Figure S3. The [66]PDSST[70] Motif in NANOG Is the Degron Recognized by SPOP (Related to Figure 3).

(A) Flag-NANOG or PDSST deletion mutant was expressed in HEK293T cells. After treating cells with cycloheximide (CHX,10 μg/ml) for indicated time intervals, protein levels of NANOG were analyzed by western blotting.

(B) The NANOG protein abundance in (A) was quantified by ImageJ and plotted as indicated.

(C) NANOG or PDSST deletion mutation was transfected with NANOG-luciferase reporter into HEK293T cells for 24 hrs. The firefly luciferase activity was measured and normalized to the renilla luciferase activity in the same sample and then normalized to vector control. Data are means ± SEM (n=3). $^{**}P<0.01$ vs WT (Student's *t*-test).

Figure S4

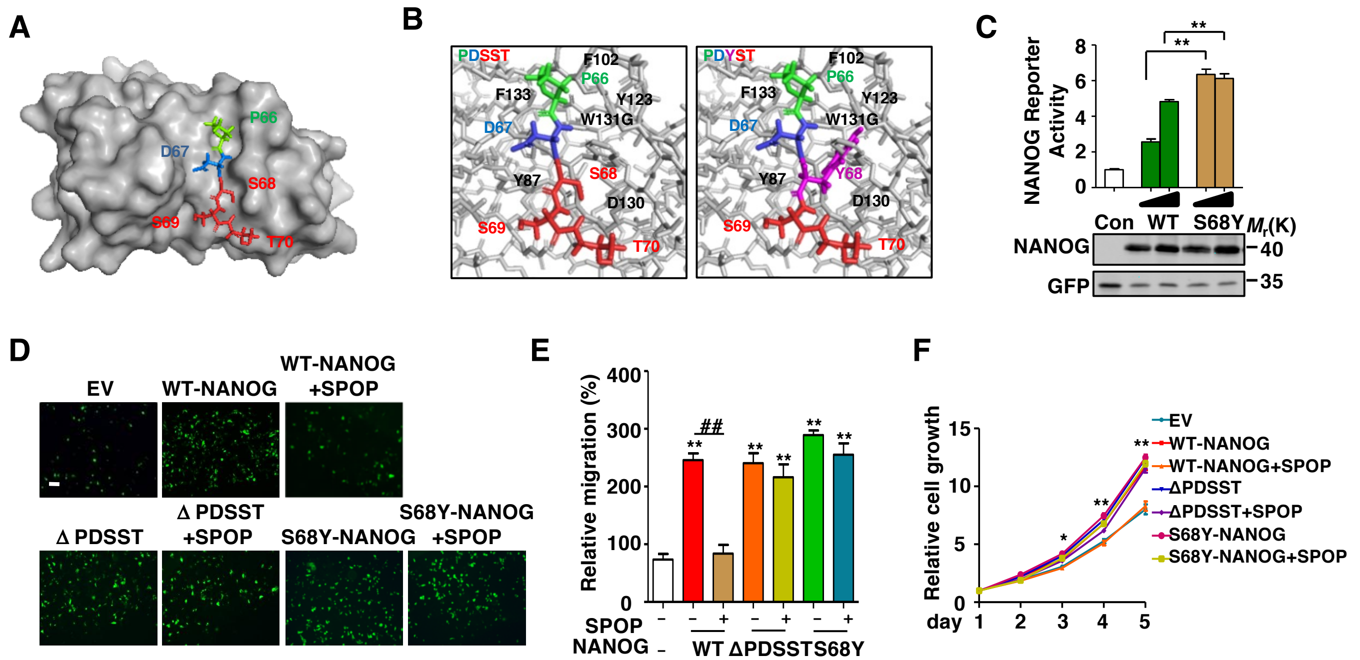


Figure S4. Oncogenic NANOG Mutations within SBC Are Resistant to SPOP-Mediated Degradation (Related to Figure 4).

(A) Computational modeling of structure of the SPOP MATH domain in complex with the NANOG SBC motif PDSST.

(B) Computational modeling of structures of the SPOP MATH domain in complex with the wild-type (WT) NANOG SBC motif PDSST and the mutant form PDYST.

(C) NANOG or mutations was transfected with NANOG-luciferase reporter into HEK293T cells for 24 hrs. The firefly luciferase activity was measured and normalized to the renilla luciferase activity in the same sample and then normalized to vector control. Data are means ± SEM (n=3). ***P*<0.01 vs WT (Student's *t*-test).

(D and E) DU145 cells infected with lentivirus carrying GFP together with SPOP or NANOG (WT or mutations) for 3 days. Representative images of migrated DU145 cells in migration assays (D). Scale bar, 100 μm. Data are means ± SEM (n=3).***P*<0.01 vs EV (Student's *t*-test); ##*P*<0.01 vs WT(-) (Student's *t*-test).

(F) MTT assay of DU145 cells infected with lentivirus expressing indicated SPOP or NANOG. Data are means ± SEM (n=3). **P*<0.05, ***P*<0.01 vs EV (Student's *t*-test).

Figure S5

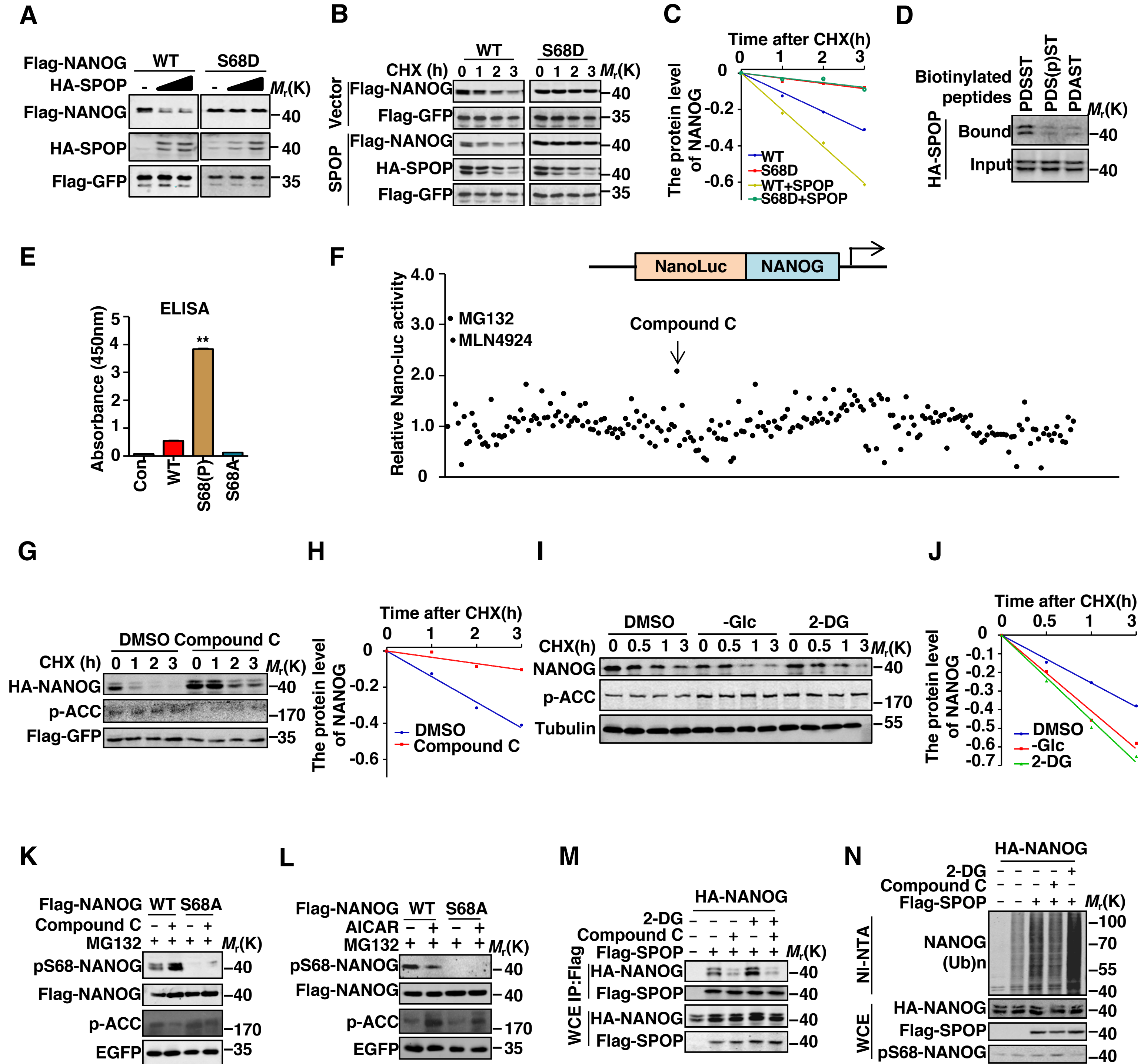


Figure S5. AMPK promotes SPOP-mediated destruction of NANOG through down-regulated Ser68 phosphorylation of NANOG (Related to Figure 5).

(A) HA-SPOP was coexpressed with Flag-NANOG or S68D in HEK293T cells. The protein levels of NANOG and SPOP were analyzed by western blotting.

(B) HA-SPOP was coexpressed with Flag-NANOG or S68D in HEK293T cells. After treating cells with cycloheximide (CHX,10 μg/ml) for indicated time intervals, protein levels of NANOG and SPOP were analyzed by western blotting.

(C) The NANOG protein abundance in (B) was quantified by ImageJ and plotted as indicated.

(D) Binding was examined using biotinylated peptide pull-down assay and analyzed using western blotting.

(E) The specificity of antibody against the pS68-NANOG in ELISA assay. ** P<0.01 vs WT (Student's *t*-test).

(F) HEK293T NanoLuc-NANOG stable cell line was treated with DMSO or the kinase inhibitors for 6 hrs, The NanoLuc luciferase activity was measured.

(G) HA-NANOG was expressed in HEK293T cells. The cells were treated with DMSO or Compoound C (6.6 μM) for 4 hrs before performing the cycloheximide (CHX,10 μg/ml) chase analysis. Protein levels of NANOG were analyzed by western blotting.

(H) The NANOG protein abundance in (G) was quantified by ImageJ and plotted as indicated.

(I) HA-NANOG was expressed in HEK293T cells. The cells were glucose starved or treated with 2-DG (25 mM) for 4 hrs before performing the cycloheximide (CHX,10 μg/ml) chase analysis. Protein levels of NANOG were analyzed by western blotting.

(J) The NANOG protein abundance in (I) was quantified by ImageJ and plotted as indicated.

(K) Flag-NANOG or S68A was transfected in HEK293T cells. The cells were treated with Compound C (6.6 μM) for 4 hrs. The protein levels of pS68-NANOG and NANOG were analyzed by western blotting.

(L) Flag-NANOG or S68A was transfected in HEK293T cells. The cells were treated with AICAR (2 mM) for 4 hrs. The protein levels of pS68-NANOG and NANOG were analyzed by western blotting.

(M) Flag-SPOP was coexpressed with HA-NANOG in HEK293T cells before treated with Compound C (6.6 μM) or 2-DG (25 mM) for 4 hrs, cell lysates were prepared for Co-IP and western blotting. Cells were treated with MG132 (10 μM) for 6 hrs before harvesting.

(N) Flag-SPOP was coexpressed with HA-NANOG in HEK293T cells before treated with Compound C (6.6 μM) or 2-DG (25 mM) for 4 hrs, cell lysates were prepared for ubiquitination assay using Ni-NTA beads and western blotting. Cells were treated with MG132 (10 μM) for 6 hrs before harvesting.

Figure S6

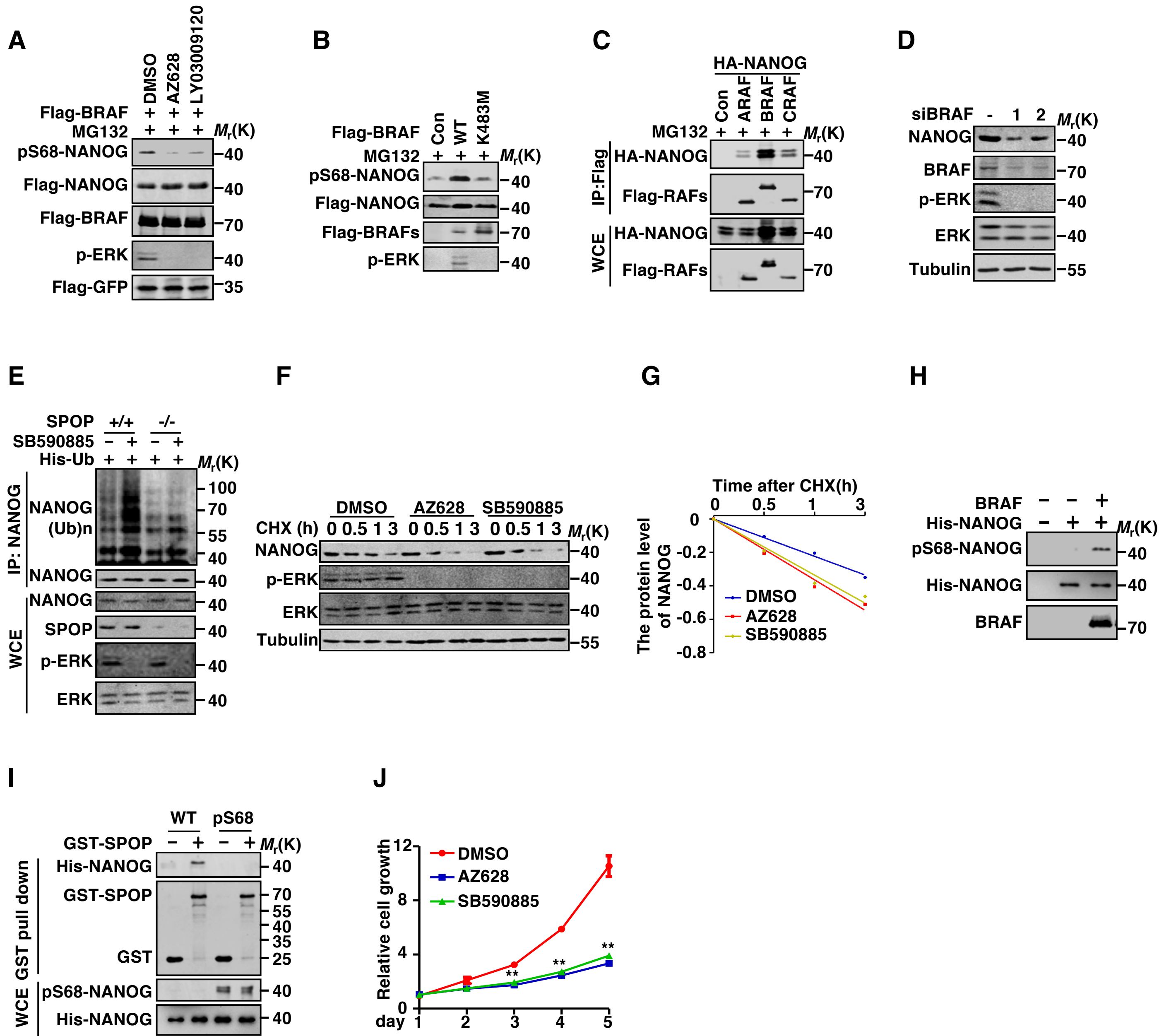


Figure S6. Phosphorylation of NANOG at Ser68 within SBC Motif by BRAF blocks SPOP-mediated destruction of NANOG (Related to Figure 6).

(A) Flag-NANOG was coexpressed with Flag-BRAF in HEK293T cells. The cells were treated with AZ628 (10 μM) or LY03009120 (2 μM) for 4 hrs. The protein level of pS68-NANOG was analyzed by western blotting. Cells were treated with MG132 (10 μM) for 4 hrs before harvesting.

(B) Flag-NANOG was coexpressed with BRAF or mutants in HEK293T cells. The protein level of pS68-NANOG was analyzed by western blotting. Cells were treated with MG132 (10 μM) for 4 hrs before harvesting.

(C) Flag-RAFs and HA-NANOG were coexpressed in HEK293T cells. Cell lysate was immunoprecipitated with anti-Flag antibody, and the associated NANOG was analyzed by western blotting. Cells were treated with MG132 (10 μM) for 6 hrs before harvesting.

(D) DU145 cells were transfected with control siRNA or BRAF siRNA. Expression level of NANOG was analyzed by western blotting.

(E) SPOP WT or KO DU145 cells were infected with lentivirus expressed His-Ub. 24 hrs later, the cells were treated with SB590885 (10 μM) for 4 hrs and the cell lysates were IP by NANOG antibody and the ubiquitinated NANOG was analyzed by western blotting. Cells were treated with MG132 (10 μM) for 6 hrs before harvesting.

(F) DU145 cells were treated with AZ628 (10 μM) or SB590885 (10 μM) for 4 hrs before performing the cycloheximide (CHX,10 μg/ml) chase analysis. Expression level of NANOG was analyzed by western blotting.

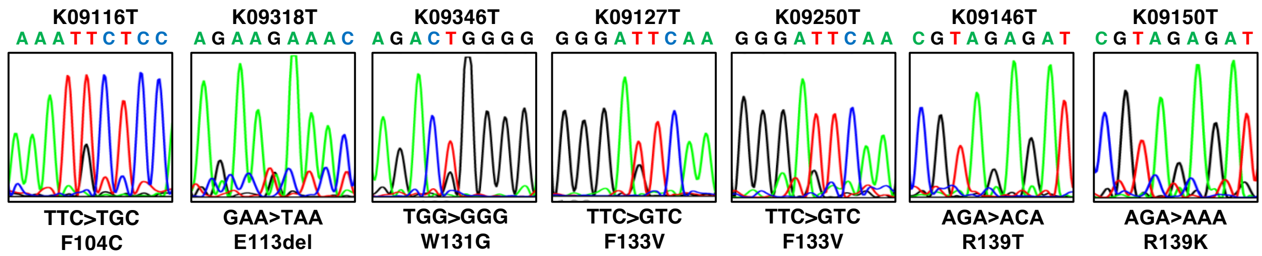
(G) The NANOG protein abundance in (F) was quantified by ImageJ and plotted as indicated.

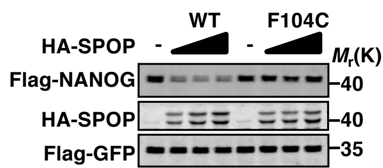
(H) *In vitro* phosphorylation of bacterial purified His-NANOG by Flag-tagged BRAF.

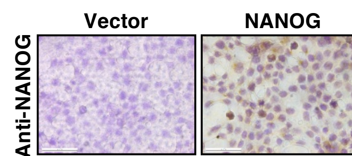
(I) Bacterially expressed His-NANOG or *in vitro* phosphorylated His-NANOG were subjected to GST pull-down assay *in vitro* by GST-SPOP recombinant proteins purified from bacteria.

(J) MTT assay of DU145 cells treated with AZ628 (2 μM) or SB590885 (10 μM). Data are means ± SEM (n=3). *P<0.05, **P<0.01 vs DMSO (Student's *t*-test).

Figure S7







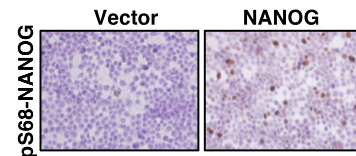


Figure S7. SPOP mutation and phosphorylation of NANOG-Ser68 contributed to elevated NANOG expression in human prostate tumor specimens (Related to Figure 7).

(A) Results of sequencing of SPOP mutations detected in prostate tumors.

(B) Flag-NANOG was coexpressed with HA-SPOP WT or F104C in HEK293T cells. The protein levels of NANOG and SPOP were analyzed by western blotting.

(C) The specificity of antibody against the NANOG for immunohistochemical assay.

(D) The specificity of antibody against the pS68-NANOG for immunohistochemical assay.