Table S1. Primer sequences

	Forward	Reverse
BS		
OPCML	GTTTTTTTGTAGGGGAAGT	TTATTAAATCACACACATAAACAA
HOP/OB1	TACAACCCTACCTAAAAAAAACCCC	TTTTTTGGGGATAGATTTGATAGAT
MCAM	TTTTTAAGTTAGGAGTTTGGGAGTT	CCATTTACTCAAAAACAAAAATAACC
PGP9.5	ACTCAAAAAACACCCACCAACAAAT	GTAGAAATAGTTTAGGGAAGA
DLC	CCAAATAAATACCTTATAACCTTTA	GAGGTGCGGTTATGTTTTGGT
NMDAR2B	ATACC AACTA TTTTC AATTC CTCTC	ATAAT GTAGA ATTTA GAGTA ATTAT
MSP (M-SP)		
B4GALT1	GGTAGAAAAAGGTTATTTAGGAAACG	AAACACAAAAATAACAACCCGTC
Trypsinogen-4	CTATACTCGAACTCCTCGCCG	TTTCGGTTTCGGTTAGCGTGC
DCC	CGTTGTTCGCGATTTTTGGTTTC	ACCGATTACTTAAAAATACGCG
APC	TTATATGTCGGTTACGTGCGTTTATAT	GAACCAAAACGCTCCCCAT
Fhit	GGGCGCGGGTTTGGGTTTTTAC	GAAACAAAAACCCACCGCCCCG
p16	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA
SSBP2	TCGGTCGTTTAGTTAGAAGGTC	GATACCCACGATTAATTTTTACGAT
β-actin	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA
MSP (UnM-SP)		
SSBP2	GTTGGTTGTTTAGTTAGAAGGTTGA	AATACCCACAATTAATTTTTACAAT
TaqMan-MSP		
SSBP2	ATTTTTGCGGTCGTAGCGGT	TTCTACGACAAATCTAACGAA
	ATATCCAAAACGCCGCGAAACTCC (Probe)	
β-actin	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA
	ACCACCACCCAACACACAATAACAAACACA (Probe)	
RT-PCR		
NMDAR2B	GCCTGAGCGACAAAAAGTTC	CATCTCCCCATCTCCAAAGA
DLC	TTGAAGCCAAGGAAGCTTGTG	TCGTCTGAATCGTCACTTCGT
SSBP2	AGTGAAGCAAAAGCCTTCCA	GACACCTCCAAGTGCCTGAT
β-actin	TGGCACCACACCTTCTACAATGAGC	GCACAGCTTCTCCTTAATGTCACGC
Real-time RT-PCR		
NMDAR2B	ACGGCAGCACAGAGAGAAAT	CCTGTTTTCAGGGAGAGCAA
DLC	TTGAAGCCAAGGAAGCTTGTG	TCGTCTGAATCGTCACTTCGT
SSBP2	AGTGAAGCAAAAGCCTTCCA	ATCTCCTGGGGGAATGTTTC
Wnt1	CTCATGAACCTTCACAACAACGA	ATCCCGTGGCACTTGCA
Wnt2	CCTGATGAATCTTCACAACAACAGA	CCGTGGCACTTGCACTCTT
Wnt3a	GCCCCACTCGGATACTTCTTACT	GAGGAATACTGTGGCCCAACA
Wnt7b	GCAAGTGGATTTTCTACGTGTTTCT	TGACAGTGCTCCGAGCTTCA
Wnt10b	GCGCCAGGTGGTAACTGAA	TGCCTGATGTGCCATGACA
β-actin	TGGCACCACACCTTCTACAATGAGC	GCACAGCTTCTCCTTAATGTCACGC

Table S2. Summary of expression levels in proteins mediating Wnt siganaling.

		TE1	MSE-Het-1A		Het-1A		HEK293	
Level		**+ pSSBP2[a]**	**M[b]**	**+ pSSBP2[a]**	**+ siR SSBP2[c]**	**+ Sulindac[d]**	**+ siR SSBP2[c]**	**+ Sulindac[d]**
mRNA	**Wnt1**	**n/s**	**↑**	**n/s**	**↑**	**n/s**	**n/s**	**n/s**
	Wnt2	**n/s**	**↓**	**n/s**	**n/s**	**n/s**	**↑**	**↓**
	Wnt3a	**n/s**	**↑**	**n/s**	**↑**	**n/s**	**↑**	**↓**
	Wnt7b	**↓**	**↑**	**↓**	**n/s**	**n/s**	**n/s**	**n/s**
	Wnt10b	**n/s**	**n/s**	**n/s**	**↑**	**n/s**	**n/s**	**n/s**
Protein	**p-LRP6**	**↓**	**n/c**	**n/c**	**↑**	**↓**		
	LRP6	**↓**	**↑**	**↓**	**↑**	**↓**		
	Dvl3	**↓**	**↑**	**↓**	**n/c**	**↓**		
	Axin1	**↑**	**↓**	**n/c**	**↓**	**n/c**		
	WIF		**↑**	**n/c**	**↓**	**n/c**		
	Naked2	**n/e**	**↓**	**n/c**	**n/c**	**↓**		
	Wnt3a	**↓**	**↑**	**n/c**	**n/c**	**n/c**		
	Wnt5a/5b		**↑**	**n/c**	**↓**	**n/c**		
	Wnt7b		**n/c**	**n/c**	**↑**	**n/c**		

Note, mRNA and protein level were assessed by real time-RT-PCR and western blot analysis, respectively.

Statistical analysis was performed to compare mRNA expression level, and *P<0.05* was considered significant (*t*-test).

↑, increase ; ↓, decrease.

n/s, not significant; n/e, not expressed; n/c, not changed; blank, not assessed.

[a]Expression compared with empty vector control-transfectants.

[b]Expression compared with control-Het-1A cells.

[c]Expression compared with control siRNA-transfectants.

[d]Expression compared with SSBP2 siRNA-transfectants in the absence of Sulindac (100 μM).

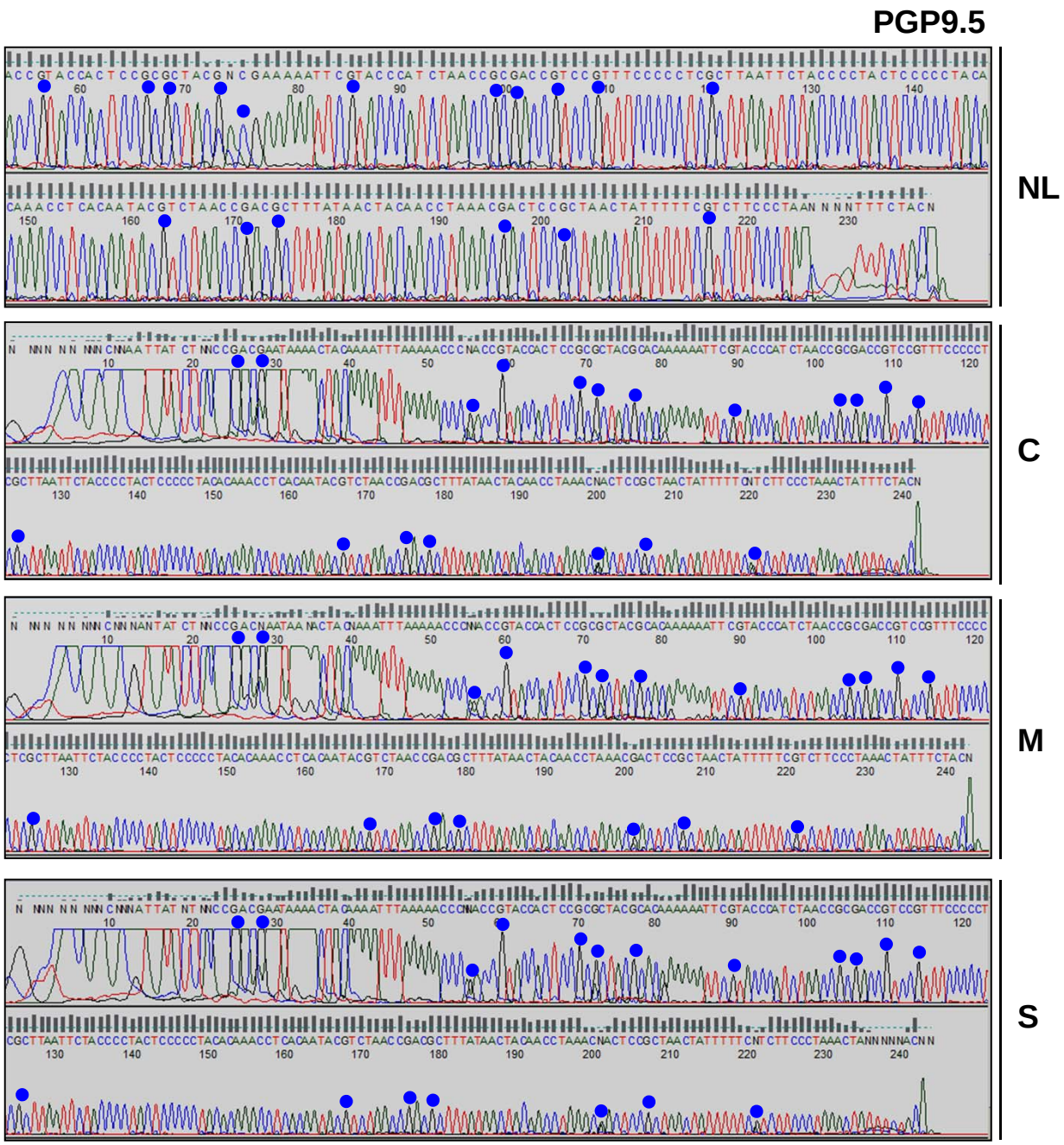
Table S3. Immunohistochemical analysis of SSBP2 in esophagus cancer tissue array with self-matching normal adjacent tissue.

Sex (Age)	Position	Pathology	Grade	Expression
F (49)				
	A1	ESCC	I	+
	A2	ESCC	I	+
	A3	PN	-	++++
	A4	PN	-	++++
M (65)				
	A5	ESCC	II	++
	A6	ESCC	II	++
	A7	PN	-	++
	A8	PN	-	++
M (69)				
	B1	ESCC	II	++
	B2	*SM	-	++
	B3	PN	-	+++
	B4	PN	-	+++
F (56)				
	B5	ESCC	II	+
	B6	ESCC	II	+
	B7	PN	-	++++
	B8	PN	-	++++
M (45)				
	C1	ESCC	III	+
	C2	ESCC	III	+
	C3	PN	-	++++
	C4	PN	-	++++
M (46)				
	C5	ESCC	II	++
	C6	ESCC	II	++
	C7	PN	-	+++
	C8	PN	-	+++

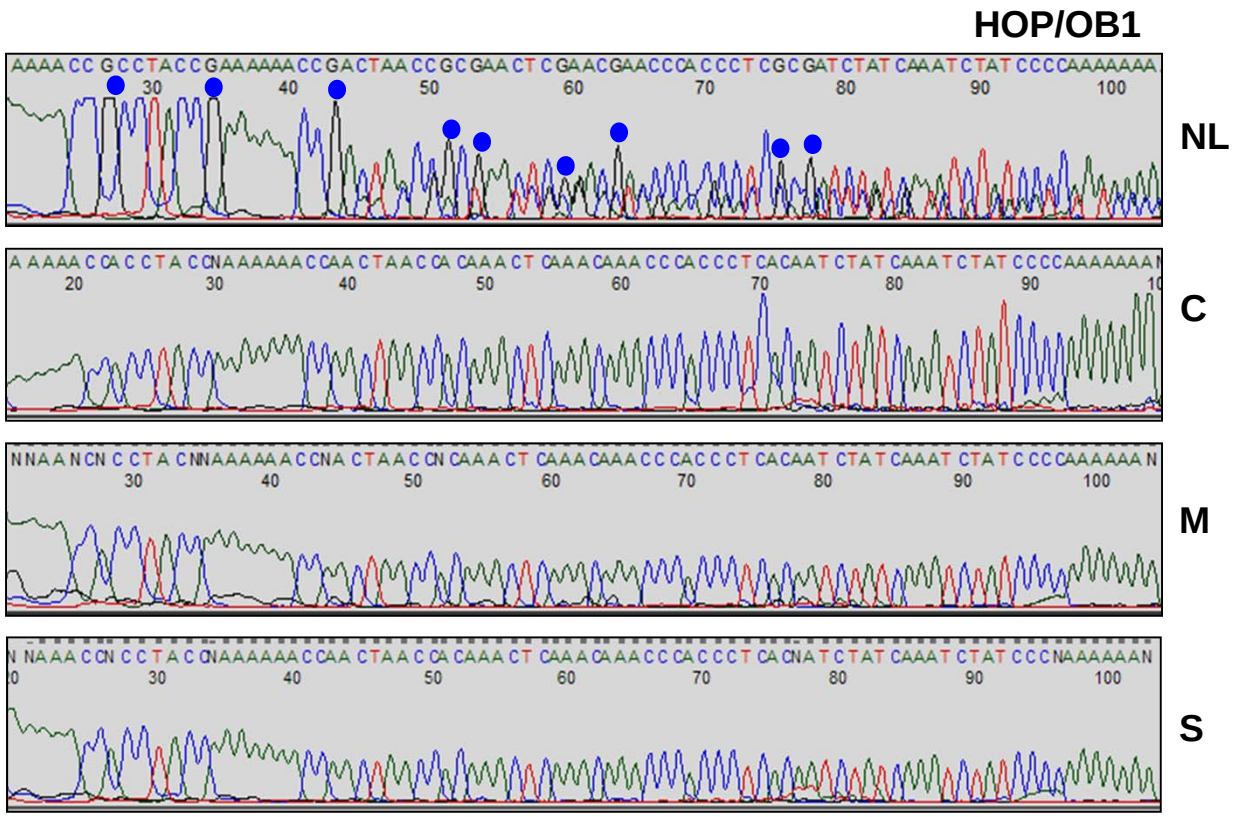
PN, cancer adjacent normal tissue; *SM, Smooth muscle tissue. -, no grading

Note, expression level is indicated as; +, no or faint expression; ++, mild expression; +++, moderate expression; ++++, strong expression.

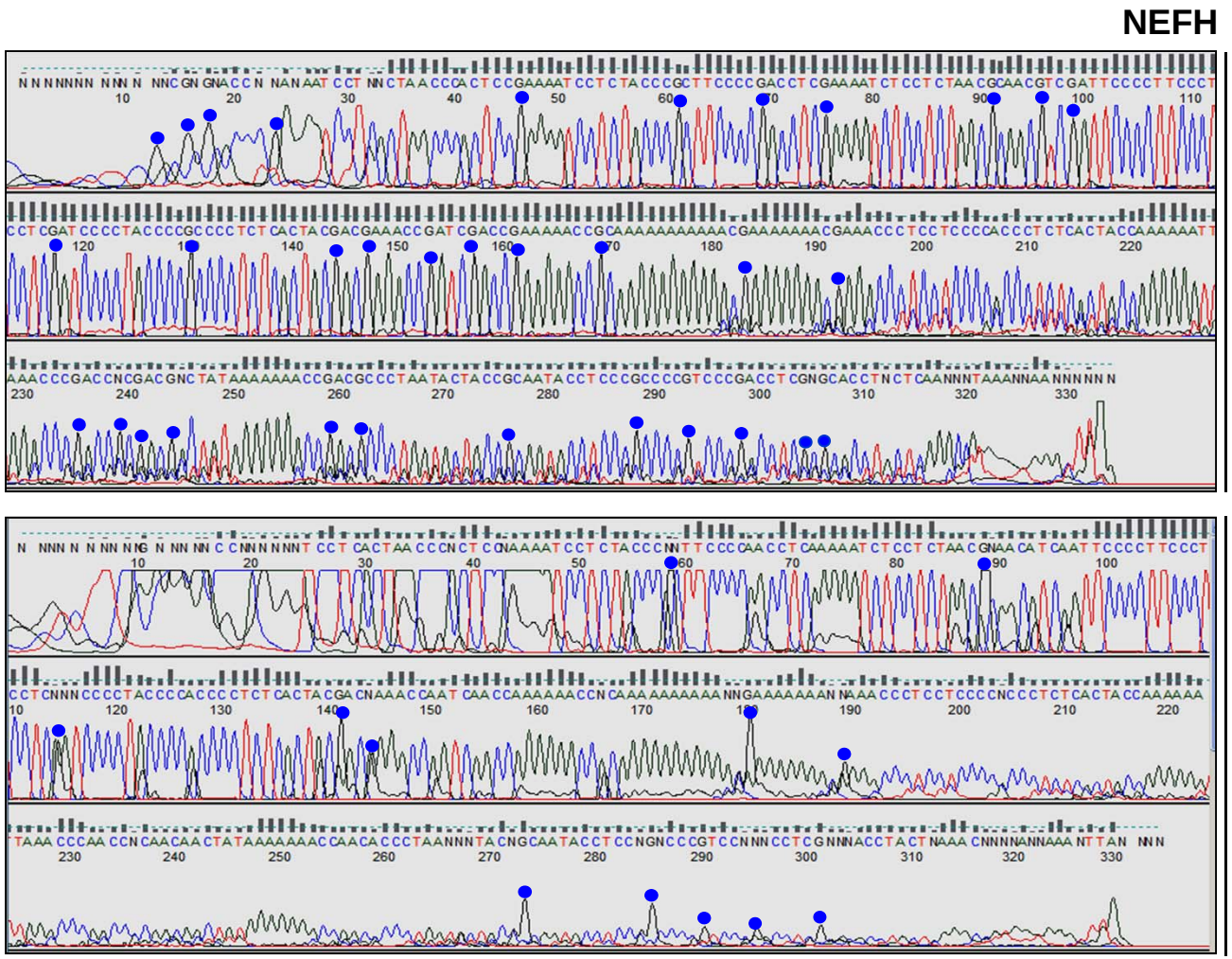
A



B

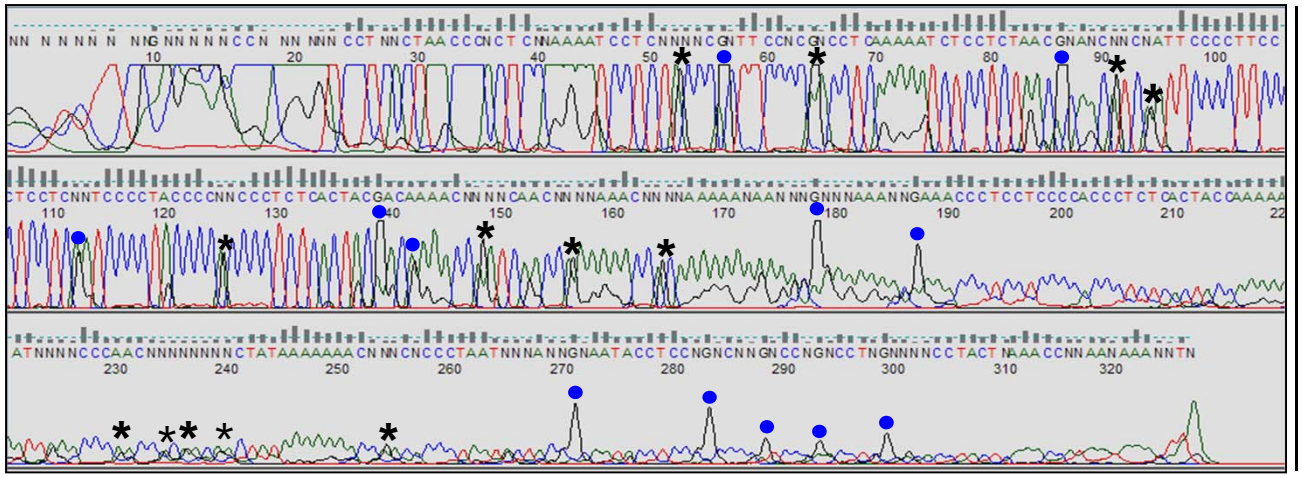


C

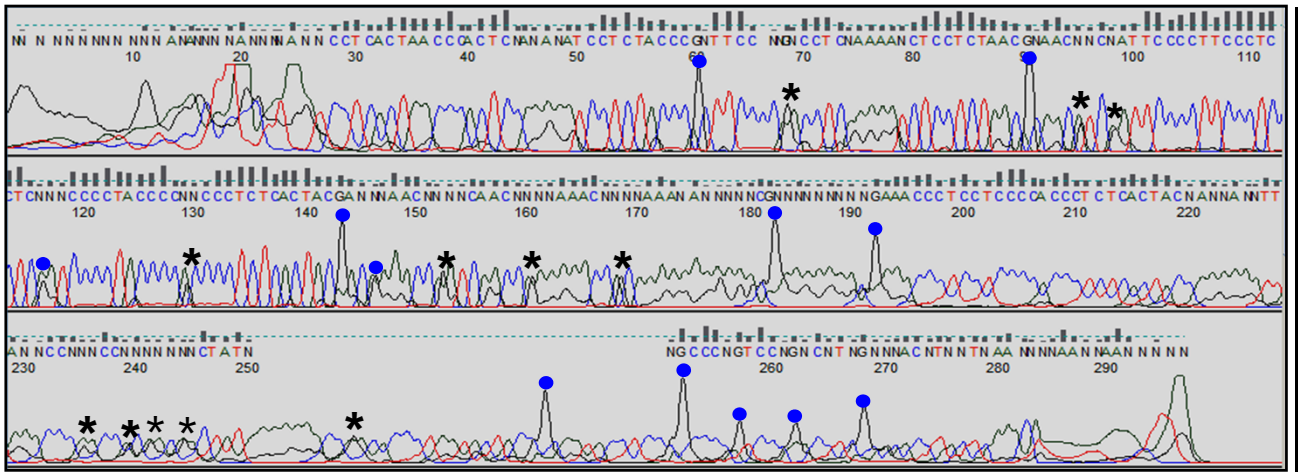


C, continued

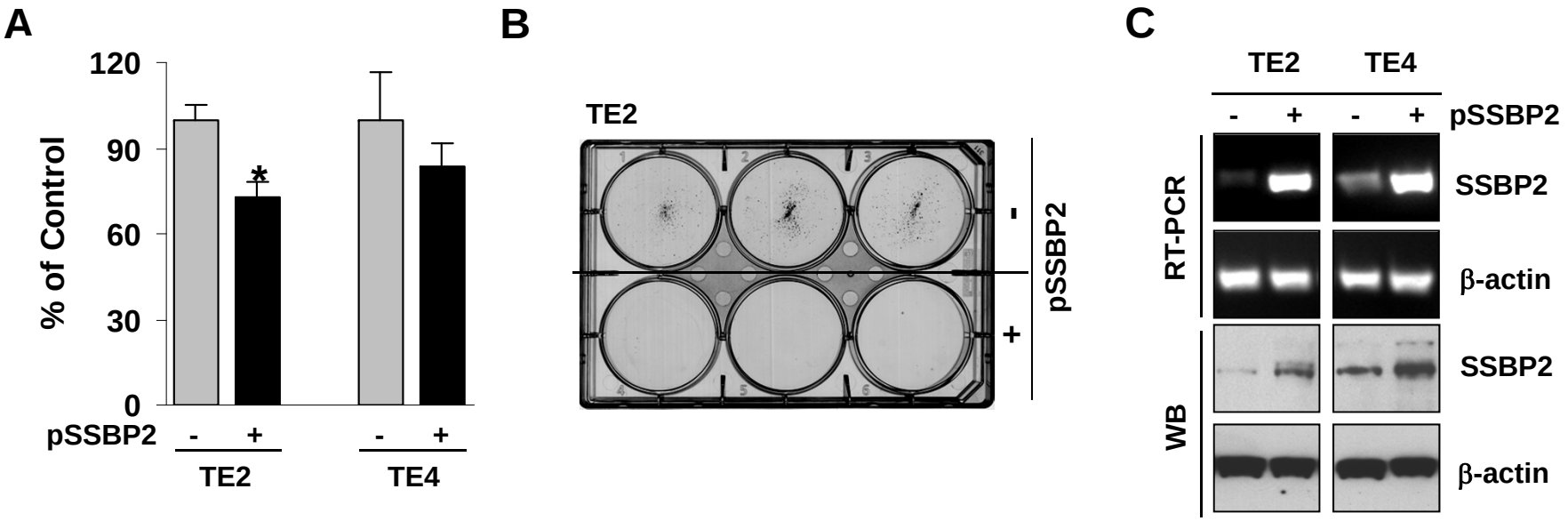
NEFH

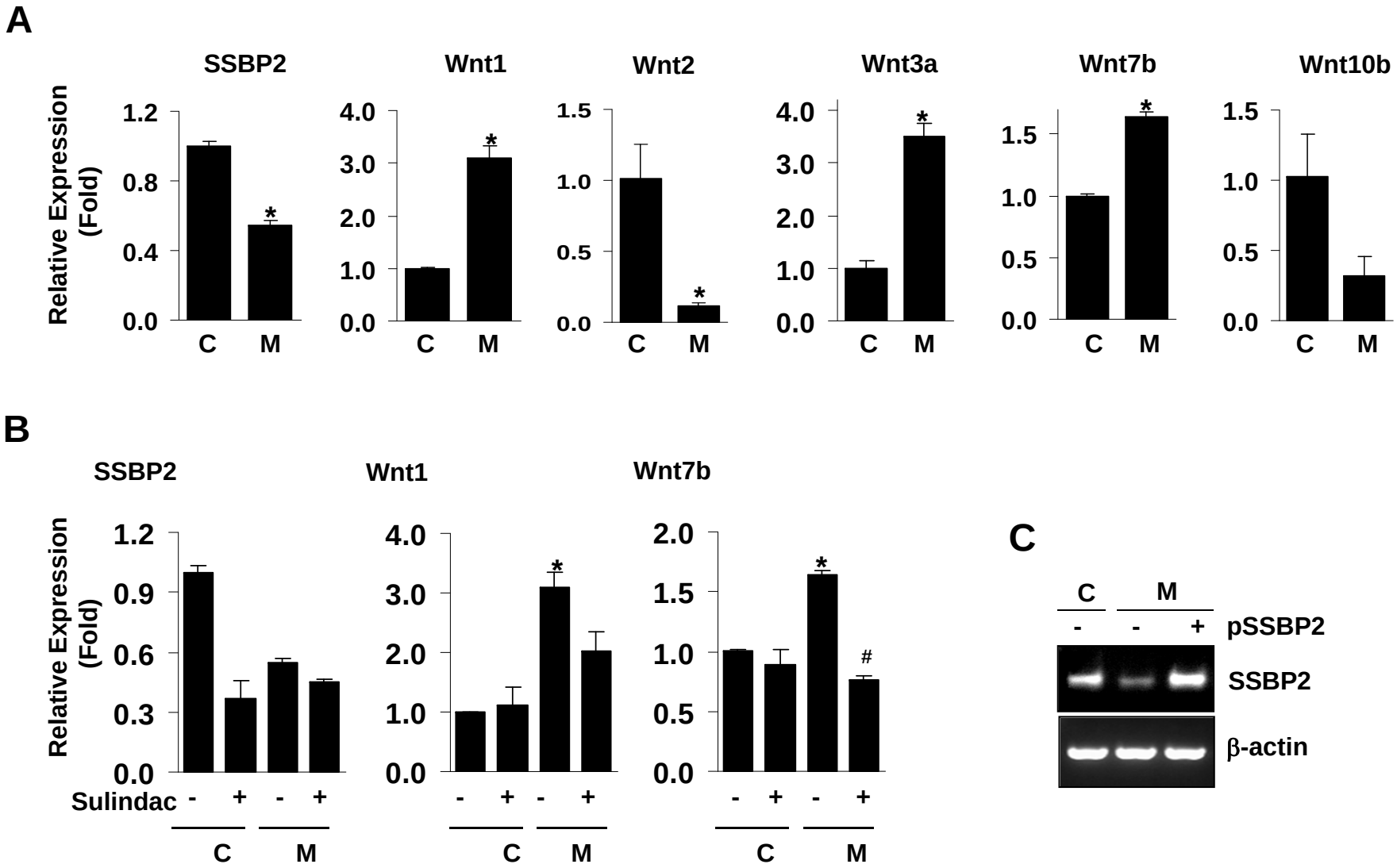


M



S


A
120
90
60
30
0
% of Control
*
pSSBP2
-
+
-
+
TE2
TE4
B
TE2
-
+
pSSBP2
C
TE2
TE4
-
+
-
+
pSSBP2
RT-PCR
SSBP2
β-actin
WB
SSBP2
β-actin


A
SSBP2
Wnt1
Wnt2
Wnt3a
Wnt7b
Wnt10b
Relative Expression (Fold)
1.2
0.8
0.4
0.0
4.0
3.0
2.0
1.0
1.5
0.5
C
M
*
B
SSBP2
Wnt1
Wnt7b
Relative Expression (Fold)
1.2
0.9
0.6
0.3
0.0
4.0
3.0
2.0
1.0
2.0
1.5
1.0
0.5
#
Sulindac - + - +
C
M
C
M
pSSBP2
SSBP2
β-actin

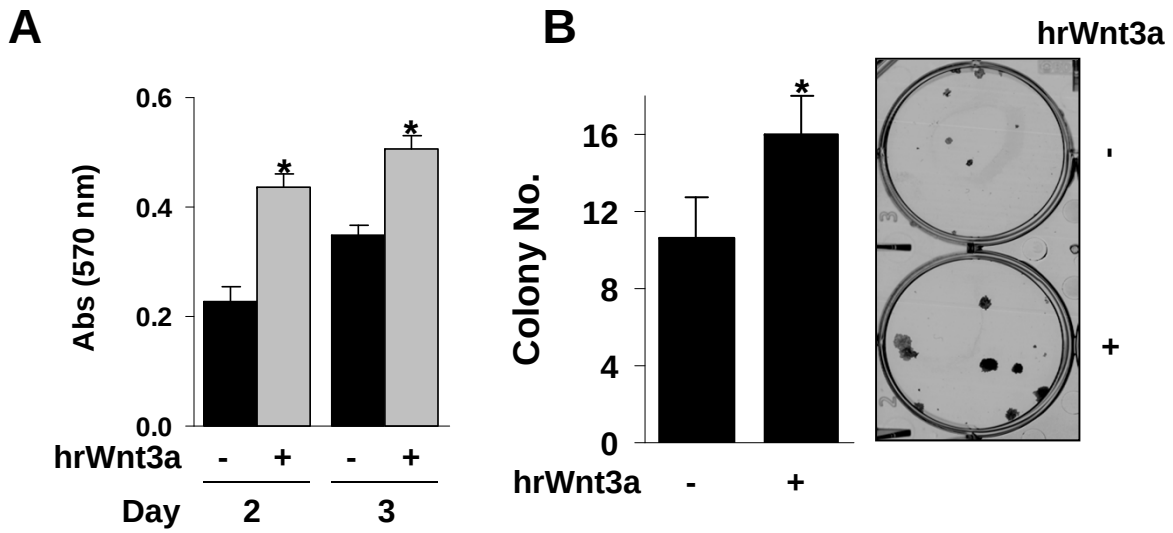

A
Abs (570 nm)
0.6
0.4
0.2
0.0
*
*
hrWnt3a - + - +
Day 2 3
B
hrWnt3a
Colony No.
16
12
8
4
0
*
hrWnt3a - +
-
+

Fig. S5

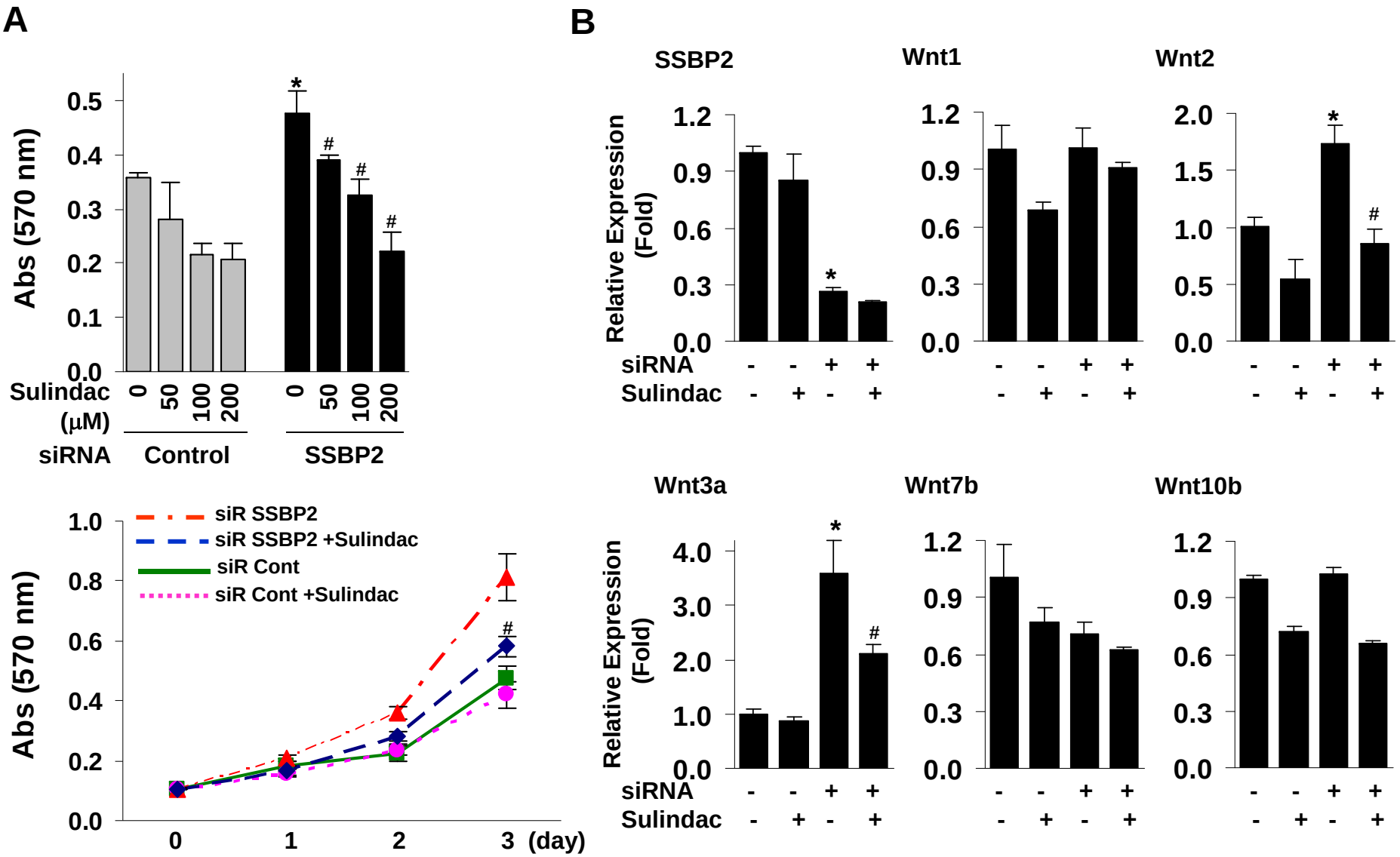
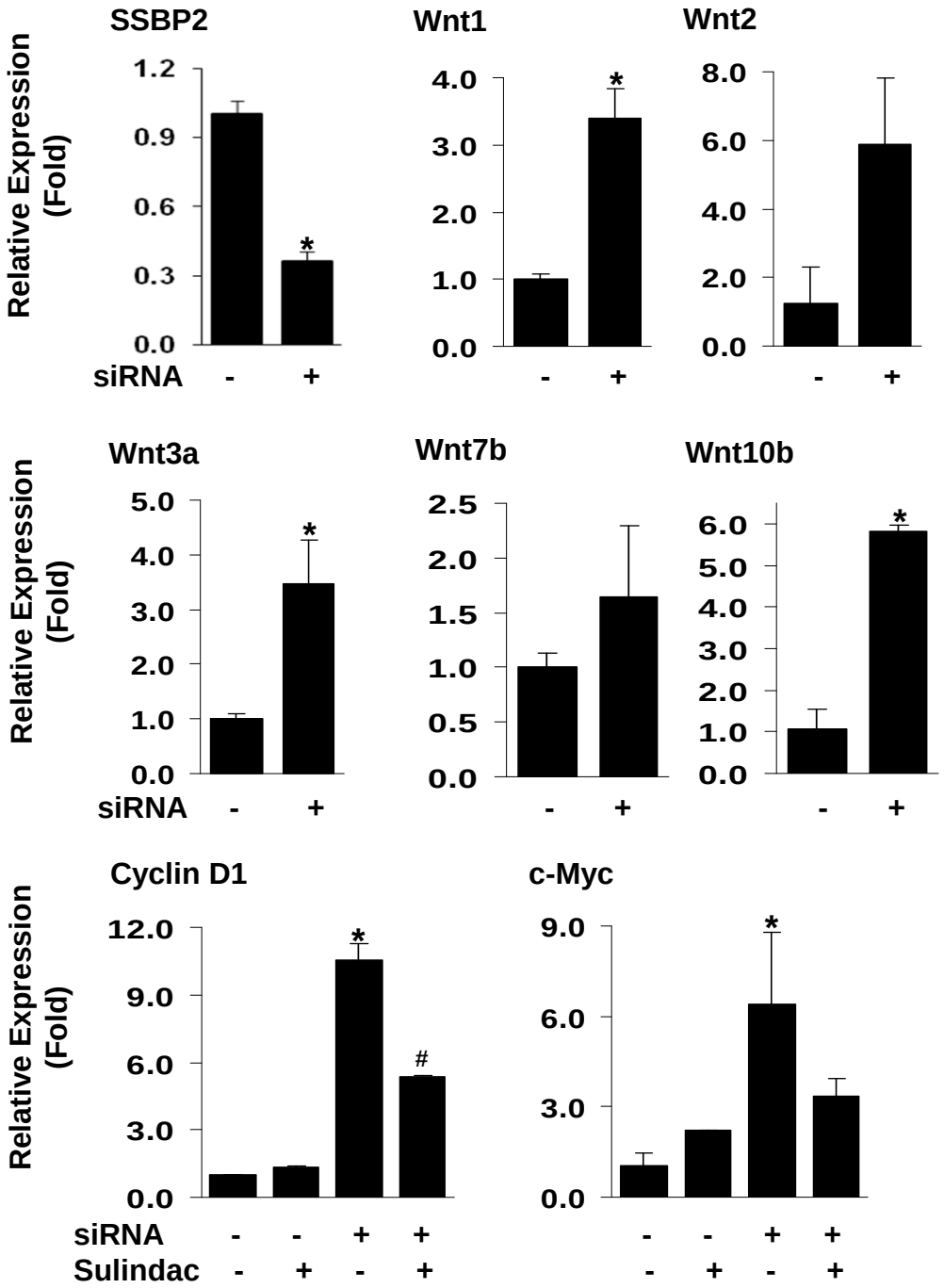

A
Abs (570 nm)
0.0
0.1
0.2
0.3
0.4
0.5
Sulindac (μM)
0
50
100
200
0
50
100
200
siRNA
Control
SSBP2
*
#
#
#
siR SSBP2
siR SSBP2 +Sulindac
siR Cont
siR Cont +Sulindac
Abs (570 nm)
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3 (day)
B
SSBP2
Wnt1
Wnt2
Relative Expression (Fold)
siRNA
Sulindac
Wnt3a
Wnt7b
Wnt10b

Fig. S6


SSBP2
Wnt1
Wnt2
Relative Expression (Fold)
1.2
0.9
0.6
0.3
0.0
4.0
3.0
2.0
1.0
0.0
8.0
6.0
4.0
2.0
0.0
*
siRNA - +
Wnt3a
Wnt7b
Wnt10b
5.0
4.0
3.0
2.0
1.0
0.0
2.5
2.0
1.5
1.0
0.5
0.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Cyclin D1
c-Myc
12.0
9.0
6.0
3.0
0.0
#
siRNA - - + +
Sulindac - + - +

Supplemental Figure Legends

Figure S1. **Bisulfite-sequencing results of PGP9.5, HOP/OB1 and NEFH**. DNA methylation in the PGP9.5 promoter (**A**), but not in the HOP/PB1 promoter (**B**) was observed in the Het-1A cells regardless of CSE exposure. In the NEFH promoter (**C**), methylated CpGs were observed in 12 of 33 CpGs analyzed in the control-Het-1A cells, so NEFH was methylation-positive in the control-Het-1A cells. *In vitro* methylated, bisulfite-treated human normal lymphocyte DNA (NL) was used as a positive control for gene methylation. C, control-Het-1A cells; M, MSE-Het-1A cells; S, SSE-Het-1A cells. Filled circle, methylated CpG; *, 12-13 new methylated cytosines by MSE or SSE exposure.

Figure S2. Tumor suppressive activity of SSBP2 in TE2 and TE4 cells. A, pSSBP2 (+) or empty vector control (-) were transfected into TE2 and TE4 cells where basal level of SSBP2 was barely (TE2) and clearly detected (TE4), and after 3 days of incubation, cell growth was examined by MTT assay. Ectopic SSBP2 expression inhibited about 30% of the TE1 cell growth, but no significant difference was observed in TE4 cell growth. *, $P<0.05$ compared to empty vector control (*t*-test). **B**, SSBP2 over-expression markedly decreased colony formation in TE2 cells. Colony focus assays were performed after incubation in the presence of G418 (500 μg/ml) for two weeks. Colonies were fixed and stained with 0.4% crystal violet solution (MeOH/Acetic acid, 3:1), and photographed. **C**, To confirm the expression of SSBP2, TE2 and TE4 cells were harvested 48 hr after transfection, and RT-PCR and western blotting were performed.

Figure S3. Expression level of proteins in Wnt signaling. A, In the control- and MSE-Het-1A cells, the transcriptional expression of indicated Wnt members was examined. The level of Wnt1, Wnt3a, Wnt7b in the MSE-Het-1A cells was significantly higher than in the control, but the level of Wnt2 and Wnt10b was decreased. C, control-Het-1A cells; M, MSE-Het-1A cells. Relative expression (Fold) was calculated by comparing the ratios of mRNA expression of genes to an internal control gene, β-actin. Experiments were done in duplicate, and independent experiments were performed twice. Values indicate means ± SD. *, $P<0.05$ compared to the control-Het-1A cells (*t*-test). **B,** Sulindac (100 μM), a Wnt pathway-specific inhibitor,[25] inhibited the expression of Wnt1 and Wnt7b increased in the MSE-Het-1A cells, whereas it did not have

significant effect on the transcriptional level of SSBP2 and other Wnt members (data not shown). **C**, SSBP2 expression in cells transfected with or without pSSBP2.
`

Figure S4. Wnt3a increases cell growth. hrWnt3a (100 ng/ml) was added for 3 days into Het-1A cells (passage +24) (**A**), and for 18 days in TE2 cells (**B**), and the MTT and colony focus assay, respectively, were performed.

Figure S5. SSBP2 gene knock-down in HEK293 cells. A, A siRNA pool targeting SSBP2 and a non-targeting control were transfected into the HEK293. After 4 hrs of transfection, Sulindac was treated at the indicated concentrations and incubated for further 48 hr (upper). Sulindac (100 μM) was added for up to 3 days (lower). Cell growth was assessed by the MTT assay. Experiments were done in four replicates, and repeated twice. Values indicate means ± SD. *, $P<0.05$ compared to cells transfected with control siRNA in the absence of Sulindac (*t*-test); $^{\#}$, $P<0.05$ compared to cells transfected with SSBP2 siRNA in the absence of Sulindac. **B,** HEK293 cells were transfected with a non-targeting control (-) or SSBP2 siRNA (+), and incubated for 48 hrs. The mRNA level of Wnt members were examined by real time-RT-PCR analysis. The mRNA level of Wnt2 and Wnt3a was increased by SSBP2 siRNA transfection, which was decreased by Sulindac treatment. The expression level of the other genes was not significantly changed. *, $P<0.05$ compared to the non-targeting control-transfected cells.

Figure S6. SSBP2 gene knock-down in Het-1A cells. Het-1A cells (passage +32) were transfected with a non-targeting control (-) or SSBP2 siRNA (+). After 4 hrs of transfection, Sulindac (100 μM) was added and incubated for a further 48 hr. The mRNA levels of Wnt members were examined by real time-RT-PCR analysis. The mRNA levels of Wnt1, Wnt3a and Wnt10b were significantly increased by SSBP2 siRNA transfection, whereas Sulindac did not have a significant effect on expression of Wnt. The levels of Cyclin D1 and c-Myc were also increased by SSBP2 knock-down, but TCF1 and MMP-7 expression was not affected by SSBP2 siRNA transfection. Relative expression (Fold) was calculated by comparing the ratios of mRNA expression of genes to an internal control gene, β-actin. Experiments were done in duplicate, and independent experiments were performed twice. Values indicate means ± SD. *, $P<0.05$ compared to cells transfected with control siRNA in the absence of Sulindac (*t*-test); $^{\#}$, $P<0.05$ compared to cells transfected with SSBP2 siRNA in the absence of Sulindac.