Supplemental Material to:

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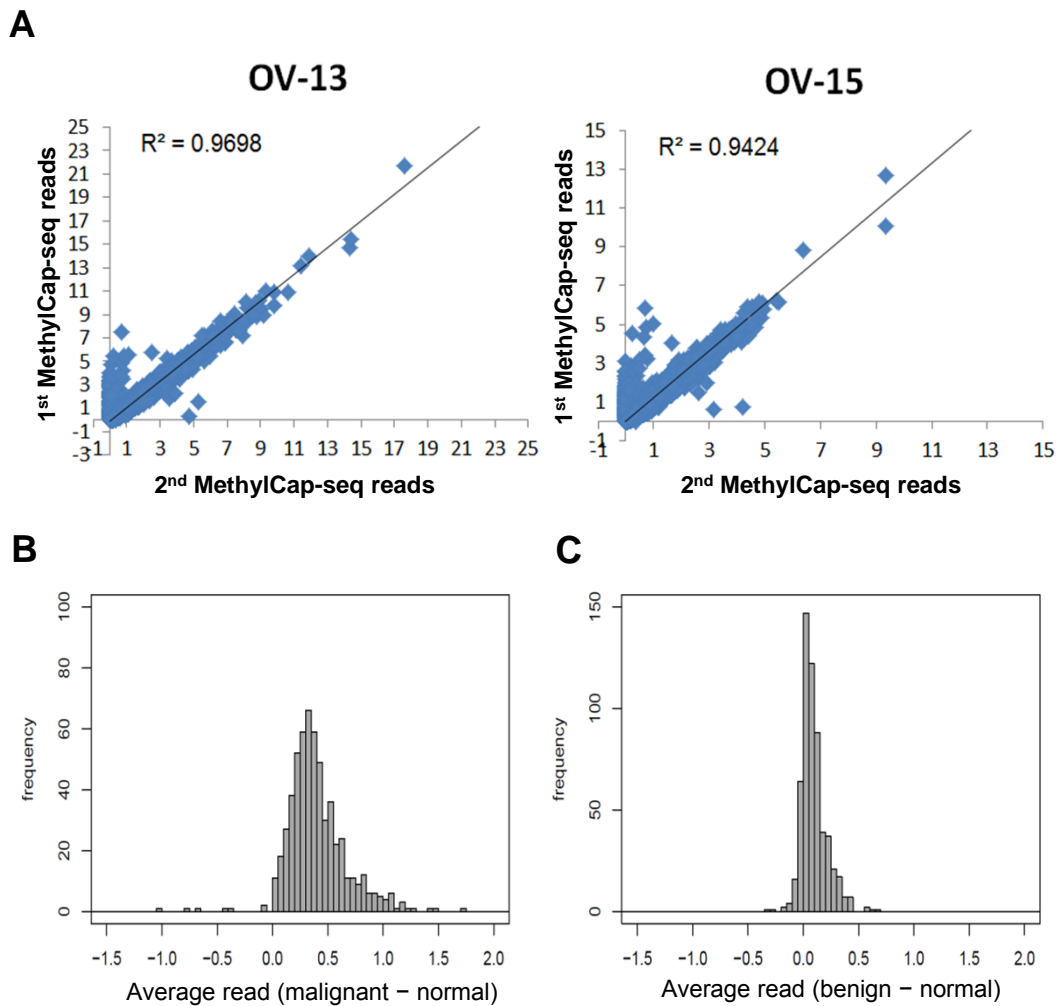
Comprehensive methylome analysis of ovarian tumors reveals hedgehog signaling pathway regulators as prognostic DNA methylation biomarkers

Supplementary Figure S1

A

B   **C**

**Supplementary Figure S1.** Global analysis of DNA methylation profiles in ovarian samples. A, Correlation between experimental replication (two samples). Each point represents the raw methylation level normalized read numbers. B, Histogram showing the differentially averaged reads between malignant and normal tissues. C. Histogram showing the differentially average reads between benign and normal tissues.

Supplementary Figure S2

A

B

Cutoff value > 17 HM-DMRs
$P < 0.001$
AUC = 0.85
95% CI = 0.74 to 0.92
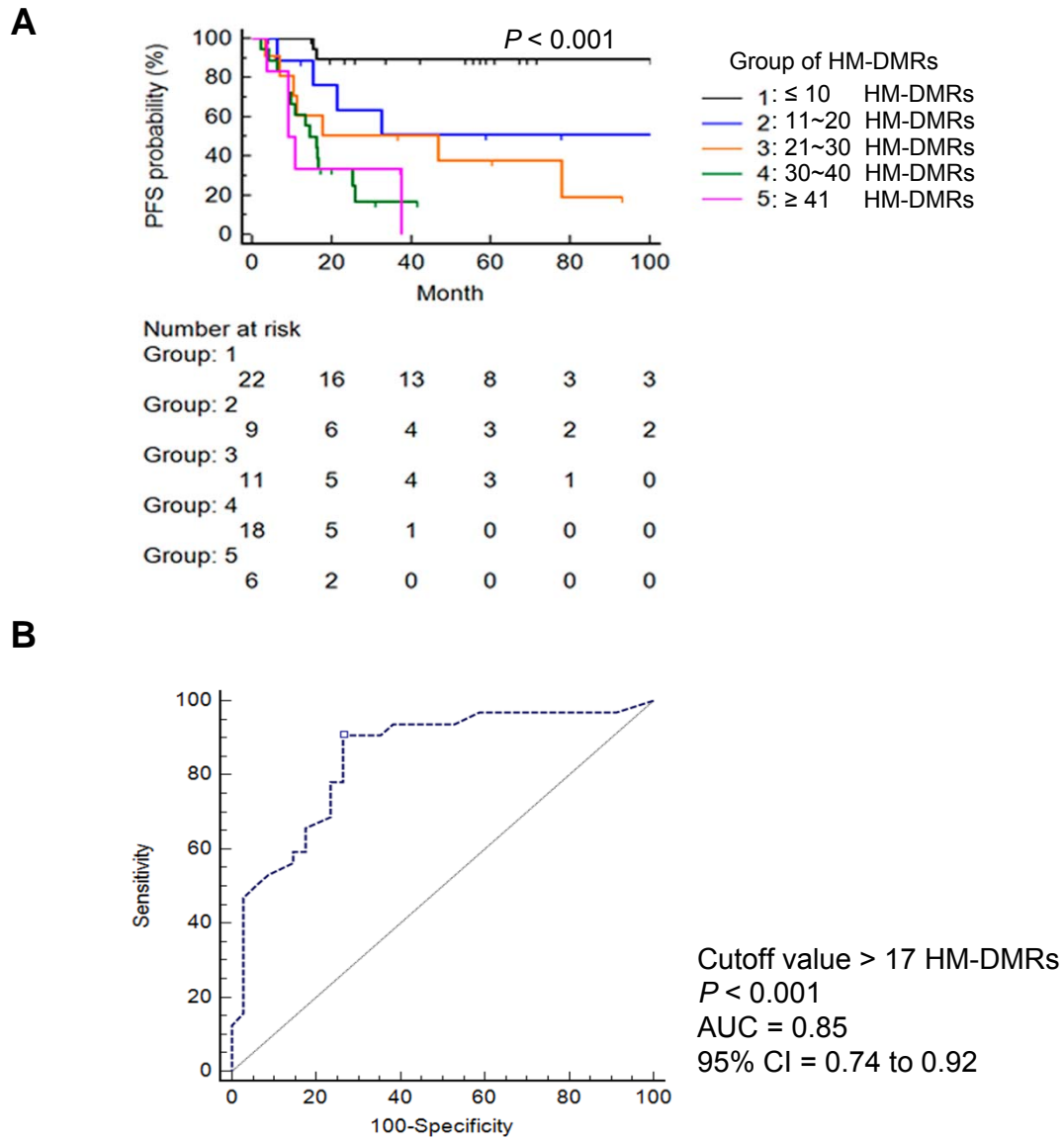
Supplementary Figure S2. Identifying recurrent patients with highest accuracy. A, Greater number of high methylation differentially methylated regions (HM-DMRs) is related to worse progression-free survival. B, Using area under the receiver operating characteristic (ROC) curve (AUC) to determine the best cutoff point to identify recurrent patients in 63 DMRs.

Supplementary Figure S3
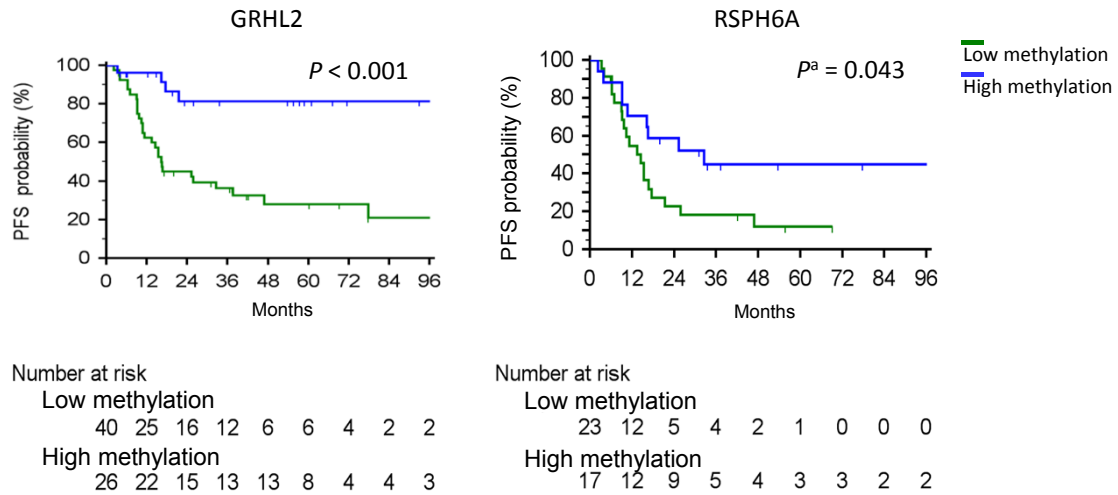
Supplementary Figure S3. Two high methylation (HM) DMRs related to good progression-free survival. [a] Adjusted stage

Supplementary Figure S4
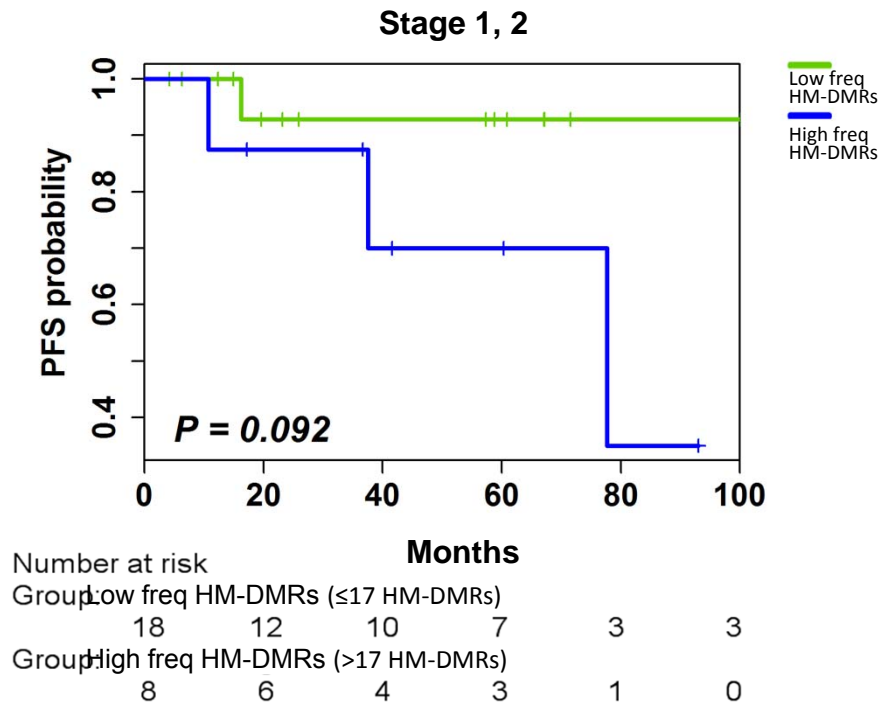
Supplementary Figure S4. High frequency HM-DMRs tended to be associated with shorter PFS and low frequency HM-DMRs with longer PFS.

Supplementary Figure S5

Supplementary Figure S5. Correlation between expression of *ZIC*s and DNA methylation in patient samples from the ovarian cancer TCGA data. Two independent CpG sites were detected by methylation bead array. A, Probe id, cg05073035 locates at +171 bp from transcription star site (TSS; arrow) of *ZIC1*. B, Probe id , cg14456683 locates at -12 bp from TSS of *ZIC1*. As the Infinium HumanMethylation27 platform does not contain probes designed for CpG sites close to the TSS of *ZIC4*, no correlation between methylation and *ZIC4* expression was observed.

Supplementary Figure S6

Supplementary Figure S6. Gene expression of *ZICs* and *GLIs*. A. Treatment with 5-aza-dc (DNA methyltransferase inhibitor) restores *ZIC1* and *ZIC4* expression. B. Expression of *GLIs* after treatment with the Hedgehog (Hh) inhibitor cyclopamine (Hhi-L = 1µM cyclopamine, Hhi-H, 8 µM cyclopamine). 10^6 OC cells were drug-treated for three days. Ctrl, no drug treatment. (*, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ by two-tailed t-test)

Supplementary Figure S7
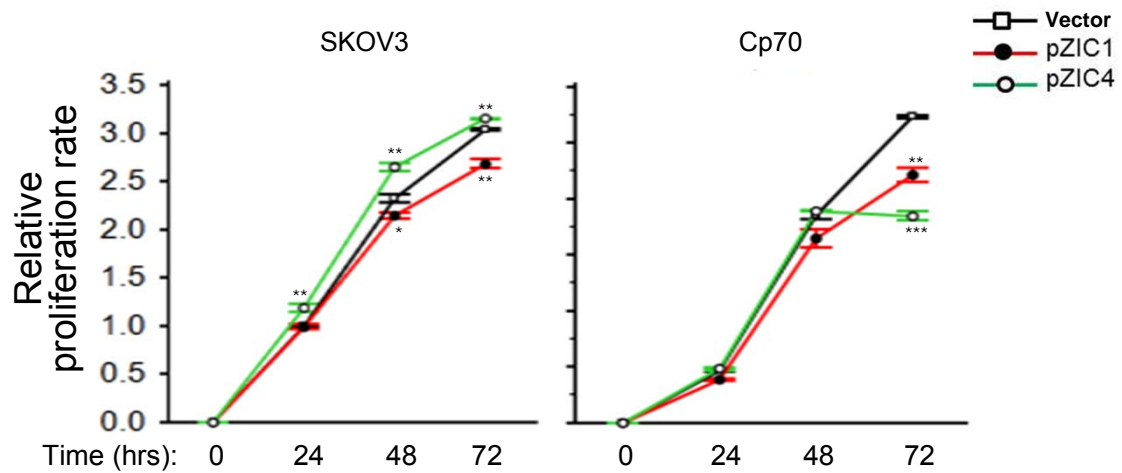
Supplementary Figure S7. *ZIC1* or *ZIC4* overexpression modulates ovarian cancer cell proliferation. A, Effect of enforced *ZIC1* or *ZIC4* on proliferation rate in SKOV3 or Cp70. Overexpression of *ZIC1* inhibited proliferation of both OC cell lines tested (SKOV3 and Cp70) compared to vector control, while *ZIC4* only reduced Cp70 cell proliferation. Vector, vector only; pZIC1, pCMV-ZIC1 plasmid and pZIC4, pCMV-ZIC4 plasmid. (*, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ by two-tailed t-test)

Supplementary Figure S8
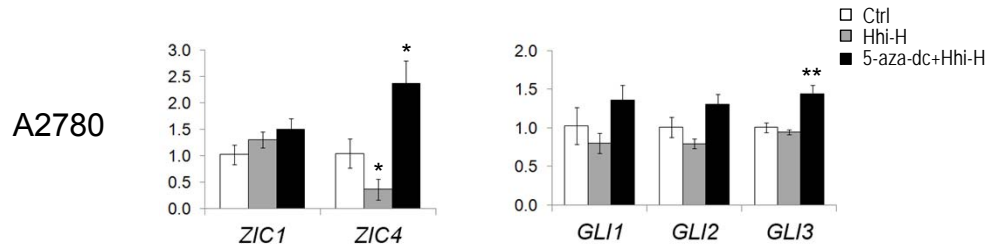
Supplementary Figure S8. Correlation of expression of *ZICs* and *GLIs* in platinum sensitive ovarian cell line (A2780). Treatment with Hh inhibitor resulted in weak repression of expression of *GLIs*. Restored *ZIC1* and *ZIC4* expression was associated with induced expression of *GLIs* in A2780 cells. (*, *P* < 0.05; **, *P* < 0.01 and ***, *P* < 0.001 by two-tailed t-test)

Supplementary Table S1. Clinical characteristics of patients and samples for MethylCap-seq analysis

	Malignant (N = 75)		Benign[a] (N = 20)		Normal (N = 6)
	Number of patients	%	Number of patients	%	Number of patients
Age (years)					
Mean±SD	57.1 ± 13.77		41.15 ± 13.38		51.33 ± 13.37
Range	29 - 90		26 - 80		28 - 77
FIGO stage					
Stage 1	18	24.0			
Stage 2	8	10.7			
Stage 3	42	56.0			
Stage 4	7	9.3			
Grading					
G1	13	17.3			
G2	18	24.0			
G3	44	58.7			
Histology					
Serous	50	66.7	7	35.0	
Mucinous	16	21.3	6	30.0	
Endometrioid	4	5.3	7	35.0	
Clear Cell	5	6.7			

Abbreviations: MethylCap-seq, methyl-CpG binding domain of the MBD2 protein to capture DNA followed by high-throughput next- generation sequencing; N, the number of patients; SD, Standard deviation; FIGO stage, it followed the International Federation of Gynecology and Obstetrics staging system to identify the stage. [a]The determined pathology of tissues is cystic adenoma.

Supplementary Table S2. Multivariate COX analysis of high methylation DMRs (HM-DMR) associated with progression free survival (N = 63)

Gene Name	HR	95% CI		P^a	Gene Name	HR	95% CI		P^a
ADAMTS5	2.43	1.14	5.18	0.021	MIR1205	3.04	1.43	6.47	0.004
ADRB1	2.20	1.05	4.59	0.036	MIR1224	2.21	1.07	4.55	0.031
AGAP2	2.39	1.14	5.00	0.020	MIR125B1	2.40	1.16	4.96	0.018
AK1	3.08	1.38	6.88	0.006	MIR25	2.27	1.07	4.83	0.033
ARHGDIA	2.27	1.11	4.63	0.024	MIR93	2.17	1.03	4.60	0.043
C16orf35	2.38	1.11	5.07	0.025	NBL1	2.33	1.11	4.87	0.025
C1orf104	2.41	1.16	5.02	0.019	NEUROG1	2.44	1.16	5.15	0.019
C1QL3	2.87	1.37	6.03	0.005	NFIX	2.18	1.04	4.60	0.040
CCDC17	2.88	1.32	6.29	0.008	NID2	2.33	1.05	5.15	0.037
DLEU7	2.77	1.32	5.79	0.007	NKX2-8	2.54	1.17	5.52	0.019
FAM131A	2.99	1.40	6.37	0.005	OLFML3	2.95	1.38	6.29	0.005
FGF12	3.52	1.64	7.54	0.001	PHF2	4.09	1.85	9.03	0.000
FOXA1	2.77	1.19	6.45	0.018	PLEKHG2	2.20	1.07	4.54	0.032
FOXL2	2.31	1.11	4.82	0.026	PRRT1	2.30	1.12	4.75	0.024
GRHL2	0.22	0.07	0.70	0.010	PTGER1	2.56	1.24	5.29	0.011
HDLBP	2.17	1.01	4.66	0.046	RORC	2.53	1.18	5.43	0.017
HIST1H2BB	2.73	1.31	5.69	0.007	RSPH6A	0.42	0.19	0.92	0.031
HIST1H3A	3.01	1.43	6.33	0.004	SHOX2	2.93	1.36	6.29	0.006
HIST1H3C	2.80	1.34	5.84	0.006	SKI	2.10	1.01	4.36	0.046
HIST1H3E	2.60	1.27	5.31	0.009	SNORA71A	2.93	1.41	6.09	0.004
HIST1H4I	2.47	1.16	5.27	0.019	SPON2	2.29	1.09	4.77	0.028
HNF1B	2.51	1.16	5.44	0.020	SRRT	2.61	1.24	5.48	0.011
HNRNPF	3.12	1.44	6.73	0.004	SSBP4	2.21	1.08	4.51	0.030
HOXB5	2.53	1.17	5.50	0.019	TAF4	3.38	1.52	7.55	0.003
IRS1	2.49	1.16	5.37	0.020	TAL1	3.01	1.38	6.57	0.006
KLF1	3.04	1.39	6.65	0.005	TBX5	2.58	1.15	5.79	0.022
LOC283392	2.33	1.11	4.87	0.025	TCFL5	2.42	1.16	5.06	0.019
LOC404266[b]	2.26	1.08	4.70	0.030	TRH	3.28	1.44	7.48	0.005
LOC404266[c]	3.13	1.40	6.99	0.005	VGLL4	2.56	1.20	5.46	0.015
MED26	4.20	2.01	8.76	0.000	WISP2	4.00	1.80	8.88	0.001
MEIS2	3.41	1.55	7.49	0.002	ZBTB12	3.09	1.49	6.38	0.002
					ZIC1	2.25	1.09	4.63	0.027

HR, hazard ratio; CI, confidence interval

[a]Adjusted for age, stage, grade, histology. [b]loci at chr17: 44022672 ~ 44026632; [c]loci at chr17: 44026338 ~ 44030298

Supplementary Table S3. Identification of recurrent patients by area under the receiver operating characteristic (ROC) curve (AUC) at CpG sites of *ZIC1* promoter

CpG site	Cutoff value	Highest accuracy	95 % CI	
CpG 1	36.9	60.7	43.5	67.2
CpG 2	49.5	63.0	48.9	72.2
CpG 3	46.9	60.8	42.1	65.9
CpG 4	74.0	62.0	48.2	71.6
CpG 5	81.6	61.9	46.4	70.0
CpG 6	52.6	68.8	54.4	77.0
CpG 7	30.0	67.4	50.5	73.7

CI, confidence interval

Supplementary Table S4. Univariate and multivariate Cox survival analysis for the association between the risk of high methylation of *ZIC1* at CpG site 7, clinicial variables and progression free survival

Variable	N	Univariate analysis				Multivariate analysis			
		HR	95% CI		*P*-value[a]	HR	95% CI		*P*-value[a]
Age									
<=60 yrs	32	1.00				1.00			
>60 yrs	41	1.35	0.68	2.66	0.391	0.85	0.34	1.72	n.s.
Methylation									
Low	36	1.00				1.00			
High	37	2.56	1.27	5.16	*0.009*	2.45	1.17	5.13	*0.017*
FIGO stage									
Early	29	1.00				1.00			
Advanced	44	12.57	3.82	41.38	*<0.001*	11.85	2.94	47.74	*<0.001*
Grade									
G1	16	1.00				1.00			
G2+G3	57	3.31	1.16	9.43	*0.025*	2.31	0.77	6.88	n.s.
Histology									
Others	20	1.00				1.00			
Serous	53	3.32	1.17	9.43	*0.024*	0.77	0.21	2.75	n.s.

Abbreviations: HR, Hazard ratio; 95% CI, 95 percent of the confidence interval; N, case number. [a]Significant permutation *P*-values are marked with boldface and italic (n.s. = not significant).

Supplementary Table S5. Correlation between methylated CpG site 6 of *ZIC1* and clinical features of ovarian cancer patients

Variables	High methylation (N= 37)	Low methylation (N=36)	P-value[a]
Age			0.061
<= 60 yrs	12	20	
>60 yrs	25	16	
FIGO stage			0.097
Early	11	18	
Advanced	26	18	
Grade			0.581
G1	7	9	
G2+G3	30	27	
Histology			1
Serous	27	26	
Others	10	10	
Recurrent			
Yes	23	12	*0.019*
No	14	24	

[a] Fisher's exact test. Significant permutation *P*-value is marked with boldface and italic.

Supplementary Table S6. Summary of sequencing reads in ovarian samples

Tissue Type	ID	Total	Uniquely Mapped	Uniquely Mapped Rate (%)	Multiple Mapped	Multiple Mapped Rate (%)	Non-Mapped	Non-Mapped Rate (%)
Malignant	OV_001	51,426,864	23,670,379	46.0	19,888,630	38.7	7,867,855	15.3
	OV_002	36,801,551	18,713,055	50.8	13,163,261	35.8	4,925,235	13.4
	OV_003	43,135,082	21,928,452	50.8	15,494,697	35.9	5,711,933	13.2
	OV_004	50,698,774	23,999,391	47.3	17,281,064	34.1	9,418,319	18.6
	OV_005	42,193,347	20,163,197	47.8	16,524,347	39.2	5,505,803	13.0
	OV_006	27,073,087	13,703,498	50.6	9,926,961	36.7	3,442,628	12.7
	OV_007	40,693,376	19,151,154	47.1	15,457,500	38.0	6,084,722	15.0
	OV_008	50,115,760	33,205,342	66.3	11,215,623	22.4	5,694,795	11.4
	OV_009	31,276,716	13,098,498	41.9	10,809,244	34.6	7,368,974	23.6
	OV_010	45,082,274	20,995,104	46.6	17,629,521	39.1	6,457,649	14.3
	OV_011	87,927,703	50,857,684	57.8	28,952,439	32.9	8,117,580	9.2
	OV_012	22,856,701	12,902,547	56.4	7,913,124	34.6	2,041,030	8.9
	OV_013	90,752,113	46,014,096	50.7	33,055,031	36.4	11,682,986	12.9
	OV_014	42,982,723	18,700,819	43.5	18,041,850	42.0	6,240,054	14.5
	OV_015	70,893,683	39,971,677	56.4	22,124,803	31.2	8,797,203	12.4
	OV_016	48,733,227	25,151,374	51.6	16,656,883	34.2	6,924,970	14.2
	OV_017	35,769,858	19,597,423	54.8	11,868,646	33.2	4,303,789	12.0
	OV_018	50,046,958	27,001,292	54.0	16,716,206	33.4	6,329,460	12.6
	OV_019	43,917,023	19,300,474	43.9	16,870,551	38.4	7,745,998	17.6
	OV_020	62,314,344	20,569,531	33.0	25,742,251	41.3	16,002,562	25.7
	OV_021	43,208,574	21,108,985	48.9	15,806,401	36.6	6,293,188	14.6
	OV_022	38,672,163	18,816,966	48.7	14,611,723	37.8	5,243,474	13.6
	OV_023	81,860,296	43,698,780	53.4	29,162,743	35.6	8,998,773	11.0
	OV_024	34,921,460	18,193,369	52.1	12,957,495	37.1	3,770,596	10.8
	OV_025	71,171,031	37,836,768	53.2	25,130,148	35.3	8,204,115	11.5
	OV_026	47,147,393	24,470,546	51.9	16,914,584	35.9	5,762,263	12.2
	OV_027	46,220,176	20,378,196	44.1	17,273,838	37.4	8,568,142	18.5
	OV_028	52,321,071	22,017,509	42.1	19,342,036	37.0	10,961,526	21.0
	OV_029	37,242,826	17,087,466	45.9	15,332,083	41.2	4,823,277	13.0
	OV_030	48,932,669	27,090,154	55.4	14,988,156	30.6	6,854,359	14.0
	OV_031	38,350,420	17,430,828	45.5	14,915,322	38.9	6,004,270	15.7
	OV_032	40,229,206	19,700,916	49.0	14,693,880	36.5	5,834,410	14.5
	OV_033	49,031,011	22,607,232	46.1	19,582,097	39.9	6,841,682	14.0
	OV_034	46,974,803	22,627,727	48.2	17,959,034	38.2	6,388,042	13.6
	OV_035	41,486,536	21,116,730	50.9	13,243,702	31.9	7,126,104	17.2
	OV_036	50,650,061	20,339,308	40.2	21,243,380	41.9	9,067,373	17.9
	OV_037	40,939,788	17,382,787	42.5	16,891,978	41.3	6,665,023	16.3
	OV_038	43,914,694	22,051,063	50.2	15,062,016	34.3	6,801,615	15.5
	OV_039	47,570,583	29,374,685	61.7	12,582,387	26.4	5,613,511	11.8
	OV_040	45,444,974	23,954,420	52.7	15,300,730	33.7	6,189,824	13.6
	OV_041	48,814,285	21,286,959	43.6	19,352,824	39.6	8,174,502	16.7
	OV_042	47,089,054	20,648,253	43.8	16,521,171	35.1	9,919,630	21.1
	OV_043	42,220,337	21,103,783	50.0	13,104,835	31.0	8,011,719	19.0
	OV_044	52,926,334	14,525,262	27.4	15,212,109	28.7	23,188,963	43.8
	OV_045	45,115,267	19,171,780	42.5	16,563,002	36.7	9,380,485	20.8
	OV_046	47,325,198	20,132,053	42.5	18,577,464	39.3	8,615,681	18.2
	OV_047	57,226,451	22,566,315	39.4	24,019,850	42.0	10,640,286	18.6
	OV_048	47,427,666	24,909,330	52.5	13,245,767	27.9	9,272,569	19.6
	OV_049	45,081,582	21,154,435	46.9	18,809,399	41.7	5,117,748	11.4
	OV_050	40,987,704	20,585,424	50.2	13,524,106	33.0	6,878,174	16.8
	OV_051	53,327,179	23,541,458	44.1	21,345,069	40.0	8,440,652	15.8
	OV_052	44,591,388	18,274,602	41.0	18,327,786	41.1	7,989,000	17.9
	OV_053	38,109,581	18,381,164	48.2	15,200,087	39.9	4,528,330	11.9
	OV_054	43,377,479	17,968,673	41.4	14,981,000	34.5	10,427,806	24.0
	OV_055	41,393,592	16,264,905	39.3	14,124,946	34.1	11,003,741	26.6
	OV_056	44,861,398	20,877,080	46.5	16,303,070	36.3	7,681,248	17.1
	OV_057	37,612,212	20,041,638	53.3	13,372,853	35.6	4,197,721	11.2
	OV_058	33,276,817	16,437,532	49.4	11,181,260	33.6	5,658,025	17.0
	OV_059	43,637,750	21,335,858	48.9	15,813,173	36.2	6,488,719	14.9
	OV_060	36,964,466	13,699,585	37.1	11,515,327	31.2	11,749,554	31.8

Supplementary Table S6. Summary of sequencing reads in ovarian samples

Tissue Type	ID	Total	Uniquely Mapped	Uniquely Mapped Rate (%)	Multiple Mapped	Multiple Mapped Rate (%)	Non-Mapped	Non-Mapped Rate (%)
Malignant	OV_061	45,547,326	21,272,716	46.7	17,070,881	37.5	7,203,729	15.8
	OV_062	45,691,543	24,845,261	54.4	15,624,408	34.2	5,221,874	11.4
	OV_063	35,071,467	15,426,963	44.0	14,857,596	42.4	4,786,908	13.6
	OV_064	45,632,269	26,575,261	58.2	13,224,260	29.0	5,832,748	12.8
	OV_065	53,388,183	25,281,602	47.4	18,647,744	34.9	9,458,837	17.7
	OV_066	37,847,644	18,026,200	47.6	14,486,340	38.3	5,335,104	14.1
	OV_067	54,535,847	23,665,187	43.4	19,023,738	34.9	11,846,922	21.7
	OV_068	42,464,680	19,168,300	45.1	18,102,315	42.6	5,194,065	12.2
	OV_069	51,445,347	27,277,370	53.0	15,897,575	30.9	8,270,402	16.1
	OV_070	31,611,270	12,271,064	38.8	12,381,896	39.2	6,958,310	22.0
	OV_071	35,111,975	19,169,682	54.6	12,745,469	36.3	3,196,824	9.1
	OV_073	49,453,601	23,617,311	47.8	19,270,095	39.0	6,566,195	13.3
	OV_074	52,513,597	15,321,899	29.2	17,282,950	32.9	19,908,748	37.9
	OV_075	35,076,332	17,847,059	50.9	13,117,957	37.4	4,111,316	11.7
	OV_076	50,085,339	22,051,134	44.0	19,789,039	39.5	8,245,166	16.5
Benign	OV_101	35,982,439	18,905,550	52.5	13,199,791	36.7	3,877,098	10.8
	OV_102	37,199,837	19,162,457	51.5	14,375,109	38.6	3,662,271	9.8
	OV_103	40,955,993	18,611,600	45.4	16,769,945	40.9	5,574,448	13.6
	OV_104	49,336,358	18,582,516	37.7	19,303,868	39.1	11,449,974	23.2
	OV_105	44,648,838	20,856,473	46.7	17,216,106	38.6	6,576,259	14.7
	OV_106	43,434,489	21,231,549	48.9	15,480,300	35.6	6,722,640	15.5
	OV_107	35,258,170	19,131,147	54.3	12,863,735	36.5	3,263,288	9.3
	OV_108	51,700,565	18,560,364	35.9	15,081,519	29.2	18,058,682	34.9
	OV_109	44,144,137	16,732,836	37.9	17,254,924	39.1	10,156,377	23.0
	OV_110	47,173,294	21,358,552	45.3	19,678,147	41.7	6,136,595	13.0
	OV_111	42,797,234	19,197,950	44.9	18,308,895	42.8	5,290,389	12.4
	OV_112	39,499,697	17,787,453	45.0	16,179,682	41.0	5,532,562	14.0
	OV_113	91,347,082	40,621,084	44.5	34,398,620	37.7	16,327,378	17.9
	OV_114	48,934,315	22,371,215	45.7	17,272,307	35.3	9,290,793	19.0
	OV_115	48,485,468	22,177,750	45.7	15,400,110	31.8	10,907,608	22.5
	OV_116	48,740,101	21,767,413	44.7	19,236,766	39.5	7,735,922	15.9
	OV_117	45,005,117	19,214,791	42.7	18,074,570	40.2	7,715,756	17.1
	OV_118	43,338,394	19,834,840	45.8	15,785,507	36.4	7,718,047	17.8
	OV_119	41,904,360	18,953,302	45.2	17,008,211	40.6	5,942,847	14.2
	OV_120	44,175,554	23,142,617	52.4	15,266,390	34.6	5,766,547	13.1
Normal	OV_151	49,895,597	24,241,977	48.6	18,773,542	37.6	6,880,078	13.8
	OV_152	45,559,845	22,086,025	48.5	16,526,544	36.3	6,947,276	15.2
	OV_153	74,636,322	31,665,863	42.4	26,905,305	36.0	16,065,154	21.5
	OV_154	44,601,004	18,729,283	42.0	14,977,864	33.6	10,893,857	24.4
	OV_155	59,785,662	28,932,633	48.4	19,958,773	33.4	10,894,256	18.2
	OV_156	29,422,662	13,900,866	47.2	10,397,452	35.3	5,124,344	17.4

Supplementary Table S7. The summary of primers for real-time quantitative reverse transcription PCR and bisulfite pyrosequencing

Gene Name	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	PCR size (bp)
For qRT-PCR			
ADAMTS5	GCCAGCGGATGTGTGCAAGC	ACACTTCCCCCGGACGCAGA	130
AK1	GGGATGCCATGGTGGCCAAA	ACAGCAGCAGTGTGGGCTGTC	120
CCDC17	GTGCAGTGGCTGCGGCTATC	CGCCTCGGACATGGGACTCT	120
FGF12	CTCGGGGTGTTCAGCAAAGTGCG	TGTCACAATCCCTTTGAGCTGGGG	90
FOXA1	TGGAAGGGCATGAAACCAGCGAC	TGTTGCTGACCGGGACGGAG	81
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	87
GLI1	TCAACTCGATGACCCCACC	AGACAGTCCTTCTGTCCCCACA	105
GLI2	CACTCCAACGAGAAACCCTAC	CTGCTTCTTGGTGACGTGG	126
GLI3	AGGACCTCAGCAACACTACC	TGCATGAAGACTGACCACCA	111
HNF1B	CGGCCTGAACCAGTCGCACC	TGGACTGTCTGGTTGAATTGTCGGA	135
HOXB5	CCACGCACTCTGCCTGTCCG	CGCTGCCAGCTGTAGCCAGG	113
IRS1	CTCCACCCGCCGCTCAAGTG	GGTCTTCATTCTGCTGTGATGTCCA	110
PHF2	CCCACGCCTGTCACGAAGCC	GTGCAGCGAACCCTCGTCCG	78
PLEKHG2	AACCACCCTGCCTCCATCCCTG	TTTGGGGGCACAATGCAGGCT	72
SPON2	CCTTCATCCCTCCCGCCCCA	CCTCCGCACAGTCCCCAGGA	124
SSBP4	GAGCACTCCGGCGAGGCCAA	CCTGGAAGAAGCCAGCCGCCAT	127
ZIC1	TTCGCGCGCTCCGAGAATTTA	GCTTCTTGCGGTCGCTGCTGTTA	118
ZIC2	CGAGAACCTCAAGATCCACAAAA	CGTGCATGTGCTTCTTCCTGT	116
ZIC4	GCTCTGGCTACGATTCGGCTACA	CGGTGGACATCATTCGCTCAA	131
ZIC5	GTACTCATACAGGGGAAAAGCC	GGTGTGGACATGGGAATGTTT	101
For bisulfite pyrosequencing			
ZIC1_py11	GTTGAATAGGGATATTTTGGAAGTGT	#ACTTTTCCTTCTCACAATCATATACAT	183
ZIC1_py11 sequencing primer		TTTTTGTTGTGTTTATTAGTTTTAT	

The 5'-end biotinylated primer. The annealing tempature is 60℃.