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Supplemental Data

GSK-3β Targets Cdc25A for Ubiquitin-Mediated Proteolysis, and GSK-3β Inactivation Correlates with Cdc25A Overproduction in Human Cancers

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SUPPLEMENTARY FIGURE 1

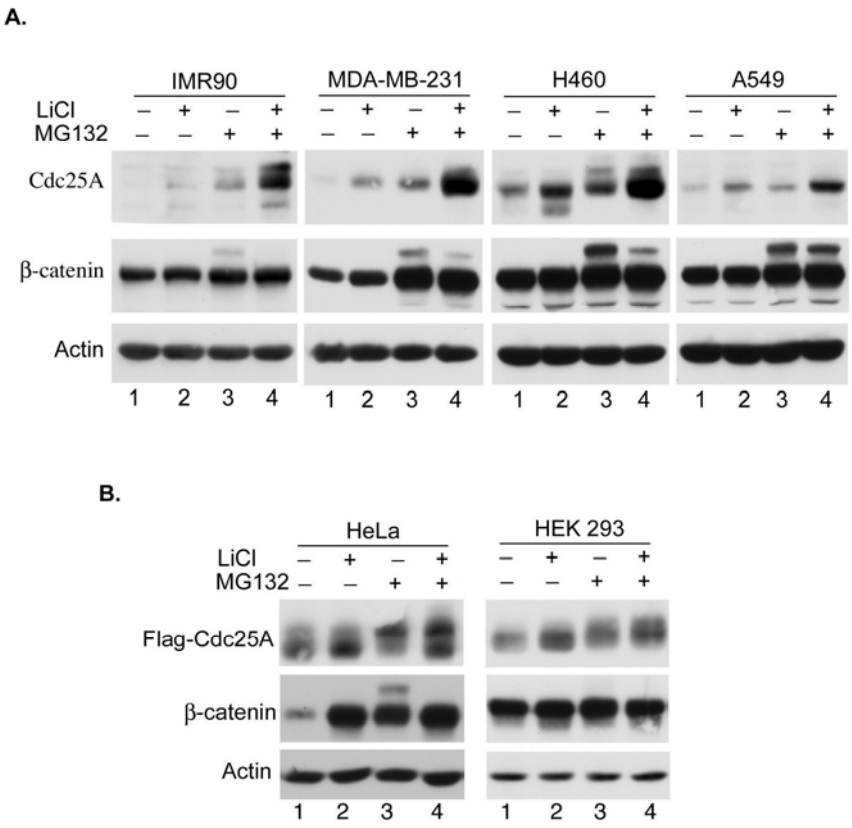
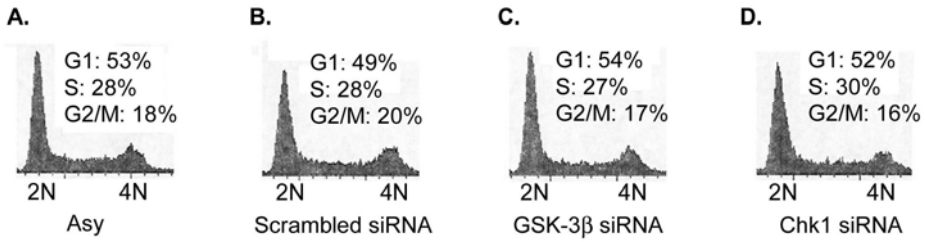


Figure S1. Cdc25A protein levels are negatively regulated by GSK-3β *in vivo*.

(**A**) The indicated cells were untreated or were incubated with 10 mM LiCl, 10 μM MG132 or both for 4 h. The indicated proteins were analyzed by Western blotting. (**B**) HeLa and HEK293 cells, transfected with Flag-Cdc25A were treated and analyzed as described in (**A)**.

SUPPLEMENTARY FIGURE 2



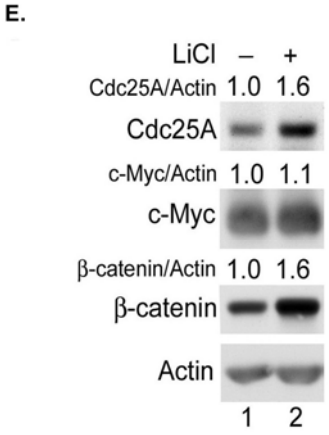


Figure S2. Effects of GSK-3β inhibition on cell cycle and Myc protein levels. (**A-D**) HeLa cells were untreated or were transfected with control siRNAs (scrambled) or siRNAs specific for GSK-3β or Chk1 for 48 h. Cells were stained for DNA content and analyzed by flow cytometry, n = 3. (**E**) HeLa cells were untreated or were incubated with 10 mM LiCl for 4 h (n=2). Relative levels of Cdc25A, Myc, and β-catenin were determined from the Western blot using ImageJ program.

SUPPLEMENTARY FIGURE 3

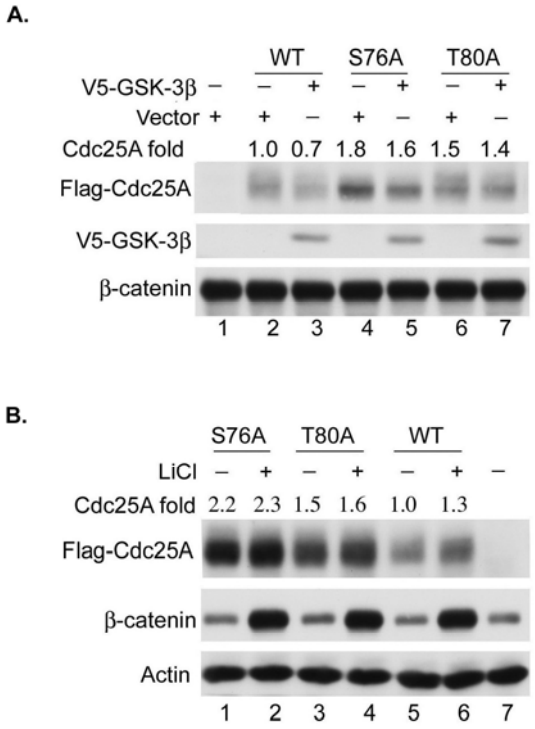


Figure S3. Phosphorylation of Cdc25A by GSK-3β regulates its stability.

(**A**) U20S cells were transfected with vector alone (lane 1) or with vector and plasmids encoding wild-type or mutant forms of Cdc25A (lanes 2, 4, 6) or with plasmids encoding various forms of Cdc25A together with V5-GSK-3β (lanes 3, 5, 7) for 24 h (n=2). Lysates were analyzed by Western blotting. Relative levels of Cdc25A were determined from the Western blot using chemiluminescence on a STORM imager. (**B**) U20S cells transfected with wild-type and mutant forms of Cdc25A for 20 h were cultured in the absence and presence of 10 mM LiCl for 4 h (n=2). Lysates were prepared and proteins were analyzed as described in (**A**).

SUPPLEMENTARY FIGURE 4

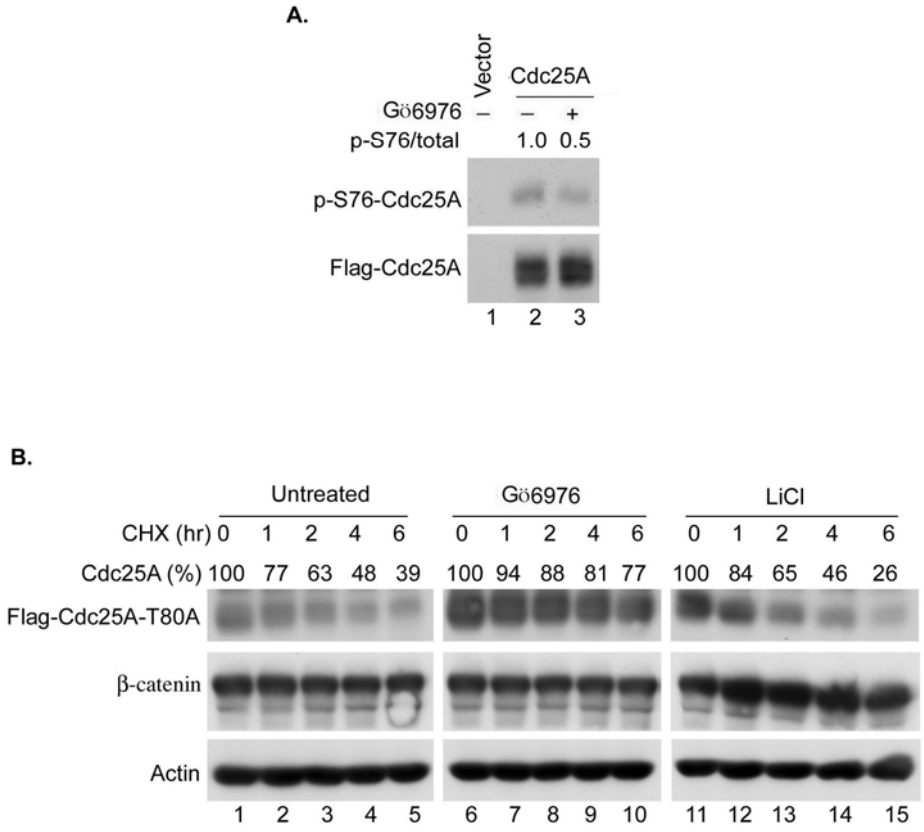


Figure S4. Regulation of Cdc25A (T80A) mutant by Chk1. (**A**) Immortalized GSK-3β null MEFs transfected with vector (lane 1) or with plasmid encoding Flag-Cdc25A (lanes 2, 3) for 20 h were incubated with 10 μM MG132 in the absence (lanes 1, 2) or presence (lane 3) of 100 nM Gö6976 for 4 h (n=2). Lysates were prepared and incubated with Flag agarose and precipitates were analyzed by Western blotting. (**B**) Asynchronously growing U2OS cells were transfected with Flag-Cdc25A(T80A) for 18 h and followed by incubation with vehicle (lanes 1-5), 100 nM Gö6976 (lanes 6-10) or 10 mM LiCl (lanes 11-15) for 2 h, and then 10 μg/ml cycloheximide (CHX) was added for indicated times. Lysates were prepared and analyzed by Western blotting.

SUPPLEMENTARY FIGURE 5

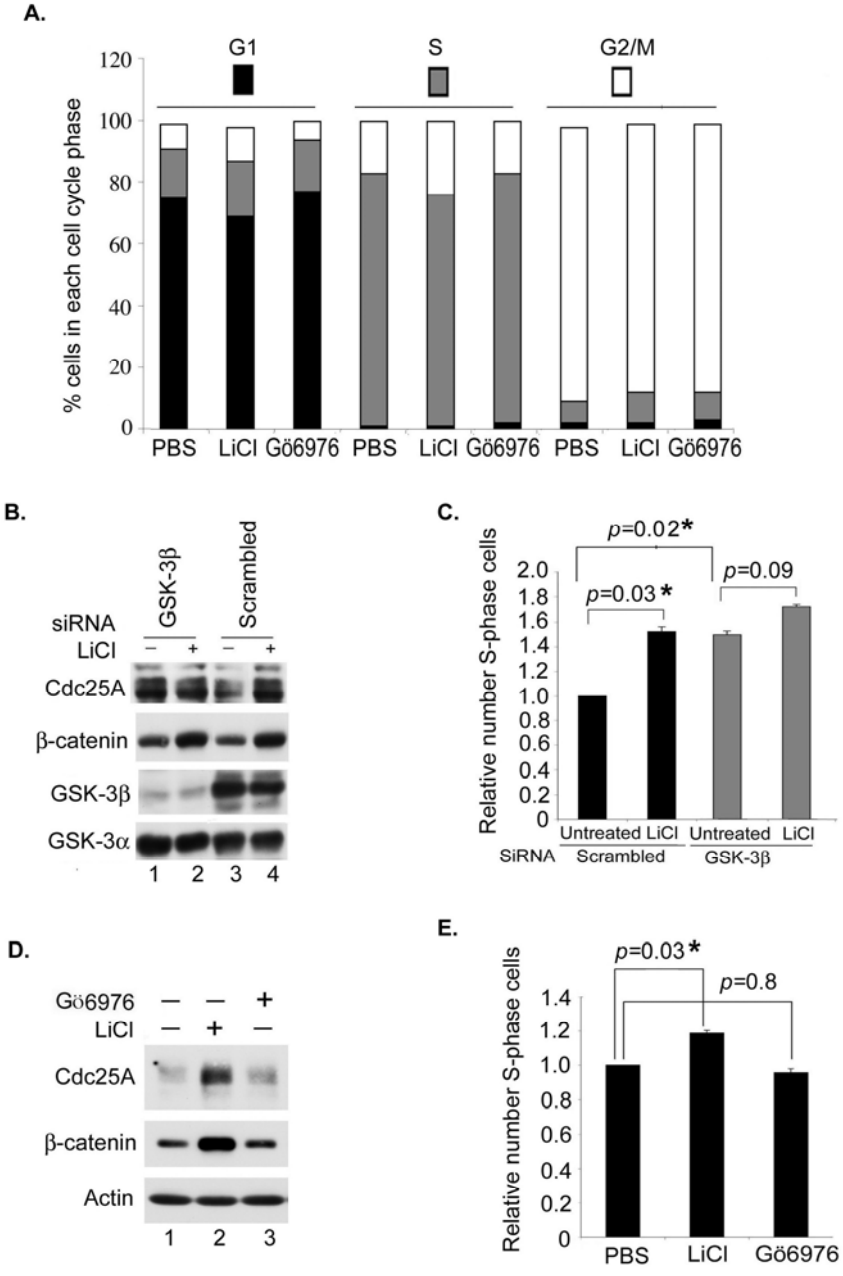


Figure S5. Cell Cycle Regulation of Cdc25A by GSK-3β.

(**A**) HeLa cells synchronized at the G1/S border using a double thymidine block and release protocol were released for 2.5 h (S phase, lanes 4-6), 6 h (G2 phase, lanes 7-9) or 11 h (G1 phase, lanes 1-3). Cells were incubated with either PBS, 20 mM LiCl, or 100 nM Gö6976 for 2 h prior to harvesting. Cells were analyzed by flow cytometry. (**B, C**). HeLa cells were transfected with control siRNAs or siRNAs specific for GSK-3β for 24 h. Cells were synchronized at the G1/S-border by a double-thymidine block and release protocol. Synchronized cells were untreated or were incubated with 20 mM LiCl or 100 nM Gö6976 immediately after release from the block. One hour later, cells were cultured in the presence of BrdU and harvested one hour later. Cells were stained with PI and for BrdU. The percentage of BrdU positive cells was determined by flow cytometry. A two-tailed t-test was performed for comparisons between groups. Standard error of the mean (SEM) for triplicate samples is shown as error bars along the y axis in panel C. * indicates statistically significant. A fraction of the cells were also lysed for Western blotting, n = 3 (**B**). (**D, E**) HeLa cells were synchronized in M-phase by incubation in nocodazole. Cells were released from the block for 9 h to allow them to enter G1 and were then treated with PBS, LiCl or Gö6976 for 1h followed by a 1h BrdU pulse. Cdc25A levels were monitored by Western blotting (**D**) and cells were stained with PI and for BrdU. The percentage of BrdU positive cells was determined by flow cytometry (**E**). Results were analyzed as described in (**C**), n= 3. A two-tailed t-test was performed for comparisons between groups. Standard error of the mean (SEM) for triplicate samples is shown as error bars along the y axis. * indicates statistically significant.

SUPPLEMENTARY FIGURE 6

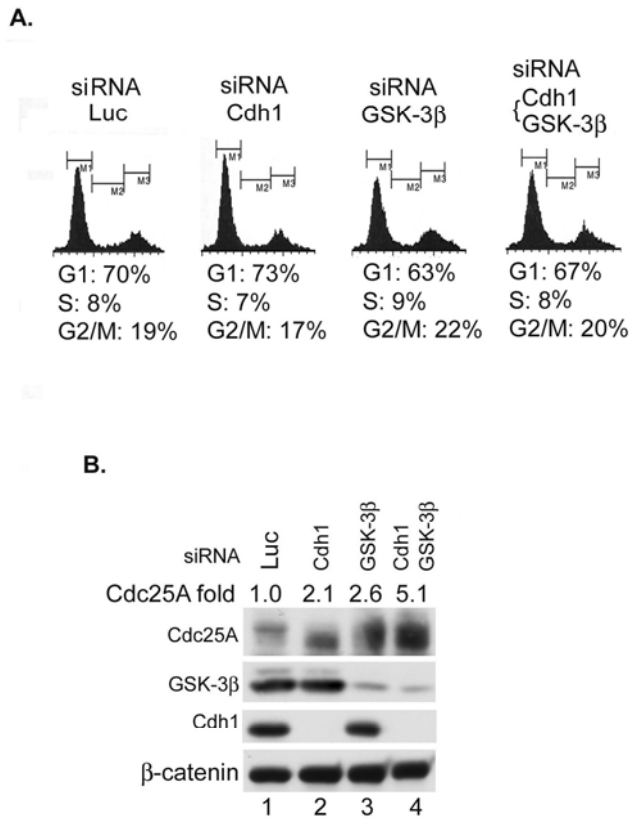


Figure S6. Regulation of Cdc25A in G1 phase by GSK-3β and Cdh1

HeLa cells transfected with luciferase siRNAs or siRNAs specific for GSK-3β and/or Cdh1 for 36 h were synchronized in M-phase by incubation in nocodazole (n=2). Cells were released from the block for 8 h and collected for analysis by flow cytometry (**A**) and Western blotting (**B**).

SUPPLEMENTARY FIGURE 7

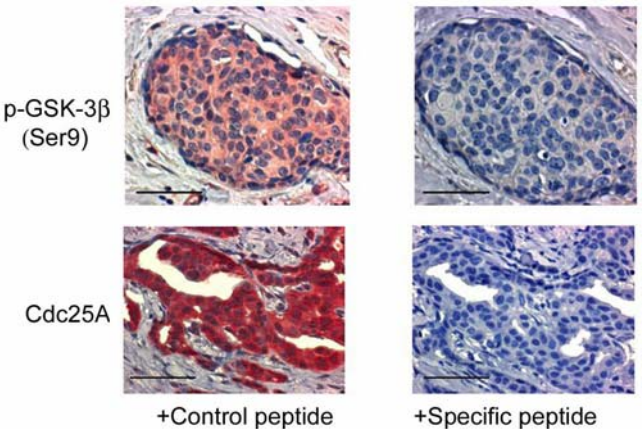


Figure S7. Specificity of p-GSK-3β(Ser9) and Cdc25A antibodies.

Representative immunohistochemical staining of p-GSK-3β(Ser9) (top panels) and Cdc25A (bottom panels) together with their control peptides (left panels) or specific peptides (right panels) in human breast carcinoma tissue using sequential slides. Scale bar: 50 μm

SUPPLEMENTARY TABLE S1

Table S1. Distribution of Cdc25A and p-GSK-3β expression in different cancer types in multiple cancerous tissues microarrays.

		Cdc25A	
Cancer Type		Non-overexpression	Overexpression
	p-GSK-3β		
Oral Cavity			
	Negative	2	0
	Positive	0	3
Nasopharynx			
	Negative	2	3
	Positive	0	0
Salivary Gland			
	Negative	1	5
	Positive	0	9
Esophagus			
	Negative	0	0
	Positive	0	3
Stomach			
	Negative	6	18
	Positive	1	9
Small Intestine			
	Negative	2	2
	Positive	0	1
Colorectum			
	Negative	8	13
	Positive	1	4
Liver			
	Negative	9	4
	Positive	0	3
Gallbladder			
	Negative	2	2
	Positive	0	2
Pancreas			
	Negative	1	4
	Positive	0	0
Larynx			
	Negative	0	7
	Positive	0	6
Lung			
	Negative	4	3
	Positive	0	2
Total		39	103

SUPPLEMENTARY TABLE S2 and S3

Table S2. Correlation between Chk1 and Cdc25A expression in multiple cancerous tissues microarrays.

	Cdc25A		
	Non-overexpression	Overexpression	P-value
Chk1			
Negative	13	21	
Positive	22	67	
			0.18

Table S3. Correlation between Cdh1 and Cdc25A expression in multiple cancerous tissues microarrays.

	Cdc25A		
	Non-overexpression	Overexpression	P-value
Cdh1			
Negative	21	60	
Positive	18	43	
			0.636