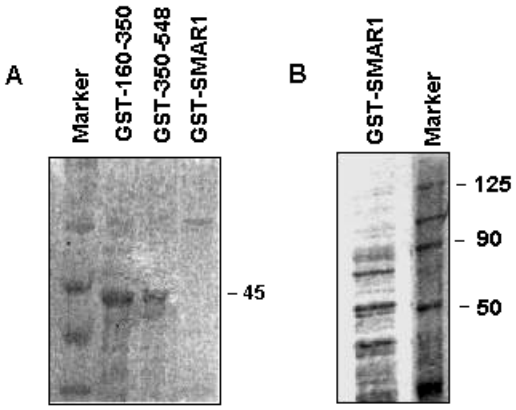
SUPPLEMENTARY DATA
(Rampalli et al.)



Supplementary Figure 1A & B; Rampalli et. al.

Figure 1: (A) Expression of GST-SMAR1 and GST tagged SMAR1 truncations were verified by Coomassie staining. **(B)** In pull down assay various proteins obtained with GST -SMAR1 versus GST alone is visualized by Coomassie staining.

EMSA:

Radiolabelling (for probe IV and probe II) and EMSA reactions were performed essentially as described in Material and methods section. For protein saturation analysis various concentrations of GST-SMAR1 (0.1 μg to 1 μg) was incubated with radiolabelled probe IV in presence of 0.5 μg of poly (dI-dC). Affinity of SMAR1 to probe IV was determined by competition experiments using unlabelled probe IV.

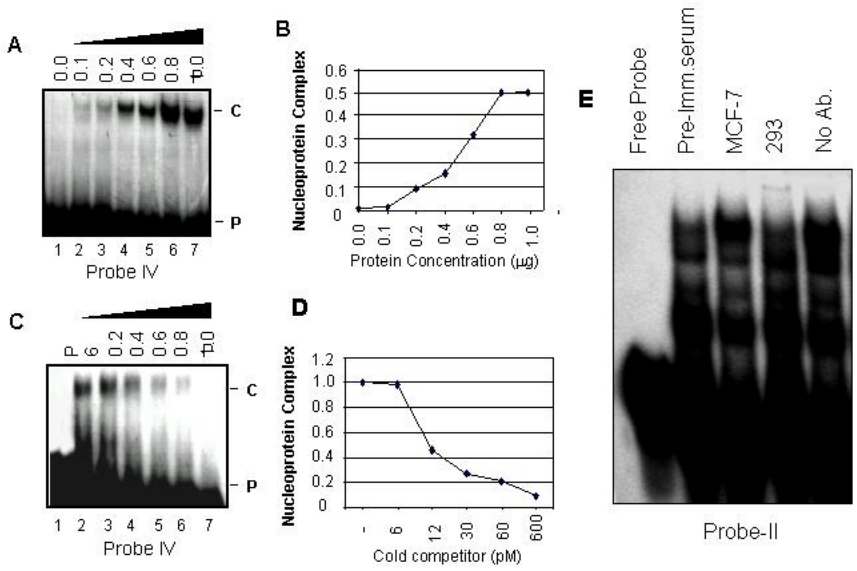
EMSAs using probe II was performed using 10 μg of MCF-7 and 293 cell lysate. Cell lysate was incubated with probe II for 15 minutes with 0.5 μg of poly (dI-dC). Super

shift assays were performed by addition of either control (control-immune sera) or SMAR1 antibody to the reaction mixture. DNA-Protein complexes were resolved on 8% polyacrylamide gel, dried and processed for autoradiography.

Results

To determine the affinity of SMAR1 to probe IV region, the complex obtained by using saturating amount of protein was competed out serially using increasing concentrations (molar excess) of unlabelled probe as specific competitor and the affinity documented (Kd = 0.48 nM-1.4 nM).

After confirming the binding of recombinant GST-SMAR1 to probe II region of Cyclin D1 promoter, we performed super shift assays for SMAR1 binding using MCF-7 and 293-cell lysate. SMAR1 super shifted complex was observed in case of 293 lysate for probe II while MCF-7 failed to show supershift using SMAR1 antibody. Control reaction with control immune sera failed to show any supershifted complex. These results further prove that endogenous SMAR1 binds to Cyclin D1 promoter.



Supplementary Figure 2A-E; Rampalli et. al.

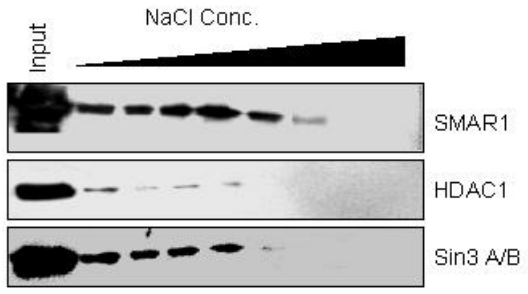
Figure 2. Affinity determination of probe IV to SMAR1. **(A)** Protein saturation analysis was done by incubating probe IV to various amount of GST-SMAR1 as indicated in the figure **(B)** and the saturation curve was plotted after densitometric anaysis. **(C)** Competition assays were performed using molar excess of cold probe IV as indicated in the figure **(D)** and the densitometric analysis was plotted against nucleoprotein complexes versus the concentration of cold probe. **(E)** Endogenous SMAR1 from 293 cell lysate binds to probe II region, control indicates the preimmune sera in a reaction of 293 cell lysate that does not show any binding with probe. Arrow indicates the super shifted band with SMAR1 antibody.

Immunoaffinity Purification

SMAR1 antibody was coupled to CNBr beads (Amersham) as per manufacturer's instructions and 293 cell lysate was passed through the column. SMAR1 and the bound proteins were eluted using various NaCl concentrations (100 mM to 1.5 M). The resultant fractions were subjected to western blot analysis for detection of SMAR1, HDAC1 and Sin 3A/B [Kellogg, D.R. and Moazed,D. Methods enzymology (2002) 351: 172-83;Vasilescu et al. Proteomics (2004), Dec 4 (12); Jack et al. Mol.Biotechnology (1994) Feb1 (1) 5986].

Result

To verify that SMAR1 is indeed associated with HDAC1 and SIN3 complex, we eluted SMAR1 associted complex from 293 cells by passing the lysate to SMAR1 antibody bound CNBr column. SMAR1 was eluted at the concentration from 100 mM to 1 M NaCl while HDAC1 and SIN3 proteins were coeluted at the concentration from 100 mM to 500 mM NaCl. Thus these results further supports that SMAR1 interacts with HDAC1 and SIN3 complex.



Supplementary Figure 3; Rampalli et. al.

Figure 3. Immunoaffinity purification of SMAR1 and associated proteins. SMAR1 and associated protein were eluted at various NaCl concentrations (lane 1- Input, from lane 2-

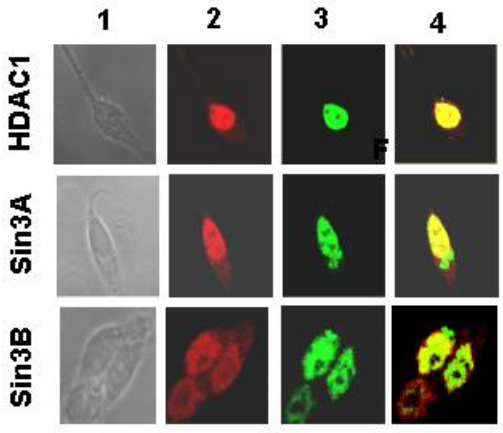
9, 100 mM to 1.5 M Nacl concentration). The eluted fractions were subjected to western analysis using anti-SMAR1, HDAC1 and Sin3A/B antibodies.

Immunofluorescence

For immunofluorescence, cells were grown on coverslip and transiently transfected with GFP-SMAR1. Twenty four hours post transfection; cells were fixed with 2% parafarmaldehyde and stained for HDAC1, Sin3A and Sin3B. Anti-mouse CY3 secondary antibody was used (Sigma). The cells mounted in anti-Fade (Sigma) were visualized with confocal laser microscope (LSN 510, version 2.01; Zeiss, Thornwood, NY).

Result:

By confocal microscopy nuclear colocalization of endogenous HDAC1, Sin3A and Sin3B with GFP-SMAR1 was detected.



Supplementary Figure 4; Rampalli et. al.

Figure 4. Nuclear colocalization of GFP-SMAR1 with HDAC1, Sin3A, and Sin3B were detected by confocal studies as indicated.

Flow Cytometry

MCF-7, 293 and HBL-100 cells were transiently transfected with either Flag-vector or Flag-SMAR1 and harvested at various time points. After trypsinization cells were washed with 1X PBS and fixed in 70% ice-cold ethanol. Cells were washed and treated with RNase A (75 U/ml) and resuspended in PBS containing 50 mg/ml Propidium Iodide (PI). PI stained cells were analyzed for cell cycle profiles by FACS Vantage (Becton Dickinson, Mountain View, CA) using Cell Quest.

Results

Cyclin D and E are the two G1 class Cyclins that are activated at G1/ S phase of the cell cycle. Downregulation of these two Cyclins have largely been correlated to G1/S phase arrest. Our studies showed a specific downregulation of Cyclin D1 and Cyclin E to a lesser extent. Therefore, we analyzed the effect of overexpression of SMAR1 on cell cycle in MCF-7 cells. As Cyclin D1 downregulation was pronounced from 24 h to 48 h, cell cycle analysis was carried out at the above indicated time points. Cell cycle was performed in asynchronous culture in accordance to our earlier studies. A time dependent increase in cell number at G1/S phase was observed. At 24 h, SMAR1 over expressing

cells showed more number of cells in G1 phase; 73 % cells in SMAR1 transfected compared to 66% cells in mock. Number of cells in S-phase also increased from 18% in mock transfected to 21 % in SMAR1 transfected cells. Thus collectively 9% of cells were seen arrested at G1/S phase of cell cycle at 24 h. However 7% population of the cells were seen in G1 phase at 36 h as indicated in the table. At 48 h only 3% cells were arrested at G1 phase, indicating re-entry of the arrested cells into cell cycle. Thus, SMAR1 overexpression causes transient cell cycle arrest in G1/S phase at 24 h in MCF-7 cell line.

As downregulation of Cyclin D1 was also seen in 293 cells, we performed a similar time course FACS analysis in 293 cell line. In contrast to MCF-7 where cells were seen arrested both in G1 and S phase, a time dependent increase in G0-G1 arrseted cells were observed in 293 cells. 7-12 % of cells were seen increased in G0-G1 phase in Flag-SMAR1 transfected cells compared to mock transfected at 24 h and 36 h respectively (Figure 2 C and D). While at 48 h only 4 % of cells were observed in G0-G1 phase in Flag-SMAR1 transfected cells compared to mock with increase in S phase population, indicating re-entry of arrested cells to cell cycle. Thus SMAR1 overexpression in 293 cells caused G0-G1 arrest that was pronounced at 24 and 36 h respectively. Thus in both 293 and MCF-7 cells G1 arrest was prominent although few % cells were seen to be arrested in S phase only in MCF-7. Cell cycle analysis in HBL-100 cell line upon Flag-SMAR1 transfection did not cause any arrest like MCF-7 and 293 (Figure 2 E and F) cells.

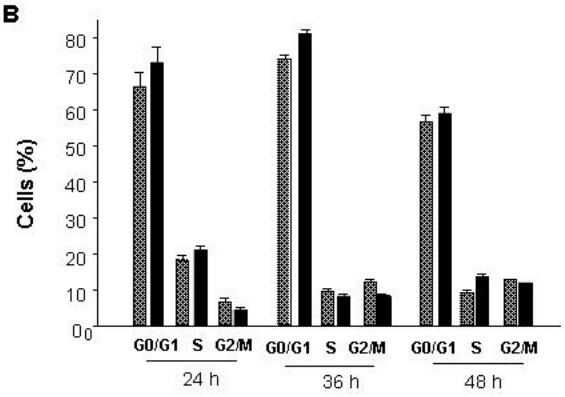
The cell lines used for the study have differential status of Rb [MCF-7 (wild type), 293, HBL-100 (inactive) and MDA MB-468 (null)] irrespective of which SMAR1

downregulated Cyclin D1. This suggests that Rb is dispensable for SMAR1 mediated Cyclin D1 repression. Functional significance of SMAR1 mediated Cyclin D1 repression and cell cycle arrest remain to be elucidated in Rb inactive lines since 293 and HBL-100 behaved differently.

A

Plasmids	Hours	1			2			3			Average (%)		
		G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
Flag	24	70	17	8	67	19	6	62	19	6	66.3	18.3	6.6
Flag-SMAR1		76	20	4	75	22	5	68	21	5	73	21	4.6
Flag	36	75	10	12	74	10	12	73	9	13	74	9.6	12.3
Flag-SMAR1		82	9	8	81	8	8	80	8	9	81	8.3	8.3
Flag	48	58	9	14	55	9	13	57	10	14	56.6	9.3	13.6
Flag-SMAR1		60	12	13	57	12	13	60	12	13	59	12	13

B

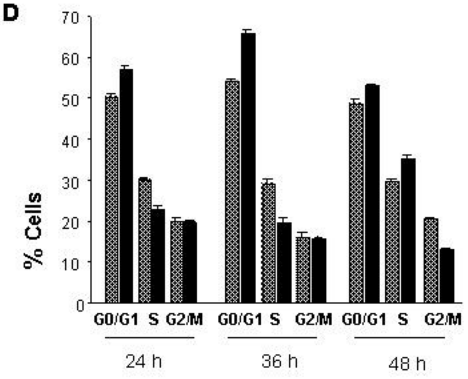


Supplementary Figure. 5A & B; Rampalli et. al.

C

Plasmids	Hours	1			2			3			Average (%)		
		G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
Flag	24	50	30	18	49	30	20	50	30	20	49.6	30	19.3
Flag-SMAR1		57	24	19	58	22	20	56	23	20	57	23	19.6
Flag	36	54	30	15	54	28	17	53	29	15	53.6	29	15.6
Flag-SMAR1		66	18	15	65	21	16	66	19	15	65.6	19.3	15.3
Flag	48	49	29	20	49	30	20	47	30	21	48.3	29.6	20.3
Flag-SMAR1		52	35	13	53	35	13	53	34	13	52.6	34.6	13

D

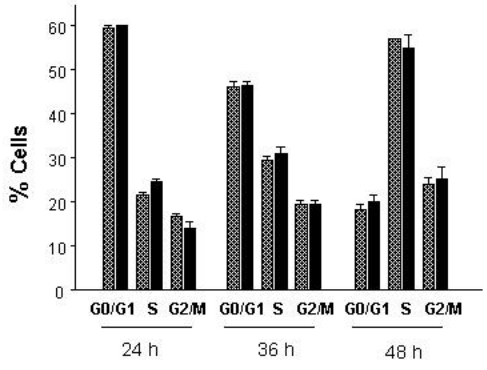


Supplementary Figure. 5C & D; Rampalli et. al.

E

Plasmids	Hours	1			2			3			Average (%)		
		G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
Flag	24	60	21	16	60	22	17	59	21	16	59.6	21.3	16.3
Flag-SMAR1		61	24	13	60	25	15	60	23	15	60.3	24	14.3
Flag	36	45	30	20	47	29	20	44	29	19	45.3	29.3	19.6
Flag-SMAR1		47	30	19	47	32	20	46	30	20	46.6	30.6	19.6
Flag	48	17	57	25	19	57	23	19	58	23	18.3	57.3	23.6
Flag-SMAR1		19	57	23	20	55	24	19	54	27	19.3	55.3	24.6

F



Supplementary Figure. 5E & F; Rampalli et. al.

Figure 5. FACS analysis of MCF-7 cells transfected with SMAR1. **(A)** MCF-7 cells were analyzed by FACS at various time points. Transfections are indicated at the left top

of the panel. The percentage of cells in each stage of cell cycle is represented in the table. **(B)** Bar graph representing the percentage of cells at various phase of cell cycle in time course experiment. **(C)** 293 cells were analyzed by FACS at various time points. Transfections are indicated at the left top of the panel. The percentage of cells in each stage of cell cycle is represented in the table. **(D)** Bar graph representing the percentage of cells at various phase of cell cycle in time course experiment. **(E)** HBL-100 cells were analyzed by FACS at various time points. Transfections are indicated at the left top of the panel. The percentage of cells in each stage of cell cycle is represented in the table. **(F)** Bar graph representing the percentage of cells at various phase of cell cycle in time course experiment.