CHAPTER 2

Coordinated Regulation of p53 Apoptotic Targets by Chromatin Remodelling protein SMAR1 through an Identical MAR Element

Fig: Open chromatin (yellow nucleosomes) are flanked by MARs/insulators (pink, blue and green spheres) that interact together to form a loop. Dorman et al., 2007

If you cannot think the absurd, you cannot do the impossible.  
-Albert Einstein
2.1 Rationale of the study

Preliminary studies from our lab have demonstrated SMAR1 as a p53 target gene and is induced upon DNA damage in a p53 dependent manner (Singh et al., 2007). Also, SMAR1 has been shown to interact with p53 and stabilize it in the nucleus by displacing its negative regulator mdm2 (Jalota et al., 2005). SMAR1 is a nuclear matrix binding transcription factor that functions as a repressor by recruiting HDAC1 (Rampalli et al., 2005). The tumor suppressor p53 also binds to the nuclear matrix in normal and stressed cells under conditions of DNA damage, but the nuclear matrix associated function of p53 is not known. Since SMAR1 and p53 are nuclear matrix associated DNA binding transcription factors, and both interact with each other, it is possible that they may influence the transcriptional activities of each other or even together as a transcriptional complex can modulate a core set of genes that are independently regulated by either SMAR1 or p53. This is important considering the fact that both SMAR1 and p53 regulates plethora of genes involved in cell-cycle and apoptosis. As tumor suppressor p53 acts as a nodal molecule in sensing DNA damage and governing cellular life or death response, it is important to understand how p53 is signaled to dictate whether a cell will survive or will undergo apoptosis. Upon mild DNA damage, p53 induces cellular arrest and initiates repair pathways to allow re-entry of damaged cells into cell-cycle. On the other hand after severe DNA damage, which is beyond the scope of cells to repair, p53 amplifies the apoptotic cascade to ensure efficient killing of damaged cells to maintain genomic integrity. Therefore, our objective was to decipher how SMAR1 can alter p53 activity and thereby modulate p53 dependent cell arrest and death mechanisms when cells are inflicted with mild and severe doses of DNA damage.

2.2 Background literature

The tumor suppressor p53 is the cellular sentinel of the mammalian cell-cycle and an indispensable component of the DNA damage response pathway. Activation of p53 in response to DNA damage results in either cell-cycle arrest or apoptosis. Although
genes that regulate cellular processes like arrest and apoptosis are essentially p53 targets, activation of p53 always results in specific and selective transcription of p53 regulated genes (Riley et al., 2008). Thus, it is likely that unique sets of p53 regulated genes operate in tandem to bring about a desired outcome in response to specific stimuli. But how p53 executes these two distinct functions in a promoter specific manner remains largely unclear. Recent reports suggest that activation of specific promoters by p53 is achieved through its interaction with heterologous transcription factors like Hzf, hCAS/CSE1L and ASPP family proteins (Das et al., 2007; Tanaka et al., 2007; Samuels-Lev et al., 2001). Also, under conditions of stress, different phosphorylation and acetylation modules stabilize p53 enhancing its sequence specific DNA binding and transcriptional activity (Sakaguchi et al., 1998). While phosphorylation of p53 at Ser15 and Ser20 are important for p53 stability, acetylation of p53 by co-activators like p300, CBP, PCAF and Tip60 is indispensable for p53 dependent activation of apoptotic targets BAX and PUMA (Sykes et al., 2006). The biological significance of p53 acetylation in regulating cell fate decisions stems from the fact that loss of acetylation impairs the ability of p53 to induce pro-apoptotic targets BAX and PUMA (Tang et al., 2008). Thus, the role of heterologous chromatin modulators and p53 co-activators assumes importance in context to p53 regulation.

2.2.1 Partners of p53 in life and death

Phosphorylation and acetylation of p53 leads to p53 stability, nuclear accumulation, increased DNA binding activity and enhanced transcriptional activation of p53 downstream targets. Activation of p53 in response to DNA damage results in either cell-cycle arrest or apoptosis depending upon the severity of DNA damage. How does p53 knows which genes to turn “on” or “off” to attain a desirable outcome has been a focus of intensive research over the last few years (Harris and Levine, 2005). It is likely that unique sets of p53 regulated genes operate in tandem to bring about a desired outcome in response to specific stimuli. How p53 executes these two distinct functions in a promoter specific manner remains largely unclear. Recent reports
suggest that activation of specific promoters by p53 is achieved through its interaction with heterologous transcription factors. It is speculated that specific DNA or chromatin binding proteins interacts with p53 and instructs p53 to activate growth inhibitory genes in response to limited (mild) damage and pro-apoptotic genes in case of irreparable (severe) damage (Figure 8). This hypothesis got a boost with two recent publications. One group reported that zinc finger protein Hzf, which is itself a p53 target gene directly interacts with p53 DNA binding domain and preferentially favors activation of \( p21 \) and \( 14-3-3\sigma \) genes while simultaneously attenuating transcription of pro-apoptotic genes such as \( BAX, PUMA \) and \( NOXA \) (Das et al., 2007). Similarly, another

![Figure 8. Differential response of tumor suppressor p53 in response to DNA damage. Upon mild stress when DNA damage is repairable, p53 triggers transient cell-cycle arrest allowing time for repair of damaged DNA, re-entry in to cell-cycle and promotes cell survival (LIFE). When DNA damage is excessive under conditions of severe stress, p53 elicits the cell suicide](image_url)
program (DEATH). The choice for these alternative fates is dictated by its interaction with specific heterologous transcriptional cofactors which are summarized in this figure.

group showed that chromatin associated human cellular apoptosis susceptibility protein (hCAS/CSE1L) selectively regulates PIG3, PUMA and p53AIP1 (Tanaka et al., 2007). Besides, there are a number of other proteins that interact with p53 and affect p53 activity in favor of either cellular survival or cell death. For example, the ASPP (ankyrin repeat-, SH3-domain and proline rich region containing proteins) family (ASPP1 and ASPP2) enhance the pro-apoptotic function of p53 by selectively promoting the binding of p53 to pro-apoptotic genes BAX, PUMA and PIG3 (Samuels-Lev et al., 2001). The chromodomain helicase DNA-binding 8 (CHD8) belongs to SNF2 superfamily of ATP-dependent chromatin remodelers interacts with p53 and inhibits p53 dependent apoptotic activity through association with histone H1 (Nishiyama et al., 2009). Recently, another chromatin binding transcription factor Bach1 (BTB and CNC homology 1, basic leucine zipper transcription factor 1) has been shown to inhibit oxidative stress induced apoptosis through binding to selective p53 promoters by forming a p53 dependent repressor complex with HDAC1 and N-coR (Dohi et al., 2008). Since, p53 transcriptional network is a complex barcode (Zmijewski et al., 2008) identifying specific targets affected by interaction of p53 with cellular cofactors remains critical for advancing our understanding of p53 function in vivo.

2.2.2 PML and p53: Bonafide partners in apoptosis

The tumor suppressor promyelocytic leukemia (PML) protein is involved in the regulation of p53 dependent and independent apoptosis. The PML protein localizes to multi-protein sub-nuclear structures termed PML nuclear bodies (PML-NBs) which act as sensors of DNA damage and cellular stress. Upon genotoxic stress, the PML protein functions as a transcriptional co-activator of p53 by recruiting it to the PML-NBs wherein PML facilitates p53 phosphorylation and acetylation through
recruitment of factors like HIPK2 and CBP (Bernardi and Pandolfi, 2007). Further, the *PML* gene itself is a transcriptional target of p53 (de Stanchina et al., 2004). The role of PML in defining programmed cell death (PCD) comes for the observation that PML knock out mice and PML^-/- cells are resistant to lethal effects of both γ-irradiation and CD95 Fas stimulation (Wang et al., 1998). Primary splenocytes, thymocytes, mouse embryonic fibroblasts (MEFs) and hematopoietic cells derived from PML knockout mice are resistant to not only γ-irradiation and CD95 Fas but also refractory to ceramide, tumor necrosis factor-α (TNF-α) and interferon (IFN). Besides, caspase activation is also impaired upon these stimuli (Wang et al., 1998; Wu et al., 2003). Since apoptosis of primary thymocytes upon γ-rays is p53 dependent while apoptosis of activated splenocytes upon CD95/Fas is p53 independent, it suggests that PML can regulate both p53 dependent and independent pathways of apoptosis. The tumor suppressor p53 is an important component of the PML-NBs and the fraction of p53 in the PML-NBs has been shown to increase under conditions of stress (Fogal et al., 2000). Various post translational modifications like phosphorylation and acetylation which are required for p53 activation are repressed in PML^-/- cells (Guo et al., 2000). This results in inhibition of p53 dependent apoptosis. PML directly interacts with p53, colocalizes with p53 in the PML-NB and facilitates p53 dependent transactivation by favoring its acetylation. Although, PML does not possess intrinsic acetyltransferase activity, it recruits acetyltransferase CBP and p300 along with p53 in the PML-NB to potentiate p53 acetylation (Guo et al., 2000; Pearson et al., 2000). PML is also induced by oncogenic RAS. The increase in PML protein levels leads to increase in size and number of PML-NBs. PML overexpression also leads to p53 induction (Ferbeyre et al., 2000). Furthermore, RAS activation triggers p53 accumulation in the PML-NBs and induce p53 acetylation and transcriptional activation. PML, p53 and CBP form a RAS inducible complex *in vivo* and colocalize in the PML-NBs (Pearson et al., 2000). Thus, PML and p53 are bonafide partners in apoptosis whose coordination is required for execution of programmed cell death (PCD).
2.2.3 Role of MARs in transcription and DNA looping

Scaffold/Matrix attachment regions (S/MARs) are regulatory DNA sequences mostly present upstream of various promoters. These cis elements act as platforms for the assembly of basal transcriptional complex and thereby control gene regulation. Matrix attachment region binding proteins (MARBPs) which bind such regulatory sequences interact with numerous chromatin modifying factors and facilitates transcription in response to diverse stimuli (Zaidi et al., 2005). Besides acting as cis elements, MARs are involved in folding of chromatin in to DNA loops and higher order DNA structures. By this mechanism, MARs can bring two different regions on the same chromosome (intrachromosomal interactions) which needs to be actively transcribed in close proximity and thereby regulate genes at large distances simultaneously (Filipski et al., 1990; Spilianakis and Flavell, 2004). The MARBP SATB1 has been shown to interact with PML-NB and organizes the MHC class I locus in to DNA loops in response to interferon stimulation (Kumar et al., 2007). The tumor suppressor p53 is also a MARBP that associates with the nuclear matrix and this association increases following DNA damage (Jiang et al., 2001). The PML-NBs are also associated with the nuclear matrix indirectly. MAR binding protein Bright (B cell regulator of IgH transcription) binds to the nuclear matrix through interaction with Sp100, a component of PML-NBs. This results in enhanced transactivation activity of Bright and IgH expression (Zong et al., 2000). However, the biological and functional significance of nuclear matrix bound p53 in context to nuclear matrix associated PML-NBs and MARBPs like SMAR1 is not known.

2.3 Results

2.3.1 SMAR1 promotes p53 deacetylation by interacting with HDAC1

We have earlier shown that SMAR1 interacts with p53 and stabilizes it in the nucleus causing p21 dependent cell-cycle arrest (Jalota et al., 2005). Here, we investigated if SMAR1 mediated p53 stabilization affects p53 apoptotic target genes. The p53
response element (p53RE) of the BAX promoter cloned in a reporter vector was tested for its responsiveness to SMAR1 expression. Surprisingly, ectopic expression of SMAR1 strongly inhibited p53 dependent transactivation of the p53RE reporter which was partially restored upon addition of HDAC inhibitor TSA (Figure 9A, bars 4 and 5). However, ectopic expression of a triple acetylated p53 mutant (p53 Lys-Arg 320/373/382) or SMAR1 showed no effect on the reporter system (Figure 9B, bars 2 and 3). These results suggest that p53 acetylation at Lys 320/373/382 residues are required for p53RE reporter transactivation and that SMAR1 in some way affect acetylation at these residues and therefore the reporter activity. Recently, we have shown that SMAR1 inhibits Cyclin D1 expression by recruitment of HDAC1-mSin3A repressor complex on the promoter MAR (Rampalli et al., 2005). Since, SMAR1 and p53 are both nuclear matrix associated transcription factors and both interact with HDAC1 independently, we investigated whether they are associated together in a complex. Double co-immunoprecipitation assay in HCT116 p53+/+ cells revealed the presence of detectable SMAR1-HDAC1-p53 complexes in vivo (Figure 9C). However, in the same eluate SMAR1 did not show binding to another MAR protein PARP, thus revealing the specificity of the interaction. Earlier, p53 has been shown to be deacetylated by its interaction with Mdm2 through recruitment of HDAC1 (Ito et al., 2000). Since p53 exists in a complex with HDAC1 along with SMAR1, it is possible that SMAR1 recruits HDAC1 to facilitate p53 deacetylation and thus keep its transactivation potential under check. This scenario is likely possible because HDAC1 itself does not have DNA binding ability and therefore its interaction with DNA binding transcription factors like SMAR1 is important for imparting gene specific regulation. To validate this, we performed reversible co-immunoprecipitation to evaluate the association of p53 with HDAC1 in presence and absence of SMAR1. While knockdown of SMAR1 reduced the amount of p53 pulled with HDAC1, overexpression resulted in strong increase in their association (Figure 9D). Finally, to confirm whether association of SMAR1 with p53 leads to p53 deacetylation in vivo deacetylation assay was done in HCT116 p53−/− cells. Cells were transfected with p53 alone or in combination with p300 and SMAR1 in presence or absence of

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Trichostatin A (TSA). Although p300 induced p53 acetylation at Lys-373/382 (Figure 9E, lane 3), presence of SMAR1 efficiently reduced p53 acetylation (lane 6) which was reversed upon treatment with HDAC inhibitor TSA (lane 5). Notably, although total p53 levels are high in SMAR1 transfected cells as SMAR1 is involved in p53 stabilization (Jalota et al., 2005), p53 acetylation levels are reduced in presence of SMAR1 and absence of TSA (lanes 5 and 6). Together, these results suggest that SMAR1 mediated p53 deacetylation is HDAC1 dependent.

**Figure 9. SMAR1 blocks p53 transactivation.**

(A-B) SMAR1 overexpression inhibits p53 dependent transactivation. The p53 response element of the BAX promoter in pGL3 enhancer vector was tested for responsiveness to p53 wt and a p53 triple acetylation mutant (lys-arg mutation at 320/373/382) in presence and absence of SMAR1 (C3 SMAR1). Bars represent standard deviation from three independent

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experiments. (C) Double co-immunoprecipitation assay to check the in vivo association of SMAR1-HDAC1 complex with p53. One milligram of cell lysate from HCT116 p53+/+ cells was immunoprecipitated sequentially with SMAR1 and HDAC1 antibodies. The eluted fraction was probed with p53 antibody. (D) Reversible co-immunoprecipitation assay in HCT116 p53+/+ cells showing differential association of p53 with HDAC1 in SMAR1 knockdown and overexpressed cells. (E) In vivo deacetylation assay of p53 by SMAR1. HCT116 p53−/− cells were transfected with p53, p300 expression plasmids in different combinations and treated with TSA (200 nM, 16 h) as given in the figure. GFP expression plasmid was transfected to monitor transfection efficiency.

2.3.2 SMAR1 promotes p300 Ubiquitination

Transcriptional activation and acetylation of p53 upon exposure to genotoxic stress is regulated by plethora of cofactors like PCAF, CBP and p300. Since, SMAR1 is a chromatin remodeling transcriptional repressor protein; it is possible that SMAR1 might affect directly or indirectly various transcriptional activators to regulate gene transcription. To explore if SMAR1 regulates p53 activity by modulating the expression of p53 coactivators, cells were transduced with GFP expressing control and SMAR1 adenoviruses followed by treatment with UV (100 J/m²). Interestingly, SMAR1 over expression strongly inhibited p300 expression which was otherwise induced upon UV treatment (Figure 10A, panel 5, lane 2 versus 3 and 4). The decrease in p300 resulted in reduced p53 acetylation at Lys 373/382 (Figure 10A, panel 3, lane 2 and 4). Under similar experimental conditions, other co-activators like CBP, PCAF and acetylation of p53 at Lys 320 remain unchanged. Conversely, we investigated the expression of p300 and p53 acetylation at Lys 373/382 upon SMAR1 knockdown. Two SMAR1 specific shRNAs (sh745 and sh1077) were generated targeting two different regions of SMAR1 mRNA. The knockdown and specificity of the shRNAs were checked by Western blotting (Figure 10B). Notably, knockdown of SMAR1 significantly induced p300 expression and p53 acetylation at Lys 373/382.
(Figure 10C). However, SMAR1 knockdown did not affect total p53 level and other cofactors like CBP and PCAF indicating the specific inhibition. Further, SMAR1 mediated downregulation of p300 was relieved upon treatment of cells with proteosomal inhibitor MG132 suggesting that SMAR1 promotes p300 endogenous proteosomal degradation (Figure 10D, lanes 3-4). Additionally, MG132 treatment in SMAR1 overexpressing cells also showed increased poly-ubiquitination of p300 (Figure 10E). Since, knockdown of SMAR1 resulted in increased p300 turnover and increased p53 acetylation, it raised the possibility that SMAR1 interacts with p300-p53 complex endogenously. Double co-immunoprecipitation assay in HCT116 p53<sup>+/+</sup> cells detected endogenous SMAR1 associating with p53-p300 complex (Figure 10F). These results suggest that SMAR1 negatively regulates p53 acetylation by proteosomally targeting p53 cofactor p300.

Figure 10. SMAR1 inhibits p300 expression.

(A) HCT116 p53\(^{+/+}\) cells were transduced with GFP expressing control and SMAR1 adenoviruses and treated with UV (100 J/m\(^2\)) for 12 hr. The levels of SMAR1, p53, p53 acetylation at lys-320, lys-373/382, p300, CBP and PCAF are shown. (B) HCT116 p53\(^{+/+}\) cells were transiently transfected with a control scrambled shRNA and two different SMAR1 specific shRNA constructs (sh745 and sh1077). Decrease in SMAR1 expression with time is shown by Western blotting. The specificity of SMAR1 knockdown by the shRNA constructs was confirmed by targeting another abundant nuclear matrix protein Lamin C. (C) Western blot analysis showing levels of p300, PCAF, CBP, p53, p53 acetylation at lys-320 and lys-373/382 upon SMAR1 knockdown. (D) SMAR1 ubiquitinites p300. Western blot showing the level and ubiquitination of p300 in HCT116 p53\(^{+/+}\) cells transduced with SMAR1 adenovirus and treated with 50 \(\mu\)M MG132. (E) The above lysates were immunoprecipitated with \(\alpha\)-p300 and immunoblotting was done using \(\alpha\)-ubiquitin. (F) Double co-immunoprecipitation assay showing in vivo association of SMAR1 with p53-p300. Two milligram of total protein was immunoprecipitated sequentially with \(\alpha\)-SMAR1 and \(\alpha\)-p53 using protein A/G beads. The eluted fraction was probed with \(\alpha\)-p300.

### 2.3.3 BAX and PUMA are transcriptional targets of SMAR1

Since SMAR1 regulates p53 acetylation, it is imperative that it would also affect the expression of p53 target genes. Indeed, overexpression of SMAR1 resulted in significant down regulation of Bax and Puma compared to control. Surprisingly, p53AIP1 and Apaf1 which are also p53 targets did not show any change (Figure 11A). However, the levels of total p53 and p21 increased significantly corroborating our earlier data that over expression of SMAR1 induces cell-cycle arrest (Jalota et al., 2005). Thus, on one hand SMAR1 enhances the expression of p21 and on the other it inhibits the expression of apoptotic genes BAX and PUMA. Interestingly, SMAR1 overexpression in HCT116 p53\(^{-/-}\) cells also repressed Bax and Puma but failed to induce p21 (Figure 11B). Thus, SMAR1 mediated induction of p21 is p53 dependent while repression of Bax and Puma is p53 independent. Since, SMAR1 mediated repression occurs through recruitment of HDAC1 repressor complex (Rampalli et al., 2007), we investigated if knockdown of HDAC1 alleviates the repression of Bax and Puma.
Puma by SMAR1. Silencing of HDAC1 not only abrogated SMAR1 mediated repression of Bax and Puma but also induced their basal expression. This suggests that the basal expression of Bax and Puma is under the control of a negative regulator and that SMAR1 mediated repression of Bax and Puma is HDAC1 dependent. Apaf1 expression remained unaltered upon HDAC1 knockdown (Figure 11C). Reporter assays using full length BAX and PUMA promoters in HCT116 p53\(^{-/-}\) cells exhibited strong inverse correlation upon SMAR1 ectopic expression and knockdown respectively while p53AIP1 promoter did not show any significant change (Figure 11D, E and F). Again, p21 reporter was induced by SMAR1 in HCT116 p53\(^{+/+}\) cells but failed to show read out in p53\(^{-/-}\) cells (Figure 11G). Thus, induction of p21 by SMAR1 is p53 dependent while transrepression of BAX and PUMA is p53 independent.

Figure 11. SMAR1 regulates Bax and Puma.

(A) HCT116 p53+/+ and (B) HCT116 p53−/− cells were transduced with GFP expressing control adenovirus (Ad-V) and SMAR1 Adenovirus (Ad-SM). Forty eight hours post transduction, the levels of p53, p21, Bax, Puma, Apaf1 and p53AIP1 were determined. (C) HDAC1 is required for SMAR1 mediated repression of Bax and Puma. HCT116 p53+/+ cells were transfected with HDAC1 siRNA and levels of Bax and Puma were determined in presence and absence of SMAR1 (C3). (D-E-F) Luciferase activity of full length promoters of BAX, PUMA and p53AIP1 upon SMAR1 overexpression by Flag-SMAR1 (FS) plasmid and knockdown using two different shRNAs (sh745 and sh1077) in HCT116 p53−/− cells. A deletion mutant of SMAR1 lacking the DNA binding and the protein interacting domain (F3) was used as a control. Bars indicate SD from three independent experiments. (G) p21 luciferase assay upon SMAR1 overexpression in HCT116 p53+/+ and p53−/− cells.

2.3.4 SMAR1 overexpression inhibits genotoxic stress induced apoptosis

To evaluate the biological significance of SMAR1 mediated p53 deacetylation and repression of Bax and Puma on cell survival, mouse embryonic fibroblasts (MEFs) and HCT116 p53+/+ cells were transduced with GFP expressing control (Ad-V) and SMAR1 (Ad-SM) adenoviruses followed by UV (100 J/m²) and Camptothecin (10 μM) treatment 48 h post transduction. Propidium Iodide staining indicated a significant reduction in the percentage of apoptotic fraction as represented by subG1 population in both MEFs (Figure 12A) and HCT116 p53+/+ cells (Figure 12B) expressing SMAR1 compared to control cells. Additionally, Annexin Cy3 surface staining of cells expressing SMAR1 also showed reduced apoptotic population in comparison to the control cells (Figure 12C). Statistical analysis of cells in over more than 50 fields (n>50) exhibited more than 50% reduction in Annexin surface staining (Figure 12D). Thus, the protective function of SMAR1 upon genotoxic stress can be attributed to its dual role. First, as a negative regulator of p53 acetylation and second by inhibiting the expression of apoptotic targets Bax and Puma.
Figure 12. SMAR1 inhibits apoptosis.

(A-B) Cell-cycle analysis of mouse embryonic fibroblasts (MEFs) and HCT116 p53+/+ cells transduced with GFP expressing control adenovirus (Ad-V) and SMAR1 adenovirus (Ad-SM). Cells were transduced with control and SMAR1 adenoviruses at an MOI of 1:100. Forty eight hours post transduction, cells were treated with UV (100 J/m²) and Camptothecin (10 μM) for a period of 12 h and thereafter stained with PI. (C) HCT116 p53+/+ cells transduced with control and SMAR1 adenoviruses are treated with Camptothecin (10 μM) for a period of 12 h and surface stained with Annexin-Cy3. (D) Statistical analysis of Annexin positive cells in more than 50 different fields (n>50) in two separate experiments.
2.3.5 SMAR1 induces an antiapoptotic signal in response to mild DNA damage

The tumor suppressor p53 induces cell-cycle arrest after mild DNA damage and apoptosis following severe irreparable damage (Aylon and Oren, 2007). To further mechanistically decipher the protective role of SMAR1, we investigated its responsiveness to mild DNA damage. Exposure of HCT116 p53+/+ cells to low dose UV (5 J/m²) led to a steady increase in SMAR1 protein level (Figure 13A, lanes 5, 6 and 7) with concomitant decrease in p300 from 24 h onwards. However, p300 levels initially increased from 4 h and peaked at 16 h which corroborated with increased p53 acetylation and Bax and Puma levels at these time points. Conversely, p53 acetylation, Bax and Puma expression came down at 36 h to 48 h during which SMAR1 protein level peaked (Figure 13A, lanes 6 and 7). This is in agreement with our data that SMAR1 represses BAX and PUMA independently of p53 and also targets p300 for proteosomal degradation. Interestingly, both p53AIP1 and PML that are p53 targets were not induced at low UV dose (Figure 13A) suggesting other post translational modifications of p53 are required for their transactivation (Oda et al., 2000). Although, Bax and Puma were inhibited by 48 h with concomitant deacetylation of p53, p21 levels increased and remain unaltered suggesting induction of cell-cycle arrest. Thus, our study supports the notion that cell-cycle arrest involves active induction of anti-apoptotic signal. Conversely, knockdown of SMAR1 by shRNA resulted in robust increase in p53 acetylation, Bax and Puma expression with subsequent PARP cleavage (Figure 13B). Cell-cycle analysis indicated that upon SMAR1 knockdown and UV treatment a large fraction of cells underwent apoptosis compared to UV alone (Figure 13C). The apoptotic pool as represented by subG1 population increased from 5% in UV irradiated cells to 21% in UV irradiated and SMAR1 knockdown cells. Notably, SMAR1 knockdown alone induced significant cell death (Figure 13C). Similar results were observed in HEK 293 cells (Figure 13D). Interestingly, we also observed that PARP cleavage was substantially reduced in presence of caspase-3 inhibitor (Figure 14A, lane 3 and lane 6). Inhibition in PARP cleavage further correlated with reduced apoptotic population in Caspase 3 inhibitor
treated HEK 293 cells (Figure 14B). This suggests that caspases are involved in SMAR1 knockdown induced apoptosis. However, shRNA mediated knock down of SMAR1 in HCT116 p53−/− cells although results in a modest increase in Bax and Puma expression (Figure 14C) no significant cell death was observed (Figure 14D). Thus, p53 is required for driving the cells towards apoptosis in a Caspase dependent manner. Finally, knockdown of SMAR1 in MEFs using SMAR1 shRNA lentivirus also induced cell death 96 h post viral infection (Figure 14E). Western blot analysis in these samples showed increased expression of Bax, Puma and P300 (Figure 14F). Thus, knockdown of SMAR1 affects the cellular apoptotic response by a dual mechanism: first, by enhancing p53 acetylation at Lys 373/382 through increased p300 turnover and second by increasing the expression of Bax and Puma.
Figure 13. SMAR1 induces anti-apoptotic signal.

(A) HCT116 p53^{+/+} cells were UV irradiated with 5 J/m^2 UV and collected at different time points as indicated. Immunoblot analysis was done for Bax, Puma, p53, acetylated p53, p21, p53AIP1 and PML. (B) Expression levels of Bax, Puma, p53, acetylated p53 and PARP cleavage upon shRNA (sh1077) mediated knockdown of SMAR1 in HCT116 p53^{+/+} cells. (C-D) Propidium Iodide staining in control HCT116 p53^{+/+} and HEK 293 cells (48 h), cells treated with UV (5 J/m^2, 48 h) and SMAR1 knockdown UV treated cells (5 J/m^2, 48 h) showing percentage apoptosis represented by SubG1 population.
Figure 14. SMAR1 knockdown induced apoptosis is p53 and caspase dependent.

(A) shRNA (sh1077) mediated knockdown of SMAR1 in HCT116 p53+/+ cells induces PARP and Caspase 3 cleavage even in absence of external DNA damage. PARP cleavage was partially inhibited in presence of Caspase 3 inhibitor (Z-DEVD-FMK). (B) HEK 293 cells showed reduced apoptosis upon SMAR1 knockdown in presence of caspase-3 inhibitor (C) Knockdown of SMAR1 by transient transfection induces apoptosis in HCT116 p53+/+ cells but not in HCT116 p53−/− cells as represented by SubG1 population. (D) HCT116 p53−/− were transfected with control shRNA vector (C-sh, lane 1) and SMAR1 shRNA sh1077 (SM-sh, lane 2). Thirty six hours post transfection cells were harvested and Western blotting done for Bax and Puma. (E) Lentiviral transduction of SMAR1 shRNA in MEFs showing apoptosis after 96 h of transduction as analysed by Flow cytometry with PI staining. (F) Western blot analysis of Bax, Puma and p300 levels in MEFs transduced with SMAR1 lentivirus.
2.3.6 SMAR1 protein drives p53 apoptotic gene target specificity through MARs

Earlier, we have shown that only BAX and PUMA were specifically repressed by SMAR1 overexpression and not other p53 targets. Since MARBPs have propensity to bind ATC rich sequences which often flank various promoters, we analyzed the promoter sequences of p53 inducible genes. Computational analysis using MARWIZ software (Singh et al., 1997) predicted potential MARs in BAX and PUMA promoter within ~700 bp upstream of p53 response element (p53RE) but not in p53AIP1 (Figure 15A, B and C). Sequence alignment of the promoters up to ~700 bp upstream of the p53RE revealed two stretch of sequences P1 (30 mer) and P2 (24 mer) in BAX which is exactly similar to PUMA promoter (Figure 15D, blue and green boxes). While the sequence encompassing the region P1 located outside the MAR, the region P2 lies within the MAR region of both promoters (Figure 15A and B). We then evaluated if SMAR1 binds to these sequences. For this, two probes of 40 mer each were designed corresponding to the two segments P1 and P2. For binding reactions, bacterially purified recombinant protein R5 (GST 350-548 aa) corresponding to the DNA binding region of SMAR1 and recombinant protein R6 representing the protein interaction domain (GST 160-350 aa) were used. Interestingly, while P1 failed to form any complex, P2 formed nucleoprotein complex with R5 (Figure 16A, lane 4). The binding specificity was further demonstrated by competition experiments showing loss of binding with the addition of 10-fold molar excess cold P2 (Figure 16B, lane 6). Under similar experimental conditions, GST and R6 failed to form any complex with P2 hinting the specific DNA binding activity of SMAR1 (Figure 16B, lane 2 and 3). To further check the specificity of SMAR1 binding to P2, we also designed two more similar sized probes P3 and P4 that lies proximal to P2 (Figure 16D, black boxes). EMSA studies with probes P3 and P4 did not show any complex formation (data not shown). Thus, SMAR1 binds to a specific and identical MAR like sequence P2 present in both the promoters. As the repressor activity of SMAR1 is attributed to its association with HDAC1, we further confirmed the binding of SMAR1 to these critical sequences.
SMAR1 and recruitment of HDAC1 by chromatin immunoprecipitation assay (ChIP). Cross-linked chromatin from HCT116 p53^{+/+} cells were pulled with SMAR1 and HDAC1.

**Figure 15A. MAR prediction of BAX promoter.**

Sequence in red denotes p53 response element (p53RE). Approximately 700 bp region of the three promoters upstream from transcription start site were analyzed using MARWIZ software. MAR potential is shown in the graph and the region corresponding to the MAR is coded yellow. The sequence in blue (corresponding to P1) and green (corresponding to P2) indicates the identical sequences of BAX and PUMA promoters. The exact location of these sequences with respect to MAR is shown in the MAR plot.

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Figure 15B. MAR prediction of PUMA promoter.

Sequence in red denotes p53 response element (p53RE). Approximately 700 bp region of the three promoters upstream from transcription start site were analyzed using MARWIZ software. MAR potential is shown in the graph and the region corresponding to the MAR is coded yellow. The sequence in blue (corresponding to P1) and green (corresponding to P2) indicates the identical sequences of BAX and PUMA promoters. The exact location of these sequences with respect to MAR is shown in the MAR plot.
Figure 15C. MAR prediction of p53AIP1 promoter.
Sequence in red denotes p53 response element (p53RE). Approximately 700 bp region of the three promoters upstream from transcription start site were analyzed using MARWIZ software. MAR potential is shown in the graph and the region corresponding to the MAR is coded yellow. The sequence in blue (corresponding to P1) and green (corresponding to P2) indicates the identical sequences of BAX and PUMA promoters. The exact location of these sequences with respect to MAR is shown in the MAR plot.

antibodies and PCR amplified for BAX and PUMA promoters using primers spanning their respective MAR regions. Both BAX and PUMA promoters gave strong signal in PCR whereas under similar experimental conditions p53AIP1 and p21 promoters were not amplified (Figure 16C). Thus, SMAR1 recruits HDAC1 to the MAR regions of both BAX and PUMA promoters but not to p53AIP1 and p21. Notably, the MAR sites and the p53RE are juxtaposed on BAX and PUMA promoters. Since, SMAR1 associates with p53-p300 complex in vivo, we reasoned that this complex might be associated with the nuclear matrix and more specifically to these MARs. To verify the co-occupancy of SMAR1/HDAC1 and SMAR1/p53 complexes on these MARs, we isolated the nuclear matrix from HCT116 cells and performed sequential ChIP using

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SMAR1/HDAC1 and SMAR1/p53 antibodies. The purity of the isolated nuclear matrix was verified using Lamin B1 and histone H1 antibodies (Figure 16D). While \textit{BAX} and \textit{PUMA} showed detectable amplification, \textit{p53AIP1} promoter was not amplified (Figure 16E, lanes 3 and 4). Further, to study the occupancy and recruitment of SMAR1 on these MARs \textit{in vivo}, cross-linked chromatin from UV treated (5 J/m\textsuperscript{2}) HCT116 p53\textsuperscript{+/+} cells at different time intervals were pulled with SMAR1, HDAC1, p53 and acetylated p53 lys373/382 antibodies. While \textit{BAX} (Figure 17A, left panel) and \textit{PUMA} (right panel) promoters were PCR detected in anti-SMAR1 pulled fraction, no amplification was observed in \textit{p53AIP1} (Figure 17B). Furthermore, the kinetics of SMAR1 occupancy showed an oscillatory pattern with initial endogenous bound SMAR1 slowly getting disappeared 4 h after UV treatment and reappeared by around 24 h. Also, the occupancy of SMAR1 and HDAC1 inversely correlated with p53 acetylation status at these loci (Figure 16A, lanes 1, 2, 6 and 7 and lanes 3, 4 and 5). Interestingly, immunostaining showed strong translocation of SMAR1 into the nucleolus at 12 h and 24 h after 5 J/m\textsuperscript{2} UV irradiation (Figure 17C, white arrows). To verify this observation, we isolated nucleoplasmic and nucleolar fraction from HCT116 p53\textsuperscript{+/+} cells after irradiation with 5 J/m\textsuperscript{2} at different time points and probed for SMAR1 in these compartments. Our data shows that SMAR1 is present in the nucleolar fraction of un-irradiated cells albeit at very low levels, but expression increases significantly 8 h onwards following UV stress. Interestingly, while SMAR1 level increases in the nucleolar fraction, it decreases in the nucleoplasmic fraction (Figure 17D). This suggests that SMAR1 translocates into the nucleolus upon UV stress. This can possibly explain the disappearance of SMAR1 from \textit{BAX} and \textit{PUMA} promoter MAR (Figure 17A) after 8 h of low dose UV treatment leading to increased expression of Bax and Puma at 8 h (Figure 10A). However, 24 h after UV stress when SMAR1 is induced, it reappears in the nucleoplasmic fraction as well as in the nucleolus. This is again consistent with our ChIP data wherein SMAR1 binding to the MAR element is restored after 24 h (Figure 17A), the time points after which Bax and Puma expression is maximally
Figure 16. **SMAR1 binds to BAX and PUMA promoter MARs.**

(A) EMSA showing specific binding of SMAR1 (R5) to probe P2 (lane 4) but not to probe P1.

(B) Purified GST (lane 2), R6 (lane 3) and R5 in increasing doses (lanes 4 and 5) were incubated with probe P2. Formation of complex (I) was visualized by autoradiography. Binding specificity of the complex in presence of 10 fold molar excess of cold competitor (cold probe P2) is shown in lane 6. Free probe is denoted as FP.

(C) Chromatin from HCT116 p53+/+ cells was immunoprecipitated with α-SMAR1 and α-HDAC1 antibodies (lane 3). PCR amplification was performed on MAR regions of BAX and PUMA and p21 promoters were used as control. Parallel immunoprecipitation with control IgG antibody is shown in lane 2. Lane 1 denotes input control.

(D) The purity of the nuclear matrix isolated from

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HCT116 p53+/+ cells was tested by Western blotting using α-LaminB1 and α-Histone H1. (E) Sequential ChIP on BAX and PUMA promoter MARs using α-SMAR1/p53 (lane 3) and α-SMAR1/HDAC1 (lane 4) antibodies in nuclear matrix fraction of HCT116 p53+/+ cells. Lane 1 and 5 shows IgG and input control respectively.

repressed as evident by immunoblot (Figure 13A). In the nucleolus, SMAR1 inhibits ribosomal gene transcription to cause cell-cycle arrest (unpublished data). The nucleolar translocation of SMAR1 facilitates p53 acetylation which then unleashes its transactivation potential on BAX and PUMA promoter resulting in pronounced expression of Bax and Puma. What post translational modifications causes SMAR1 translocation into the nucleolus and binding to the MAR is currently under investigation. Together, these data suggest that nucleolar sequestration of SMAR1 may partly facilitate p53 acetylation and allows acetylated p53 to activate BAX and PUMA. Nonetheless, once SMAR1 is induced, it facilitates p53 deacetylation through HDAC1 and inhibits the transcription of BAX and PUMA. Thus, occupancy of SMAR1 on BAX and PUMA MAR selectively regulates their expression in response to DNA damage.
Figure 17. SMAR1 translocates to the nucleolus upon low dose DNA damage.

(A-B) In vivo ChIP to detect occupancy of SMAR1 on BAX (left panel), PUMA (right panel) and p53AIP1 promoter (lower panel) upon low dose UV irradiation. Cross-linked chromatin from UV irradiated HCT116 p53<sup>+/+</sup> cells were pulled with SMAR1, p53, acetylated p53 lys373/382 and HDAC1 antibodies and bound chromatin fragments were detected by PCR.

(C) Immunostaining of SMAR1 with nucleolar marker Nucleolin (C23) after low dose (5 J/m<sup>2</sup>) UV irradiation showing nucleolar localization of SMAR1 (D) Western blotting of SMAR1 in nucleolar and nucleoplasmic fraction of UV irradiated (5 J/m<sup>2</sup>) HCT116 p53<sup>+/+</sup> cells at different time points.

2.3.7 Apoptotic DNA damage translocates SMAR1 into PML nuclear bodies

Since low dose UV DNA damage induced cell-cycle arrest wherein an apoptotic signal triggered by p53 acetylation was superseded by an anti-apoptotic signal...
through induction of SMAR1, we now treated the cells with 100 J/m² UV to evaluate the effect of SMAR1 on p53 acetylation. At this dose of DNA damage cells inadvertently goes towards apoptosis. Immunostaining analysis of UV irradiated cells showed SMAR1 forming discrete speckled structures colocalizing with Promyelocytic Leukemia (PML) nuclear bodies. PML nuclear bodies are involved in regulation of p53 dependent and independent DNA damage induced cellular apoptosis (Wang et al., 1998). This colocalization was very weak and not distinct in control cells and in cells treated with low dose 5 J/m² UV (Figure 18A). Notably, colocalization of SMAR1 and PML becomes distinctly visible after 12 h of UV (100 J/m²) treatment when the size and number of PML-NBs increases (Figure 18B, white arrows). Interestingly, SMAR1 was also found translocated to the nucleolus in cells exposed to high dose UV irradiation (Figure 18B, red arrows) as in case with low dose. This is consistent with the fact that PML protein is not induced at low dose UV damage. This raised the possibility that induction of PML at high dose enhances PML-SMAR1 complex formation with subsequent sequestration of SMAR1 into the PML-NBs. To evaluate this, we performed immunoprecipitation experiments. HCT116 p53+/+ cells were either treated or left untreated with UV and binding complexes were immunoprecipitated with SMAR1 antibody and analysed by Western blotting with anti-PML monoclonal antibody. Control cells showed a faint pulled band suggesting that SMAR1 and PML crosstalk endogenously (Figure 19A, lane 7). However, the PML-SMAR1 complex increased stochiometrically at 12 h and 24 h when the level of both SMAR1 and PML increases (Figure 19A, lanes 8 and 9). We confirmed this specific interaction by transiently overexpressing HA-PML-IV and then immunoprecipitating the binding complexes with SMAR1 polyclonal antibody. Immunoblot analysis with HA antibody detected large amounts of SMAR1-PML complexes in PML-IV transfected but not in control cells (Figure 19B). Further, we find that upon overexpression of PML-IV, SMAR1 completely translocates into the PML nuclear bodies (Figure 19C, upper panel). Because sumoylation of PML has
been shown to be important for nuclear body formation and interaction with various transcription factors (Zhong et al., 2000) we used a triple sumoylation mutant PML construct to check its ability to sequester SMAR1. As expected, ectopic expression of this mutant failed to sequester SMAR1 into nuclear bodies and therefore showed a diffused distribution pattern (Figure 19C, lower panel). To identify the PML domain involved in interaction with SMAR1, pull down assays were performed with recombinant GST-SMAR1 and various HA tagged deletion mutants of PML-IV. HA-PML-IV (Figure 19D, lanes 2 and 3) and HA-PML-IV (200-453) (lanes 6 and 7) encompassing the coiled coil domain of PML showed strong binding to GST-SMAR1. These results suggest that PML interacts with SMAR1 endogenously through its coiled coil dimerization domain. However, upon apoptotic stress when the level of PML and SMAR1 increases, their association also increases proportionately. Functionally, these data suggest that PML negatively regulates the activity of SMAR1 by sequestering it into the nuclear bodies to positively regulate the effector functions of p53. Because at high apoptotic UV dose (100 J/m^2) p53 is invariably acetylated to bring about cell death, we suspected that during this condition SMAR1 dissociates from p300 to facilitate p53 acetylation. To confirm this, we performed a reciprocal quantitative co-immunoprecipitation assay using low and high dose UV by pulling the SMAR1-p53 functional complexes with respective antibodies. As expected, we find that although SMAR1-p53 complex increases in cells treated with UV dose of 5 J/m^2 (Figure 19E and F, lane 1 and lane 2), but at 100 J/m^2 UV the interaction remains same as in control cells (lane 1 and lane 3). Conversely, while SMAR1-p300 association becomes weaker, p53-p300 interaction gets stronger at high UV dose. Also, immunostaining of PML and p53 after apoptotic UV damage failed to show p53 in the distinct PML punctuate structures (Figure 20A). This highlights the fact that translocation of p53 to PML nuclear bodies is dispensable for its acetylation at least in response to UV damage. Collectively, our data shows that by sequestering SMAR1 into PML nuclear bodies, PML facilitates p53 acetylation through enhanced association with p300.
Figure 18. SMAR1 colocalizes with PML-NBs after apoptotic DNA damage.
Immunofluorescence analysis showing SMAR1-PML co-localization in HCT116 p53+/+ cells at (A) low dose 5 J/m² and (B) high dose 100 J/m² UV. (C) Co-localization of SMAR1 with Sp100 at high dose. Cells were stained with SMAR1 (green), PML (red) and Sp100 (red) antibodies and analyzed by confocal microscopy. Nuclei were stained with DAPI (blue). Co-localization of SMAR1 and PML bodies are shown in white arrows. Red arrows indicate SMAR1 in nucleolus. Images shown are representative of more than 50 images (n>50) taken in different fields from two independent experiments.
**Figure 19. PML interacts with SMAR1.**

(A) Five hundred micrograms of total cell extract from control and UV irradiated cells (100 J/m²) at different time points were immunoprecipitated (IP) with α-SMAR1 (IS) and control α-IgG (PIS) and detected by immunoblotting (IB) with antibody against PML (left panel). 10% of total cell lysate was used for input (left panel). (B) Co-immunoprecipitation of SMAR1 and PML in HA-PMLIV transfected HCT116 p53+/+ cells as detected by α-HA. (C) Immunostaining demonstrating SMAR1-PML colocalization after overexpression of PML-IV and PML-IV sumoylation mutant construct. PML is stained with red (Cy3) and SMAR1 is stained with green (FITC). Nucleus is stained with blue (DAPI). (D) GST pull down assays were performed using purified GST-SMAR1 and GST in HCT116 cells overexpressing full length HA-PML-IV and truncated HA-PML-IV proteins as shown in figure. Specific binding was detected using α-HA. (E-F) Cell extracts (0.5 mg) from control and UV treated with 5...
J/m² and 100 J/m² were immunoprecipitated with α-SMAR1 and α-p53 antibody and amount of p53, p300 and SMAR1 in the immunoprecipitated complex were detected by antibodies against p53, p300 and SMAR1.

![Image](image.png)

**Figure 20. PML knockdown impairs apoptosis.** (A) Immunostaining of PML and p53 in control HCT116 p53³/³ cells and cells treated with low dose (5 J/m²) and high dose (100 J/m²) UV irradiation. p53 is stained with FITC (green), PML with Cy3 (red) and DNA is stained with DAPI (blue). (B) Cell-cycle analysis in HCT116 p53³/³ cells by propidium iodide staining depicting percentage apoptosis upon PML knockdown by siRNA in presence and absence of high dose apoptotic UV treatment (100 J/m²) for 12 h.

### 2.3.8 Silencing of PML expression enhances SMAR1 mediated transrepression of Bax and Puma

To establish whether SMAR1-PML interaction is indeed indispensable for induction of SMAR1-p53 target genes BAX and PUMA, endogenous PML was depleted by specific siRNA and then treated with UV. We found that silencing of PML by specific siRNA does not affect p53 acetylation up to 12 h of UV treatment as reported earlier (Bernardi et al., 2004) (Figure 21A, lanes 1, 2, and lanes 4, 5). The lower levels of SMAR1 in PML knockdown cells could be because of the fact that PML regulates the activities of many transcription factors like Sp1 which possibly...
could regulate SMAR1 basal transcription. Further, PML also regulates p53 protein levels and stability and the fact that SMAR1 is a p53 target gene (Singh et al., 2007) could account for the lower levels of SMAR1. Indeed, our data also shows reduction in p53 levels in PML knock down (Figure 21A, lanes 4, 5 and 6). Consequently, upon PML knockdown p53 acetylation levels should be comparatively less. This is however not observed because of lower levels of SMAR1. However, SMAR1 induced at around 24 h after UV treatment not only reduces p53 acetylation as described earlier but also inhibited Bax and Puma expression (Figure 21A). The inhibition of Bax and Puma was reflected in reduced apoptotic population as shown by PI staining (Figure 20B). Interestingly, we found that PML was strongly induced after apoptotic UV DNA damage. Of note, the 60 kDa band corresponding to PML isoform IV was significantly induced in comparison to 90 kDa band representing PML isoform III (Figure 21A). Other isoforms of PML close to the 60 kDa and 90 kDa also increased but not to significantly high levels. This corroborated with previously published data suggesting the involvement of PML III and PML IV in p53 regulation. Surprisingly, earlier studies (Seker et al., 2003) did not find changes in PML protein level after UV stress. This could be because the studies were done with low UV dose and also for lesser time points which is consistent with our own data wherein no significant induction of PML protein was found at 5 J/m² low UV dose. Even at high apoptotic dose the increase in PML protein and the number of nuclear bodies was significant only after 12 h. Notably, the observed increase in PML and SMAR1 was p53 dependent (Figure 21B). Further, we performed quantitative real time PCR to analyze if the differential response of \textit{BAX} and \textit{PUMA} at high apoptotic dose is reflected at transcript level. SMAR1 overexpression inhibited not only endogenous transcript level of \textit{BAX} and \textit{PUMA} but under conditions of UV treatment (Figure 21C and D). Also, in the same experimental samples \textit{p21} was induced synergistically by UV and SMAR1 (Figure 21E). These results are in accordance with our previous data. Importantly, SMAR1 induced by high dose UV treatment failed to repress \textit{BAX} and \textit{PUMA} (Figure 21F, C and D, compare lane 2). It is possible that at high apoptotic dose SMAR1 is sequestered into the PML-NBs as earlier
demonstrated and therefore unable to exert its repressive function on BAX and PUMA. Immunostaining of SMAR1 in PML knockdown cells suggest that SMAR1 does not form discrete speckle structures as it did in presence of PML (Figure 21G) although SMAR1 was found to be translocated to the nucleolus (white arrow). Furthermore, when cross-linked chromatin derived from PML siRNA and UV treated cells were immunoprecipitated with α-SMAR1 and α-HDAC1, we found that

![Image of Figure 21A: Comparative expression levels of SMAR1, Ac-p53 and p53 in HCT116 p53+/+ cells transfected with scrambled siRNA (left panel) versus PML siRNA (right panel) upon UV (100 J/m²) treatment.](image)

![Image of Figure 21B: HCT116 p53−/− cells were UV irradiated with apoptotic dose (100 J/m²) and Western blotting was done for SMAR1, Bax and PML.](image)

**Figure 21. Knockdown of PML impairs Bax and Puma expression.**

(A) Comparative expression levels of SMAR1, Ac-p53 and p53 in HCT116 p53+/+ cells transfected with scrambled siRNA (left panel) versus PML siRNA (right panel) upon UV (100 J/m²) treatment. (B) HCT116 p53−/− cells were UV irradiated with apoptotic dose (100 J/m²) and Western blotting was done for SMAR1, Bax and PML. (C-D-E-F) Quantitative real time
PCR analysis showing transcript levels of Bax, Puma and p21 in SMAR1 over expressing cells with and without UV (100 J/m$^2$) treatment. The results are average of three independent experiments. Bars represent standard deviation. (G) Confocal staining of SMAR1 and PML in PML siRNA transfected and UV treated cells. Images are representative of more than 50 fields (n>50) from two independent experiments.

SMAR1 was selectively bound onto the MAR regulatory regions of BAX and PUMA promoter along with HDAC1 (Figure 22A). To further demonstrate the repressive effect of the MAR element, a deletion mutant of BAX promoter lacking the MAR region but having intact p53 binding site was used to test its transactivation potential upon low dose and high dose UV irradiation in comparison to the full length promoter. While the full length BAX promoter (pRE+MAR) was effectively repressed even below its basal level, the MAR deleted mutant of BAX promoter (p53RE) was mildly repressed and was still activated upon low dose 5 J/m$^2$ UV irradiation (Figure 22B and C). This mild repression of the reporter lacking the MAR could be because of factors like Hdm2 which can also regulate p53 acetylation through interaction with HDAC1 (Kobet et al., 2000; Ito et al., 2002). Similarly, upon apoptotic dose of 100 J/m$^2$ UV DNA damage the full length BAX promoter harboring the MAR (p53RE+MAR) exhibited greater degree of repression in comparison to the p53RE reporter in absence of PML (Figure 22D). Nonetheless, upon PML knockdown, the p53RE reporter showed mild repression similar to seen in case of low dose irradiation. This is because, recruitment of p53 cofactors like CBP and p300 which are recruited by PML into PML-NBs to facilitate p53 acetylation are impaired in absence of PML (Hofmann et al., 2003). Notably, the activity of the p53RE promoter is weaker than that of the p53RE+MAR. However, p53RE+MAR reporter activity should have been less because of the negative effect of MAR. This is not observed because the p53RE reporter contains only the 35 mer sequence containing the p53 consensus binding site in the BAX promoter. The p53+MAR reporter is the full length BAX promoter (~700 bp) and contains the core promoter including AP1, SP1, p53 and TATA box binding elements in addition to the MAR element. Because

of this reason the basal activity of the p53RE+MAR is more than that of p53RE only. Collectively, these data suggests that knockdown of PML results in persistent occupancy of SMAR1 on to the BAX and PUMA promoter MAR and hence the repression. Taken together these results confirm that PML is required for complete transactivation of BAX and PUMA by releasing the negative effects of SMAR1 through sequestration into the PML-NBs.

![Figure 22](image)

**Figure 22. Responsiveness of MAR containing and MAR deleted reporter vectors upon PML knockdown.**

(A) In vivo chromatin immunoprecipitation assay in HCT116 p53+/+ cells exposed to low dose (5 J/m²) and high dose (100 J/m²) UV with and without transfection with scrambled siRNA and PML siRNA. (B) Luciferase activity of the full length BAX (pBaxLuc) and (C) MAR deleted (p53RE) reporters in HCT116 p53+/+ cells treated with low dose 5 J/m² UV. (D) Promoter read out of pBaxLuc and p53RE reporters in HCT116 p53+/+ cells transfected with two different PML siRNAs and treated with high dose (100 J/m²) UV.

_Surajit Sinha Thesis, September 2009_
2.3.9 Double knockdown of SMAR1 and PML attenuates Survivin repression and abrogates apoptosis

We have earlier shown that during UV induced apoptosis, PML protein forms a complex with SMAR1 and sequesters it into the PML-NBs. Further, silencing of PML impairs the expression of Bax and Puma as SMAR1 occupies their promoter MAR and exerts repression through HDAC1. Therefore, to finally confirm the requirement of SMAR1 in regulating the expression through MAR, we knocked down both PML and SMAR1 using specific siRNAs and evaluated the expression pattern of Bax and Puma upon apoptotic UV DNA damage. As expected, double knockdown resulted in increased expression of Bax and Puma (Figure 23A). Interestingly, repression of Survivin expression was abrogated when both PML and SMAR1 was knocked down. Survivin is an inhibitor of apoptosis protein (IAP) and is overexpressed in most human tumors. Survivin blocks apoptosis by directly binding and inhibiting caspase-3 and caspase-7, which act as terminal effectors in apoptotic cascade (Ambrosini et al., 1997; Shin et al., 2001). Moreover, this anti-apoptotic gene has been shown to be a direct p53 target and is repressed by p53 in response to UV DNA damage induced apoptosis (Hoffman et al., 2002; Mirza et al., 2002). Additionally, the promyelocytic leukemia protein (PML-IV) has been reported to induce apoptosis by inhibiting Survivin expression (Xu et al., 2004). Although, the PML protein itself does not have a DNA binding domain and does not interact with DNA directly, it regulates transcription by interacting with various DNA binding transcription factors. Since, PML and SMAR1 forms a complex during UV DNA damage induced cellular apoptosis, it raised the possibility that PML recruits SMAR1 to repress Survivin expression during damage induced apoptosis. To verify this we first evaluated the effect of SMAR1 and PML on Survivin promoter. While overexpression of either PML or SMAR1 lead to a strong repression of Survivin promoter read out, the reporter activity was further reduced when both PML and SMAR1 were used together in combination suggesting a synergistic effect (Figure
We further confirmed this by Western analysis wherein we show that overexpression of either PML or SMAR1 leads to repression in Survivin expression (Figure 23C and D). Interestingly, sequence analysis of Survivin promoter revealed an identical MAR element similar to what is present in BAX and PUMA promoters. However, this MAR element is about 17 bp compared to 25 bp in BAX and PUMA. To see the biological significance of Survivin repression, we scored the cell death population upon knockdown of either SMAR1 or PML or both SMAR1 and PML together after apoptotic DNA damage by PI staining. Indeed, knockdown of SMAR1 greatly reduced cell death very similar to what is observed in PML knockdown cells. Likewise, knockdown of SMAR1 and PML together also showed reduced apoptosis similar to knockdown of SMAR1 or PML alone (Figure 23E). This suggests that SMAR1 and PML both are required for apoptosis and SMAR1-PML complex formation facilitates apoptosis upon apoptotic DNA damage partly by inhibiting Survivin expression. Finally, promoter analysis of different cell-cycle and apoptotic genes revealed that this identical MAR element is present in DR4, DR5, Topoisomerase IIα, CDC25C, CDC2, Cyclin B1, FADD and Trp53.
Figure 23. Double knockdown of SMAR1 and PML abrogates Survivin repression.

(A) Expression analyses of Bax, Puma and Survivin upon double knockdown of PML and SMAR1 in HCT116 p53+/+ cells. (B) Survivin promoter assay upon overexpression of SMAR1 and PML. (C-D) Expression analysis of Survivin upon overexpression of SMAR1 and PML. (E) Propidium iodide staining of UV irradiated HCT116 p53+/+ cells upon knockdown of SMAR1, PML and both together.

2.3.10 SMAR1 inhibits the expression of p53 regulated miRNAs

miRNAs are a recently discovered class of small regulatory RNAs that play fundamental role in gene regulation (Bartel and Chan, 2004). They are 18-24 nucleotide RNAs that negatively regulate the translation and stability of partially complementary target messenger RNAs (Kim, 2005). However, miRNAs themselves are also regulated by transcription factors but the underlying mechanisms are poorly understood.
understood. To identify key miRNAs regulated by SMAR1, we performed genome scale miRNA microarray analysis using Agilent technology platform in HCT116 colon cancer cells upon SMAR1 overexpression and knockdown. Keeping two fold change in the expression of miRNAs as the baseline, we sorted only those few miRNAs which showed reciprocal relationship with SMAR1 expression. The lists of these miRNAs are summarized in Figure 24.

Figure 24. List of miRNAs affected by SMAR1 overexpression and knockdown.

Figure 25. SMAR1 inhibits miR-34a expression. Real time PCR of miR-34a in HCT116 p53+/+ and p53-/- cells upon overexpression and knockdown of SMAR1.

Interestingly, we found that miR-34a showed more than four fold change with respect to SMAR1 expression. miR-34a has recently been shown to be a p53 target and is implicated to have role in apoptosis (Chang et al., 2007). Indeed, by real time PCR, we found that SMAR1 overexpression and knockdown repressed and enhanced miR-34a expression respectively in a p53 dependent manner (Figure 25). Since miR-34a is known to repress Bcl-2 and considering the fact that SMAR1 overexpression induces Bcl-2 expression (Figure 23C), it is possible that SMAR1 modulates the apoptotic response by regulating p53 dependent miRNAs. Further work is undergoing to elucidate the full mechanism of SMAR1 mediated miRNA regulation during apoptosis.

2.3.11 SMAR1 expression is altered in different grades of colon cancer cells

Studies from our lab have demonstrated that SMAR1 expression is downregulated in higher grades of breast tumors. Since our data shows that SMAR1 directly regulates the endogenous expression of Bax and Puma, we evaluated the expression profiling of SMAR1 with respect to Bax and Puma in five different colon cancer cell lines derived from different grades of colon tumors. HCT15 cell line is derived from a primary tumor. HT29 and SW480 are derived from a slightly metastatic colon tumor while Colo320 and Colo205D are derived from highly aggressive, metastatic and invasive tumors. Western blot analysis reveals that SMAR1 expression is high in cells derived from primary tumors, but the expression gradually decreases in metastatic and invasive tumor derived cells. Interestingly, we also observe the appearance of an alternatively spliced product or a different isoform of SMAR1 in these high grade derived tumor cells (Figure 26, panel 1). Thus, the level of Bax and Puma is low in HCT15 where SMAR1 expression is high but the levels increases in SW480 where SMAR1 expression is drastically reduced (Figure 26, panel 2 and 3). Also, in Colo320 SMAR1 level is high and this correlated with lowered Bax and Puma expression. However, this inverse correlation was not true in case of Colo205D. This
suggests that in highly metastatic and invasive cells like Colo205D, other mechanisms exist to counteract the levels of apoptotic molecules. One mechanism could be increased expression of an isoform of another MAR binding protein CDP/Cux. CCAAT-displacement protein/cut homeobox (CDP/Cux) is a transcription factor which can also bind MARs (Banan et al., 1997). CDP/Cux exists in different isoforms and a particular isoform of ~75 kDa has been shown to be overexpressed in higher grades of breast tumors where it activates TGF-β mediated tumor cell proliferation and migration (Goulet et al., 2002; Michl et al., 2005; Aleksic et al., 2007). Western blot of CDP/Cux does reveal an increase in the 75 kDa isoform in Colo205D cells (Figure 26, panel 4). This kind of mechanism is absolutely possible because it gives cancer cells selective advantages by being resistant to apoptosis and at the same time can proliferate and disseminate. Preliminary experiments from our lab suggest that CDP/Cux can transcriptionally repress Bax and Puma expression. However, other experiments are needed to validate this observation and hypothesis.

![Western blot analysis of SMAR1, Bax, Puma and CDP/Cux in different colon cancer cell lines.](image)

**Figure 26. SMAR1 expression is altered in colon cancer cells.** Western blot analysis of SMAR1, Bax, Puma and CDP/Cux in different colon cancer cell lines.
2.4 Discussion

2.4.1 SMAR1 and p53 in apoptosis
The tumor suppressor p53 is regulated by plethora of transcription factors. Many heterologous transcription factors interact with p53 after DNA damage and tilt the balance of cell towards either survival or apoptosis. How these specific heterologous factors are able to channelise and streamline the function of p53 towards either arrest or apoptosis is an important area of research. We have tried to understand this regulation of p53 by a matrix attachment protein SMAR1 because of two molecular reasons. First, we have shown that SMAR1 interacts with p53 and therefore bound to affect the transcriptional ability of p53 and second because of the fact that SMAR1 itself is a p53 target gene. We have used UV induced DNA damage at two different doses to study the function of p53 under these conditions. Mild damage (UV 5 J/m²) was given to activate p53 and induce cellular arrest while an apoptotic dose (UV 100 J/m²) was used to induce apoptosis. We show that p53 target gene SMAR1 is induced during UV DNA damage but is differentially compartmentalized into the PML nuclear bodies only at high dose DNA damage and therefore have different functional outcome. Unlike proteins such as Mdm2, Pirh2 and COP1 which regulate basal levels of p53 through ubiquitination (Brooks and Gu, 2006), SMAR1 modulates the basal p53 acetylation status by regulating the levels of acetyltransferase p300 through ubiquitination. This kind of basal regulation of p53 acetylation is important to prevent sudden cell death arising out of mild metabolic stresses which cells are always encountered with. Whether SMAR1 itself has any ubiquitin ligase activity or recruits them for p300 ubiquitination is currently under investigation. Recently, Skp2 has also been shown to suppress p53 dependent apoptosis by promoting p300 proteosomal degradation (Kitagawa et al., 2008). While, transcription factors like Hzf, hCAS and ASPP family members modulate p53 function by directly binding to selective p53 target genes under conditions of stress, SMAR1 modulates the transactivation potential of p53 through p300 ubiquitination apart from imparting specific regulation.
of p53 apoptotic targets Bax and Puma. We show for the first time that both BAX and PUMA promoter harbor a similar 25 bp MAR element through which SMAR1 anchors these genes to the nuclear matrix making them transcriptionally inactive. Although, mild DNA damage triggers p53 acetylation and transcription of p53 apoptotic targets BAX and PUMA, induction of SMAR1 results in p53 deacetylation through inhibition of p300. Further SMAR1 is recruited to BAX and PUMA promoter MAR resulting in abatement of their transcription. Thus, induction of SMAR1 generates an anti-apoptotic signal that prevents cellular apoptosis after mild DNA damage. While low dose UV DNA damage induced around 8% cell death, the percentage of apoptosis increased to 16% in SMAR1 knock down cells treated with low dose UV. This suggests that absence of SMAR1 preferentially shifts the balance of cells towards apoptosis in response to DNA damage. Thus, we establish SMAR1 as an important apoptotic check point regulator.

Figure 27. Model showing the MAR element (blue box) of BAX and PUMA promoter juxtaposed to each other causing the looping out of the intervening sequence. SMAR1 (orange) binds to this identical MAR element along with HDAC1 (green) to p300 (purple) and p53 (red) forming a repressor complex switching off transcription after mild DNA damage.
damage (suppression of apoptosis). After severe apoptotic DNA damage, SMAR1 is no longer bound to the MAR element facilitating p53 acetylation (p53-Ac) by p300 and transcription of BAX and PUMA (induction of apoptosis).

It is intriguing as to why SMAR1 needs to repress BAX and PUMA by binding to a MAR element when it can inhibit p53 acetylation through p300 and thereby block p53 downstream effectors. One possible explanation is that p53 deacetylation occurs at protein level and is kinetically much slower process compared to direct repression through MAR. The MAR site in BAX and PUMA promoter act as negative regulatory element that ensures timely repression and safeguard mechanism from commitment of cells towards apoptosis when damage is repairable. This is the reason why after mild DNA damage p21 and SMAR1 remains induced till 48 h while BAX and PUMA are suppressed considering all of them are p53 target genes. This suggests that the drastic repression of BAX and PUMA is a direct effect of the MAR element coupled to p53 deacetylation. Moreover, genes which harbor MARs and are attached to the nuclear matrix are more repressed than genes in matrix without MARs (Rudd et al., 2004). Thus, MARs serves as an additional layer of transcriptional regulation for BAX and PUMA besides p53 in response to DNA damage. This is important since even mild external stress can trigger apoptosis through Bax and Puma (Chipuk et al., 2005). Interestingly, Bax and Puma are mitochondrial proteins, genes of which are located in the same locus 19q13.3 and both are transcriptionally regulated by a single MAR element present in both promoters. Thus, it is possible that binding of SMAR1 to the identical MAR element brings the promoters of BAX and PUMA in close proximity of each other to confer regulatory effects by allowing the intervening sequence (1.7 Mb) between the promoters to form intrachromosomal loops (Figure 27). This also explains as to why BAX and PUMA behave very similarly under conditions of UV stress. Such kind of long range intrachromosomal interactions have been reported for T\(_H\)2 cytokine locus (Spilianakis and Flavell, 2004).
It is demonstrated that in PML−/− cells, induction of pro-apoptotic targets like BAX is impaired (Guo et al., 2000). However, mechanism how PML regulates the expression of Bax is not known. In this study, we showed that PML is induced at high dose 100 J/m² UV in a p53 dependent manner. PML induction is associated with sequestration of SMAR1 with concomitant increase in p53 acetylation. We have shown that SMAR1 forms a repressor complex with p53 and HDAC1 and this repressor complex is associated with BAX and PUMA MAR. Therefore, sequestration of SMAR1 by PML not only facilitates p53 acetylation but enhances the expression of Bax and Puma by allowing acetylated p53 to transactivate them. Further, the occupancy of SMAR1 on BAX and PUMA promoter MAR in UV treated PML knock down cells abrogates UV induced apoptosis. This suggests that displacement of SMAR1 from MAR regulatory element by PML is indispensable for p53 acetylation and expression of BAX and PUMA leading to apoptosis (Figure 28). These findings firmly establish SMAR1 as bonafide PML partner required for effective tumor suppression. In this context, it is worth mentioning that tumor suppressor PML protein is downregulated in most of the cancers (Scaglioni et al., 2006). Although we have earlier shown that SMAR1 is downregulated in higher grades of breast cancer where p53 function becomes defective, SMAR1 responds positively to various DNA damaging and chemotherapeutic agents in cells harboring wild type p53 (Singh et al., 2007). Thus, it is tempting to speculate that loss of PML expression coupled to the responsiveness of SMAR1 to various drugs in tumors harboring wild type p53 might explain their increased refractory nature to various chemotherapeutic treatment modules. Thus, SMAR1 can be a potential target for cancer therapy where p53 function is not compromised.

2.4.2 SMAR1 and miRNAs in cancer

miRNA expression is frequently disregulated in cancer cells. Specific miRNAs have also been shown to regulate cell-cycle and apoptosis (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006; Hammond, 2006). Since tumor suppressor p53
acts as a nodal molecule and transactivate myriads of genes involved in cell-cycle arrest and apoptosis in response to DNA damage, it is possible that p53 also regulate the expression of many miRNAs which might function in influencing cell fate decisions. The miR-34 family has been demonstrated to be direct targets of p53. Ectopic expression of miR-34 induces apoptosis, cell-cycle arrest or senescence (Hermeking, 2009). Therefore, transcription factors which can alter p53 stability and activity can also modulate p53 regulated miRNAs. We have demonstrated that SMAR1 can increase p53 stability in the nucleus by displacing mdm2 and thus

\[\text{Figure 28. Model depicting the occupancy of SMAR1 on BAX and PUMA promoters along with p53, p300 and HDAC1. The binding of this repressor complex is maintained after mild DNA damage leading to inhibition in apoptosis and cell-cycle arrest. This binding is however lost after severe DNA damage wherein SMAR1 is sequestered by PML and other co-activators like hCAS and ASPP comes in to activate apoptosis.}\]
facilitating p53 phosphorylation at Ser-15. Simultaneously, SMAR1 also promotes p53 deacetylation by recruiting HDAC1 and interacting with p53 in a complex. This regulation is further complicated by the fact that SMAR1 can also promote p300 ubiquitination which acts as a cofactor for p53 acetylation upon DNA damage. In line with this, our miRNA microarray analysis in colon cancer cells revealed that SMAR1 can regulate the expression of miR-34a in a p53 dependent manner which we further validated by real time PCR. miR-34a targets a broad range of cell-cycle and apoptotic molecules including Bcl-2, Cyclin D1, Cyclin E2, CDK4, CDK6, c-MYC, N-Myc, SIRT1 and others. Thus, SMAR1 by targeting miR-34a can modulate p53 dependent cell-cycle arrest and apoptosis. Genome wide miRNA microarray analysis in presence and absence of SMAR1 suggest that SMAR1 regulates many miRNAs important for tumor metastasis and stem cell pluripotency. Since most of the cancers originate from stem cells, it is possible that dysregulation of SMAR1 regulated miRNAs can provide the stem cells oncogenic properties with increased renewal potential. Another interesting facet of SMAR1 regulation is its level in different grades of cancer cells. Studies from our lab have shown that SMAR1 expression is downregulated in higher grades of breast and other tumors. Considering the fact that SMAR1 regulates the expression of apoptotic molecules like Bax and Puma directly, the lowered expression of SMAR1 should be reflected in increased levels of Bax and Puma and therefore tumor cells should be sensitized to go to apoptosis. Infact we do observe in SW480 colon cancer cells that SMAR1 expression correlates with the levels of Bax and Puma. However, these tumor cells remain refractory to chemotherapeutic agents and are resistant to apoptosis. One possible explanation can be the fact that in most tumors proliferative signals driven by oncogenes override apoptotic signals which could be the apt reason for metastatic spread. Also, as previously explained molecules like SMAR1 although absent in high grade tumors are induced upon drug treatments which can then act on promoters like \textit{BAX} and \textit{PUMA} and contribute to resistance. Therefore, to develop effective therapeutic modules for treatment of a heterogeneous diseases like cancer, combinatorial approaches are necessary.