P53-DEPENDENT INDUCTION OF PVT1 AND MIR-1204
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Running head: p53 induces miR-1204
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Capsule
Background: p53 regulates myriad target
genes including non-coding RNAs that effect
cellular outcomes consistent with tumor
suppression.
Results: p53 induces expression of the Pvt1
locus which encodes both long non-coding
RNA and several microRNAs, one of which,
mir1204, is directly regulated by p53.
Conclusion: miR1204 may regulate key p53
outcomes including cell death.
Significance: p53-regulated miR1204
expression may contribute to tumor
suppression.

p53 is a tumor suppressor protein whose function
acts as a transcription factor to regulate (either
positively or negatively) a plethora of
downstream target genes. While its ability to
induce protein coding genes is well-
documented, recent studies have implicated p53
in the regulation of non-coding RNAs,
including both microRNAs (e.g. miR-34a), and
long non-coding RNAs (e.g. lincRNA-p21). We
have identified the non-protein coding locus
PVT1 as a p53-inducible target gene. PVT1, a
very large (>300 kb) locus located downstream
of c-Myc on chromosome 8q24, produces a wide
variety of spliced non-coding RNAs, as well as a
cluster of 6 annotated microRNAs: miR-1204,
mir-1205, mir-1206, miR-1207-5p, miR-1207-
3p and mir-1208. Chromatin
immunoprecipitation (ChIP), electrophoretic
mobility shift assay (EMSA) and luciferase-
assays reveal that p53 binds and activates a
canonical response element within the vicinity
of miR-1204. Consistently, we demonstrate the

p53-dependent induction of endogenous PVT1
transcripts and consequent up-regulation of
mature miR-1204. Finally, we have shown that
ectopic expression of miR-1204 leads to
increased p53 levels and causes cell death in a
partially p53-dependent manner.

p53 is a tumor suppressor protein whose function
is inactivated (by mutations or by other means)
with a high frequency in a wide spectrum of
human cancers (1,2). Several functions of p53
have been shown to contribute to its tumor
suppressive function. For example, the ability of
p53 to cause cell cycle arrest, senescence and cell
death affords protection against carcinogenesis
(3,4). To achieve such cellular programs, p53
predominantly acts as a sequence-specific
transcription factor. In response to cellular stress,
various signaling pathways converge to both
stabilize p53 levels and direct its transcriptional
activity toward specific target genes (4). The
protein products of many such target genes
directly participate in processes that arrest cell
cycle proliferation or result in apoptosis. For
example, p21/CDKN1A halts progression through
the cell cycle (5-7). PUMA and Noxa, on the other
hand, are p53-inducible targets that facilitate
apoptosis (8).

In addition to genes encoding protein
products, p53 also induces transcription from non-
protein coding genomic loci, including both
microRNAs and long intergenic non-coding RNAs
(lincRNAs). Accordingly, the regulation of such
non-protein coding genes by p53 is also thought to
contribute to p53’s ability to modulate various
cellular processes. For example, p53-inducible
miR-34a participates in cell cycle arrest,
senescence and apoptosis (9-12) by
downregulating the expression of proteins that
inhibit apoptosis (e.g. Bcl-2), promote cell cycle progression (e.g. b-Myb) or inhibit p53 activity (e.g. SIRT1) (13-15). p53 induces the transcription of various other microRNAs, including miR-145, miR-192 and miR-107 (16-20). These microRNAs counteract cell cycle progression, pluripotency, and angiogenesis through the regulation of c-Myc, Oct4/Sox2/Klf4, dihydrofolate reductase, and hypoxia-inducible factor 1-alpha. Recent work has revealed that p53 also induces the transcription of select long non-coding RNAs such as lincRNA-p21, which represents a key mediator of p53-dependent repression of pro-survival genes (21,22).

PVT1 represents a non-protein coding locus that yields a wide variety of non-coding RNAs, including a cluster of six microRNAs. In addition, extensive alternative splicing produces a complex array of mature transcripts that may function independently of the PVT1-encoded microRNAs. Despite the complex regulation of PVT1 RNAs, no protein product has yet been identified (23,24).

Many studies have implicated the PVT1 genomic region in cancer biology. PVT1 is a large (>300 kb) locus located adjacent to the c-Myc locus on human chromosome 8q24 (mouse chromosome 15). Translocation breakpoints within either the c-Myc or PVT1 locus are the characteristic lesions associated with Burkitt’s lymphoma and mouse plasmacytomas (23,24). Furthermore, global analysis of copy number alterations and gene expression changes in human tumors have identified PVT1 as a candidate oncogene. PVT1 copy number gain (e.g. double-minutes, amplifications) or overexpression of PVT1 has been demonstrated in breast cancer, ovarian cancer, pediatric malignant astrocytomas, acute myeloid leukemia and Hodgkin’s lymphoma (25-30). In congruence with such observations, PVT1 expression has been reported to be low in normal tissue, but highly expressed in many transformed cell lines (31). In addition, further work has demonstrated that the deregulation of PVT1 contributes to tumor survival and chemoresistance (30,32-34).

However, with no protein product or consensus long non-coding RNA, the functional implications of PVT1-deregulation has been difficult to discern. A study in 2008 identified seven microRNAs encoded by the PVT1 locus, six of which are annotated in miRBase (24). The characterization of these microRNAs may provide otherwise elusive mechanistic insights into biological functions of PVT1.

The six annotated microRNAs of the PVT1 locus are as follows: miR-1204, miR-1205, miR-1206, miR-1207-5p, miR-1207-3p and miR-1208 (23,24). The mature form of these microRNAs are expressed to varying extents in several cancer cell lines, including Burkitt’s lymphoma, breast and colon cancer cell lines (24). miR-1207-5p was also reported to be among the top 30 overexpressed microRNAs in a collection of colon cancer specimens (20). Nevertheless, the function of the PVT1-encoded microRNAs is largely unexplored, and two studies have suggested a potential role for miR-1204 in development/differentiation and senescence (24,35).

Given the frequency of alterations and contribution of PVT1 to human cancer and the ability of p53 to transcriptionally regulate non-coding RNAs, we have explored the potential relationship between the tumor suppressor p53 and the PVT1 locus. While PVT1 pro-tumorigenic roles have been described, surprisingly we have found that p53 acts to positively regulate transcription of this locus through a canonical p53-binding site.

Experimental procedures:
Cell culture and transfections: HCT116 (colorectal carcinoma), HCT116 p53-/-, RKO (colorectal carcinoma), SK-HEP1 (hepatocellular carcinoma) and WI38 (normal human fibroblasts) cells were maintained in DMEM/10% FBS. HCT116 wild type cells and their derivatives HCT116 p53-/- were generously provided by B. Vogelstein. EW36 (Burkitt’s lymphoma) cells were generously provided by Riccardo Dalla-Favera and maintained in RPMI/10%FBS. HCT116, HCT116 p53-/-, SK-HEP1, RKO, WI38, and EW36 cells were treated with Daunorubicin (0.22 μM; Sigma) or Nutlin-3a (10 μM; Sigma-Aldrich) for 24 hours unless otherwise noted.

microRNA mimics purchased from Qiagen were used individually at 20 nM (unless otherwise noted) and transfected into cells using Dharmafect 1 reagent (Dharmacon) according to the manufacturer’s instructions. Control microRNA (Qiagen All-Stars) represents a
negative control microRNA mimic. The microRNA inhibitor to miR-1204 was purchased from Qiagen (miScript inhibitors) and used at 50 nM and transfected into cells using Dharmafect 1 reagent (Dharmacon).

Western Blot Analysis: Cell extracts were analyzed according to standard Western blotting procedures through use of the Odyssey system (LI-COR). The monoclonal antibodies DO1 or 1801 were used to detect p53. The following polyclonal antibodies were purchased from either Santa Cruz, (sc-502, anti-p21; C-19), or Sigma: (Anti-Actin; A2066).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) to assess changes in the expression of PVT1 transcripts: To quantify relative changes in PVT1 ncRNA and primary microRNA expression RNA was isolated from cell cultures using the Qiagen RNasy Mini Kit. cDNA was synthesized by the use of the Qiagen QuantiTect Reverse Transcription Kit. PCR was carried out on an Applied Biosystems Step One Plus using the SYBR Green dye (Applied Biosystems). RNA expression was assayed in at least three experiments and normalized to RPL32 mRNA expression. Relative levels were calculated by the Comparative-Ct Method ($\Delta \Delta C_t$ Method). Error bars are derived from the standard deviations of multiple experiments. Levels of significance were calculated using a two-sided paired student’s t-test. Statistical significance ($p<0.05$) is denoted with an asterisk.

Primers were designed with Primer Express (Applied Biosystems):

| Primer   | Sequence                     |
|----------|------------------------------|
| RPL32 F  | TTCTTGGTCCACACCTCAAG         |
| RPL32 R  | TGTTAGCGATCTCGGCAC           |
| primiR-1204 F | GGCACAAGGCGCCAACCT   |
| primiR-1204 R | TCCCTCTGGGAATCTCAAG |
| primiR-1205 F  | CTTTCTGTCAACCCCTGTCT   |
| primiR-1205 R  | GCTTGCATATGGTTTGTG   |
| primiR-1206 F  | CAGTTATCATGCTCAGTTTTGTG |
| primiR-1206 R  | GCATAATTGCGCCGGTCA     |
| primiR-1208 F  | TGTTGGGCAACAATGAATCA     |
| primiR-1208 R  | CGGTGCTCCGGGTCTCAGTA    |
| PVT1 Exon 1A F | CGGCACCTTCCAGTGGAT |
| PVT1 Exon 1A R | CCGTGCTCCACAGTGTCACA |
| PVT1 Exon 1B F | TCCCGGAAAGCTCAGGAAG |
| PVT1 Exon 1B R | CAGGCCCAGGGTTTCTC |
| PVT1 Exon 4 F  | TGGCACAACAGCCCATATGA |
| PVT1 Exon 4 R  | ACGTGCCAAGCAGCTCAAA |

qRT-PCR to assess expression changes of mature microRNAs:

HCT116 cells were plated to ~50% confluency (24 hours) in 6 well plates with at least 3 replicates per sample. At 24 hours, cells were treated with 0.22 μM Daunorubicin (Sigma) or untreated to a total volume of 5 ml of RPMI1640 plus 10% HBS. After addition of Daunorubicin, cells were harvested for RNA at 24 hours using the mirVana miRNA isolation protocol (Ambion #1560). Mature miRNA was assayed by synthesis of cDNA using the miScript PCR system protocol (Qiagen) at 20 μl final volume (37°C, one hour). miRNA specific real time fluorescence amplification was carried out with 5’ gene specific primers and a 3’ universal primer annealing to the synthetic oligo dT sequence (Denaturation 15s, 94°C, Annealing 30s, 55°C, Extension 30s, 70°C) for 35 cycles. Data-points used are the average of 3-4 values and are normalized to Hs_RNU6B_3 (Qiagen MS00029204). Gene specific primers were obtained from Qiagen and are the following: Hs_miR-1204_1 (MS00014161) and Hs_miR-1207-5p_1 (MS00014189).

Flow Cytometry Analysis: Cell pellets were washed with PBS and fixed/permeabilized with 50% ice-cold ethanol. Pellets were washed and resuspended in 50 μg/ml Ribonuclease A and 62.5 μg/ml propidium iodide. Samples were analyzed on the FACSCalibur Becton Dickinson. The percentages of cells in various phases of the cell cycle were quantified using the ModFit LT Version 3.0 program. Error bars are derived from the standard deviations of multiple experiments. Levels of significance were calculated using a two-sided paired student’s t-test. Statistical significance ($p<0.05$) is denoted with an asterisk.

Chromatin Immunoprecipitation Analysis:
ChIP analysis was carried out as previously described (36). Briefly, p53 was immunoprecipitated with monoclonal antibodies DO1 and 1801. A no-antibody control was also included to confirm the specificity of the immunoprecipitation.

ChIP DNA was isolated with the Qiaquick PCR Purification Kit (Qiagen). Subsequent qPCR using 1/50th fraction of ChIP-enriched DNA, and 100 nM primers in a total volume of 20 μL was conducted to assess the amount of DNA that had been precipitated. Standard curves from 0.1–100 ng of sonicated genomic DNA were also amplified by qPCR as a reference. The following primers were used for qPCR:

PVT1 RE F: TGCACTCTGGCAGCGACAAG
PVT1 RE R: TTCGCTATAGCACCAGGACTGT
PVT1 -398 F: GGTAGAGGGCTACGGGAAAGA
PVT1 -398 R: GTGATGCCAGCCTGCGTTGT
PVT1 Exon 8 F: CCGGCTCAGGATCTACA
PVT1 Exon 8 R: TACAGCCTCAAGCTTCTCT
p21 3’RE F: CTGTCCTCCCGAGGTCA
p21 3’ RE R: ACCACAAAAGATCAAAGGTGAGTG

Luciferase Assays:
The following 70 nucleotide oligonucleotides were purchased from IDT and used to generate EMSA probes. The top and bottom strands were annealed to create double-stranded DNA. PVT1 RE wild-type probes were labeled with IRDye800 at the 5’ end of both top and bottom DNA strands. The region in bold denotes the p53-response element (PVT1 RE). Italicized nucleotides indicate mutations introduced in the invariant region of the PVT1 RE.

Wild-type PVT1 RE: 44 bp
Top strand:
CTCAGACCTATTTTGCTACACTGCGAGCGAC
AAGTTGAGACTTTGCTCAACTGACACAGTC
CTGTTGGCTACAT

Wild-type PVT1 RE: 44 bp
Bottom strand:
CTGTTGTCAAGTTGAACAGTCTCAACTTCTG
CGCTCAGAGTATGC

Mutant PVT1 RE: 44 bp
Top strand:
GCATACTGGCAGCGAAGTTGAGACTTT
GTCTCAACTTTGACACAG

Bottom strand:
CTGTTGTCAAGTTGAACAGTCTCAACTTCTG
CGCTCAGAGTATGC

Flag-tagged p53 protein was purified as previously described (37). Flag-tagged p53 (0 ng-50 ng) was incubated with 10 ng labeled wild-type PVT1 RE probe. Reaction mixtures were then run on 4% native acrylamide gels to assess the formation of p53-DNA complexes (up-shifted products). The labeled probe was visualized with fluorescence detection through use of the Odyssey system (LI-COR). In competition assays, unlabeled competitor probe was added in a range of 0-250 ng.

RESULTS

p53 induces transcription from the PVT1 locus.
To investigate the potential link between the tumor suppressor p53 and the \textit{PVT1} locus, we employed the isogenic colon carcinoma HCT116 and HCT116 p53-/- cell lines (38). HCT116 cells harbor wild-type p53 whose levels can be stabilized by a variety of mechanisms, including cellular stress (e.g. DNA-damage) or Nutlin (39) that specifically inhibits p53 binding to MDM2, the major negative regulator of p53 stability (Figure 1A). In HCT116 cells, activation of p53 by one such compound, Nutlin-3a, led to increased RNA levels of the \textit{PVT1} non-coding RNA (\textit{PVT1} ncRNA) (Figure 1B). The DNA-damaging agent Daunorubicin also caused an upregulation of \textit{PVT1} ncRNA, to an even greater extent than observed after Nutlin. Both Nutlin and Daunorubicin caused p53-dependent induction of \textit{PVT1} ncRNA, as HCT116 p53-/- cells either failed to induce, or showed only partial induction, of \textit{PVT1} ncRNA. Notably, the basal steady-state levels of \textit{PVT1} ncRNA are also reduced in HCT116 p53-/- cells.

As previously mentioned, \textit{PVT1} ncRNA undergoes extensive alternative splicing to produce a wide variety of mature transcripts. Figure 1B represents the induction of \textit{PVT1} ncRNA as measured by the RNA levels of exon 4, an internal exon of \textit{PVT1}. The fold change of \textit{PVT1} ncRNA induction as measured by other internal \textit{PVT1} exons was consistent with those changes observed for RNA molecules containing exon 4 (data not shown). However, a difference in exon usage after p53-dependent induction of \textit{PVT1} ncRNA can be delineated through the use of PCR primers that differentiate between the alternative exons 1A and 1B. Daunorubicin treatment led to a greater fold induction of Exon 1B than Exon 1A (Figure 1C). Nonetheless, the increase in both Exon 1A and Exon 1B-containing transcripts was abrogated in the absence of p53 (Figure 1C).

In order to further characterize the relationship between p53 and the \textit{PVT1} locus, we asked whether activated p53 could also induce \textit{PVT1} ncRNA transcripts in additional cell lines that harbor endogenous wild-type p53. As shown in Figure 1D, both Nutlin and Daunorubicin treatment led to increased \textit{PVT1} ncRNA levels in SK-HEP1 cells (hepatocellular carcinoma), WI38 cells (normal human fibroblasts), RKO cells (colon carcinoma) and EW36 cells (Burkitt's lymphoma). Thus, the regulation of \textit{PVT1} by p53 is not confined to a single tissue or cell type.

\textit{p53 binds a canonical response element at the \textit{PVT1} locus}

Given our observation that \textit{PVT1} ncRNA is induced in a p53-dependent manner, we investigated the mechanism by which p53 upregulates \textit{PVT1} ncRNA. To this end, we identified a putative p53-binding site using the p53 MH algorithm (40) which closely adheres to the p53 canonical consensus binding sequence. Depicted in Figure 2A, this binding site is located roughly 1200 base pairs (bp) downstream from the \textit{PVT1} transcriptional start site (TSS), and 172 bp upstream of the miR-1204 stem-loop sequence. Therefore, the binding site is positioned in between exon 1A and exon 1B, and in very close proximity to the miR-1204 hairpin structure. The \textit{PVT1} p53-response element is also conserved in mice, with only a slight variation of sequence and position (Figure 2A). Furthermore, an independent study that performed global analysis of p53-binding previously identified this region of the genome to be bound by p53 after 5-flurouracil treatment of HCT116 cells (41) (http://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=197390211&c=chr8&g=wgEncodeGisChipPetAll).

Using chromatin immunoprecipitation (ChIP) analysis, we found that this putative binding site was bound by endogenous p53. Furthermore, p53’s binding to the \textit{PVT1} p53-response element (\textit{PVT1} RE) was enhanced by Daunorubicin treatment (Figure 2B). p53 bound the \textit{PVT1} RE with an even greater affinity than another canonical p53 binding site, the p21 3’ RE (as measured by primers located -1391 bp upstream of the p21 transcription start site). However, p53 bound the p21 5’ RE (the stronger of the two well-established p53 REs in the p21 promoter) with two-fold greater affinity compared to the \textit{PVT1} RE (data not shown). The analyses of two regions within \textit{PVT1} where no consensus p53-binding site has been identified, one adjacent to the transcription start site (-398) and one at a site within a downstream exon (exon 8) revealed no significant p53 binding. Assessment of an additional negative control site 11 kilobase-pairs (kb) downstream of the p21 transcription start site further confirmed the specificity of the p53-immunoprecipitation (Figure 2B).
To confirm that the ability of p53 to bind to the PVT1 locus depended on the PVT1 RE that we had identified, we used electrophoretic mobility shift assays (EMSA) (Figure 2C,D). In order to perform this assay, we fluorescently labeled a 44-bp DNA fragment consisting of the PVT1 RE (20 bp) flanked by the DNA sequence from the surrounding genomic context (Figure 2, bottom panel). Using this probe, we assayed for the ability of purified Flag-tagged p53 to form a complex with the PVT1 RE. Increasing amounts of purified p53 caused an increased curve of up-shifted PVT1 RE, indicative of direct binding by p53 to the PVT1 RE. This p53-DNA complex could also be super-shifted by anti-Flag antibody, further confirming that this up-shifted band represents the p53/PVT1 RE complex (Figure 2C).

To test our hypothesis that p53 binding of the PVT1 RE required sequence specificity, we generated a PVT1 mutant RE. This mutated PVT1 RE was altered at four nucleotide residues out of 20, the invariant G/Cs of the consensus p53-binding site (Figure 2, bottom panel). We used excess amounts of either unlabeled wild-type PVT1 RE or unlabeled mutant PVT1 RE as potential binding competitors to the p53-PVT1 RE complex. These assays revealed that an increasing amount of unlabeled wild-type probe is able to compete away the interaction between p53 and the labeled PVT1 RE. However, an increasing amount of mutant PVT1 RE was unable to compete for p53 binding (Figure 2D). Thus, the interaction between p53 and the PVT1 RE requires sequence specificity.

p53 facilitates transactivation from the PVT1 response element

We extended our in vitro binding results by cloning either the wild-type or mutant PVT1 RE into luciferase-reporter constructs. These constructs were co-transfected with empty-vector or p53-containing plasmids into the p53-null H1299 lung-cancer cell line. As can be seen in Figure 3A, the addition of ectopic p53 caused a stimulation of transcription from the PVT1 RE, thus resulting in an approximate 15-fold increase in luciferase activity. However, no increase in luciferase activity was detected in cells co-transfected with p53 and mutant PVT1 RE, confirming that only the wild-type PVT1 RE is competent for transcriptional activity mediated by p53.

We also assessed the ability of endogenous p53 to activate transcription that is dependent on the wild-type PVT1 RE (Figure 3B). To do so, we transfected HCT116 or HCT116 p53--/- cells with the luciferase constructs containing wild-type or mutant PVT1 RE. Notably, transcription mediated by the wild-type PVT1 RE generated ~5-fold greater luciferase activity in HCT116 cells compared to their p53 null counterparts (HCT116 p53--/--). In addition, the luciferase activity driven by the wild-type PVT1 RE was ~24-fold higher than the mutant PVT1 RE in HCT116 cells. This difference in steady-state luciferase activity is primarily due to the presence of p53, as only a ~two-fold difference in luciferase activity between wild-type and mutant PVT1 REs was observed in HCT116 p53--/-- cells. Thus, the basal levels of endogenous p53 are sufficient to activate transcription from this response element in a sequence-specific manner.

Furthermore, stabilization of endogenous p53 levels by Nutlin treatment caused a greater than 2-fold increase in luciferase activity of the wild-type PVT1 RE, but no increase in activity of the mutant PVT1 RE. In addition, Nutlin treatment of HCT116 p53--/-- cells did not result in up-regulation of luciferase activity driven by the wild-type PVT1 RE (Figure 3B). Surprisingly, however, treatment of HCT116 cells with Daunorubicin failed to induce luciferase activity from the PVT1 RE (data not shown).

p53 up-regulates miR-1204

Given the observations that p53 both binds and stimulates transcription at the PVT1 locus, we wished to explore the function of p53-mediated activation of this locus. While p53 induces the transcription of PVT1 ncRNA (Figure 1), the PVT1 locus also encodes a cluster of microRNAs, miR-1204, miR-1205, miR-1206, miR-1207 5p, miR-1207 3p and miR-1208 (whose positions relative to PVT1 exons are depicted in Figure 4A). As these microRNAs are likely to play biologically significant roles, we investigated the ability of p53 to induce their transcription. To do so, we designed qPCR primers that amplify cDNA sequences corresponding to the primary microRNA molecules.
Treatment of HCT116 cells with either Nutlin or Daunorubicin resulted in an increase of all the primary microRNAs of this locus with the exception of miR-1208 (Figure 4B). Notably, HCT116 p53-/- cells failed to induce or only moderately induced primary microRNA levels. Although the different stimuli (Nutlin and Daunorubicin) used to activate p53 in HCT116 cells resulted in different levels of primary microRNA induction, within the context of either treatment the fold-induction of the primary microRNAs (except miR-1208) was similar, perhaps indicating that these primary microRNAs are transcribed in a polycistronic fashion.

As microRNAs function as mature 19-22 nucleotide species, we aimed to determine whether the transcriptional induction of the primary transcripts was sufficient to up-regulate mature microRNA levels. To do so, we employed a qPCR strategy specific for mature microRNA detection (Qiagen miScript). Consistent with the induction of primary miR-1204, Daunorubicin treatment of HCT116 resulted in a ~7.5 fold-induction of mature miR-1204. In contrast, miR-1207-5p was not induced under these conditions (Figure 4C). However, the induction of other microRNAs of the PVT1 locus, including miR-1206, miR-1207-3p and miR-1208, was observed following daunorubicin treatment (Supplemental Figure S1). Finally, Daunorubicin also caused induction of the canonical p53-inducible microRNA miR-34a, but did not alter the levels of an unrelated small nucleolar RNA, SnoRD48 (data not shown).

As miR-1204 is strongly induced by p53 in HCT116 cells, we assessed the ability of p53 to regulate primary miR-1204 transcripts in other tissue types. To do so, we again employed SK-HEP1, WI38, RKO and EW36 cells as in Figure 1D. Similar to HCT116 cells, all such cell lines displayed up-regulation of primiR-1204 in response to Daunorubicin (Figure D). Nutlin-treatment in the absence of DNA-damage also caused a significant increase in the levels of primiR-1204 in these cell lines (except in SK-HEP1 cells), thus confirming that primiR-1204 induction can be facilitated by activated p53 (Figure 4D). Furthermore, Daunorubicin treatment of EW36 cells led to increased levels of mature miR-1204 (as well as miR-1207-3p and miR-34a (Figure 4E)), again suggesting that an increase of primiR-1204 levels is sufficient to increase levels of the mature miR-1204.

**Ectopic miR-1204 expression causes anti-proliferative phenotypes**

Since levels of mature miR-1204 are increased by activation of p53, we next examined the phenotypic consequence of increased miR-1204 expression, as well as the other microRNAs of the PVT1 locus. In order to do so, we transfected microRNA mimic molecules into various cancer cell lines, including HCT116 cells. In HCT116 cells, ectopic expression of miR-1204 resulted in a significant amount of cell death, as indicated by the percentage of cells measured in sub-G1, compared to cells transfected with negative control RNA (Figure 5A). Notably, this level of cell death is comparable to that observed after ectopic expression of miR-34a. Two additional observations strengthen the conclusion that miR-1204 induces death of HCT116 cells: First, the overexpression of an unrelated microRNA, miR-21, did not result in cell death (Supplemental Figure S2A), and second, the increase in cell death upon introduction of ectopic miR-1204 could be neutralized by an antisense microRNA inhibitor of miR-1204, suggesting that miR-1204 kills cells via specific pathways, and not through toxic accumulation of the RNA-based mimic (Supplemental Figure S2B). In addition, microRNA mimics for miR-1205, miR-1207-5p and miR-1207-3p also induced substantial cell death. Interestingly, however, the miR-1206 mimic did not induce cell death, nor did it alter the cell cycle profile of HCT116 cells (Figure 5A).

As miR-34a is known to enhance p53 levels and cause cell death that is at least partially dependent upon expression of p53 (15), we investigated whether or not ectopic miR-1204 could also increase p53 protein levels. Western blot analysis revealed that ectopic miR-1204 indeed increased p53 protein levels (~1.7 –fold induction), though to a lesser extent than the increase observed after ectopic miR-34a expression (Figure 5B). Nonetheless, ectopic miR-1204 caused significantly greater cell death in HCT116 cells than in HCT116 p53-/- cells (Figure 5C).

Note that in other cancer cell lines, the ectopic expression of miR-1204 did not lead to cell death, but in fact resulted in cell cycle arrest at
the G1 phase. For example, two diverse cancer cell lines, RKO (colon cancer) and SK-HEP1 (liver cancer), were arrested in G1 after miR-1204 transfection (Supplemental Figure S3A). In these settings, a miR-1206 mimic again showed no phenotypic effect on the cell cycle profile. Finally, the overexpression of miR-1204 also decreased the viability of normal human fibroblast cells (WI38), suggesting that its anti-proliferative effects are not a consequence of interactions with cellular pathways that have become deregulated through the process of tumorigenesis (Supplemental Figure S3B).

DISCUSSION

We have characterized a novel p53-inducible target: the non-protein coding PVT1 locus. p53 stimulates transcription of PVT1 ncRNA (exon-containing) and primary microRNAs through a canonical response element adjacent to the PVT1 Exon 1B and miR-1204. The binding of p53 to this site is likely to explain the marked p53-dependent induction of both the PVT1 Exon 1B and the mature miR-1204. That is, transcription from this specific genomic region would be enhanced by p53 binding with corollary up-regulation of miR-1204; perhaps p53-binding results in the usage of an alternative transcription start site directly upstream of Exon 1B.

In addition, p53 may regulate mature miR-1204 by directing microRNA biogenesis events. Intriguingly, p53 has been linked to processing efficiency of microRNAs through an interaction with Drosha (42), and thus it is tempting to speculate that p53 binding to the genomic region adjacent to miR-1204 somehow facilitates co-transcriptionally-dependent microRNA processing events. As microRNA processing is heavily regulated, levels of primary microRNA do not necessarily dictate levels of mature microRNA. In fact, our data demonstrate that the induction of primiR-1207 does not lead to induction of mature miR-1207-5p in HCT116 cells. However, this could be explained by preferential incorporation of miR-1207-3p into the RISC complex, with subsequent degradation of miR-1207-5p. Consistent with this hypothesis, we do observe that mature miR-1207-3p levels are increased by Daunorubicin treatment in both HCT116 and EW36 cells.

Irrespective of the mechanism by which p53 operates, our results have revealed that mature miR-1204 is induced by DNA-damage. Yet, HCT116 colon cancer cells contain significantly lower levels of endogenous mature miR-1204 than miR-34a (data not shown). Thus a ~7.5 fold-induction represents a drastic change in the cellular levels of miR-1204 that may significantly impact cellular fate. As ectopic miR-1204 stabilizes p53 and causes cell death, basal levels of miR-1204 may be tightly regulated. Alternatively, miR-1204 may be expressed more highly in other cell lineages and exert its biological function(s) within those settings. Future work will determine the expression pattern of miR-1204 across multiple tissue types in an effort to formulate hypotheses about miR-1204 function(s).

One such hypothesis is that miR-1204 functions in B-cell differentiation, as primary miR-1204 levels rise with increased differentiation (24) and miR-1204 and light chain immunoglobulin expression levels appear to be correlated as well (24). Intriguingly, p53 is also involved in B-cell differentiation (43-45). Specifically, gamma-irradiation causes accumulation of p53 in pre-B-cells that leads to further differentiation that is consistent with increased immunoglobulin kappa light chain expression (45). As such, the p53-induced expression of miR-1204 may play a key role in B-cell differentiation and the regulation of immunoglobulin light chain expression.

Future work will also address the hypothesis that p53-dependent induction of miR-1204 leads to cellular senescence. A recent study found miR-1204 to be the microRNA most induced by the onset of replicative senescence in fibroblasts (35). As such, p53 transcriptional regulation of the PVT1 locus may represent an additional means by which p53 limits the replicative lifespan of normal cells.

Delineating the extent of p53-dependent PVT1 induction in response to different stimuli has already begun to provide insight into both PVT1 regulation and function. Our data reveals that DNA-damage activates p53 more robustly to upregulate PVT1 ncRNA/primary microRNAs than does stabilization of p53 in the absence of genotoxic stress (Nutlin-treatment). As DNA-damage cooperates with p53 to induce the PVT1 locus, PVT1-encoded transcripts (microRNA or
otherwise) may function in DNA-damage repair pathways, or in apoptosis-related pathways that initiate when resulting cellular insults have become too severe. Surprisingly, Daunorubicin did not stimulate transcriptional activity from a luciferase construct containing the PVT1 p53 RE (data not shown). This suggests that additional factors that bind outside of the immediate region of the PVT1 RE contribute to the p53-mediated activation of endogenous PVT1.

As deregulation of the PVT1 locus is observed in a wide array of cancers, our observations implicating p53 in the control of this putative oncogenic locus are seemingly contradictory. However, pro-survival roles for p53 have been previously described. For example, p53 is able to induce the transcription of death decoy receptors, DcR1/TRID and DR2/TRUNDD (46) which compete with functional death receptors (like p53-inducible KILLER/DR5 (47)) and thereby inhibit extrinsic apoptosis. Furthermore, p53 can blunt apoptosis by the intrinsic pathway as well. Here, p53 induces the transcription of Slug, a protein that suppresses transcription of the p53-inducible target, and potent pro-apoptotic gene, PUMA (48,49). Finally, p21, a major p53 target, also plays anti-apoptotic roles (50).

Thus, it is not without precedence that p53 induces genes that counteract cell death, and all indications to date suggest that the PVT1 gene would fall under this category. However, the mechanism by which PVT1 opposes cell death has not been explored. Many non-coding RNAs interact with and direct gene silencing through interaction with the polycomb repressive complex (51) and recent work has suggested that the deregulation of ncRNA-directed gene silencing contributes to tumorigenesis (52). As such, the deregulation of PVT1 in cancer cells may contribute to aberrant gene silencing.

Due to the close proximity of the PVT1 and c-Myc loci, gross alterations of PVT1 could affect c-Myc expression (23). While many examples of plasmacytomas with translocations of PVT1 are accompanied by up-regulated expression of c-Myc, there are nevertheless, examples that lack this correlation (53,54). However, computational analysis has suggested that PVT1 and c-Myc operate within the same oncogenic pathway and expression studies with T cell lymphomas have uniformly shown up-regulated expression of c-Myc with either PVT1 or c-MYC based insertions (23). While such evidence points toward cooperation between PVT1 and c-Myc, the observations that ncRNAs often repress transcription warrants further exploration into the functional and mechanistic interface between PVT1 ncRNA and c-Myc expression. For example, silencing experiments of PVT1 in ovarian and breast cancer cell lines have shown induction of apoptosis in the absence of any perceived changes of expression in c-Myc (30). As p53 is known to repress c-Myc through multiple mechanisms (17,55), future studies using antisense PVT1 RNA will aim to determine whether p53-dependent induction of PVT1 ncRNA positively or negatively regulates c-Myc expression. Examining the consequences of PVT1 ncRNA induction on c-Myc expression (as well as genome-wide mRNA expression changes) will help to elucidate the mechanism by which PVT1 promotes cell survival.

Previous experiments linking PVT1 with cell survival have utilized an RNA interference approach targeting exonic regions of the PVT1 ncRNA (30,34). It is uncertain (and perhaps unlikely) that this approach inhibited expression of the PVT1-encoded microRNAs. In contrast to a pro-survival role for PVT1, our data suggests that miR-1204 plays an anti-proliferative role, inducing cell death apoptosis or cell cycle arrest. These phenotypes are more in line with the well-established roles of p53, and as such miR-1204 may represent a more obvious functional target of p53 at the PVT1 locus. Intriguingly, miR-1204 expression also increases p53 levels, suggesting a positive feedback loop that may serve to enhance or fine-tune p53 activity.

If miR-1204 is the functional target of p53 at the PVT1 locus, the additional ncRNA transcripts (those that include exonic PVT1 sequences or other miRs) may represent either transcriptional by-products of miR-1204 regulation, or may represent additional functional targets. Thus, activation of the PVT1 locus may represent a double-edged sword in which induction of miR-1204 may promote cell death (or arrest), while the PVT1 ncRNAs may prevent cell death. Thus, p53 may exert control over the ratio of such transcripts to fine tune cellular responses to certain stimuli. In this way, the PVT1 locus...
could be viewed as a molecular switch between life and death. While still speculative, cancer cells may have manipulated the balance of such transcripts through deregulation of the locus, producing more PVT1 pro-survival ncRNAs than anti-proliferative miR-1204 (and perhaps the other microRNAs of this locus). Future experiments will begin to test this hypothesis in order to understand the precise interplay between the microRNAs and other ncRNAs of the PVT1 locus, and ultimately how they function within the context of the p53-pathway.

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FOOTNOTES
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FIGURE LEGENDS

Figure 1. p53 stimulates transcription at the PVT1 locus. A. HCT116 or HCT116 p53-/- cells were treated for 24 hours with either Nutlin-3a (10 µM) or Daunorubicin (0.22 µM). Levels of p53, p21 and actin (loading control) were detected by immunoblotting. B. Quantification of PVT1 ncRNA by qRT-PCR of HCT116 cells (hatched bars) and HCT116 p53-/- cells (black bars) as treated in (A.) Asterisks represent significant induction (p<0.05, two-sided paired student’s t-test) compared to DMSO-treated HCT116 cells. C. Quantification of PVT1 alternative exons 1A (hatched bars) and 1B (black bars) by qRT-PCR in HCT116 and HCT116 p53-/- cells treated as in (A). Asterisks represent significant induction (p<0.05, two-sided paired student’s t-test) compared to DMSO-treated HCT116 cells. D. SK-HEP1, WI38, RKO and EW36 cells were treated for 24 hours with either Nutlin-3a (10 µM) or Daunorubicin (0.22 µM). Quantification of PVT1 ncRNA by qRT-PCR following treatment with DMSO (hatched bars), Nutlin (gray bars) or Daunorubicin (black bars). Asterisks represent significant induction (p<0.05, two-sided paired student’s t-test) compared to DMSO-treated cells.
Figure 2. p53 directly binds to a canonical response element within the \textit{PVT1} locus in a sequence specific manner. \textit{A}. Schematic diagram of the p53 RE in both the human and mouse \textit{PVT1} loci. Gray nucleotides in the human sequence represent deviations from the canonical p53 response element. Italicized nucleotides in the murine sequence denote changes from the human sequence. \textit{B}, HCT116 cells were untreated (hatched bars) or treated for 8 hours with 0.22 μM Daunorubicin (black bars) and subjected to Chromatin Immunoprecipitation (ChIP). qPCR was used to quantify DNA fragments co-immunoprecipitated with p53, or with beads alone (gray bars). Three primer pairs within the \textit{PVT1} locus were used to assay for p53 binding, -398, \textit{PVT1} RE, and Exon 8. Two additional primer pairs at the \textit{CDKN1A}/p21 locus, the 3’ p53 RE (-1391), and +11 kb (downstream of the \textit{CDKN1A}/p21 gene), were used as positive and negative controls, respectively. The extent of relative p53-binding to each genomic region is graphed. \textit{C}. EMSA assay: an increasing amount of purified, flag-tagged p53 was incubated with labeled \textit{PVT1} RE probes. Such reactions were loaded onto 4% native polyacrylamide gels. Only the up-shifted probe is displayed. Flag antibody was added into one reaction to ensure the specificity of p53-DNA complexes. \textit{D}. Competition EMSA: Excess unlabelled \textit{PVT1} RE (either wild-type or mutant) probes were added in increasing amounts to reactions described in (C). The bottom panel displays the sequences of both wild-type and mutant \textit{PVT1} RE. Gray nucleotides indicate mutations of the probe at invariant residues of the p53-consensus sequence.

Figure 3. p53 facilitates transcriptional activity from the \textit{PVT1} RE. Reporter constructs were engineered to contain either wild-type or mutant \textit{PVT1} RE that drive the transcription of the Firefly luciferase gene. \textit{A}. Empty vector or ectopic p53 were co-transfected with the luciferase reporter constructs in p53-null H1299 cells. Relative luminescence is quantified: empty vector (hatched bars), p53 (black bars). Asterisks represent significant induction of luciferase activity (p<0.05, two-sided paired student’s t-test) compared to H1299 cells transfected with empty vector. \textit{B}. HCT116 (hatched bars) or HCT116 p53-/- cells (black bars) were transfected with either wild-type or mutant \textit{PVT1} RE containing-luciferase constructs. Relative luminescence is quantified. Asterisks represent significant induction of luciferase activity (p<0.05, two-sided paired student’s t-test) compared to DMSO-treated HCT116 cells.

Figure 4. p53 induces primary and mature miR-1204. \textit{A}. Schematic of the human \textit{PVT1} locus that depicts the location of primary microRNAs relative to \textit{PVT1} exons. \textit{B}. HCT116 or HCT116 p53-/- cells were treated for 24 hours with either Nutlin-3a (10 µM) or Daunorubicin (0.22 µM). Relative levels of the primary microRNA transcripts were quantified by qRT-PCR: The relative quantification of primiR-1204 (hatched bars), primiR-1205 (black bars with white dots), primiR-1206 (horizontal striped bars), primiR-1207 (gray bars) and primiR-1208 (black bars) is displayed. Asterisks represent significant induction (p<0.05, two-sided paired student’s t-test) compared to DMSO-treated HCT116 cells. \textit{C}. HCT116 cells were treated with 0.22 µM Daunorubicin. The microRNA enriched RNA fraction was subjected to Qiagen miScript cDNA synthesis and qRT-PCR. Relative quantification of mature miR-1204 and miR-1207-5p is displayed and represents the fold induction of each RNA species after Daunorubicin treatment. The asterisk represents a significant difference (p<0.05, two-sided paired student’s t-test) between the induction ratio of miR-1204 compared to miR-1207-5p. \textit{D}. SK-HEP1, WI38, RKO and EW36 cells were treated for 24 hours with either Nutlin-3a (10 µM) or Daunorubicin (0.22 µM). Quantification of \textit{PVT1} ncRNA by qRT-PCR following treatment with DMSO (hatched bars), Nutlin (gray bars) or Daunorubicin (black bars). Asterisks represent significant induction (p<0.05, two-sided paired student’s t-test) compared to DMSO-treated cells. \textit{E}. EW36 cells were treated for 24 hours with Daunorubicin (0.22 µM). Quantification of mature miR-1204, mature miR-1207-3p and mature miR-34a (miScript PCR) is displayed.
Figure 5. Ectopic miR-1204 leads to increased p53 levels and causes cell death. A. HCT116 cells were transfected with 20 nM microRNA mimics. Cells were harvested 72 hours post-transfection and subjected to propidium iodide staining and FACs analysis. The cell cycle profile following transfection of each mimic is shown. The percentage cell death, as quantified by Sub-G1 content, is graphed. Asterisks represent significant induction of cell death (p<0.05, two-sided paired student’s t-test) compared to negative control transfected HCT116 cells. B. HCT116 cells were transfected as in (A). The levels of p53 and actin (loading control) were detected by immunoblotting. Quantification using the LI-COR Odyssey system is provided and represents that average fold-induction of p53 protein levels. Error bars represent that standard deviation of multiple experiments. Asterisks represent significant induction of p53 protein levels (p<0.05, two-sided paired student’s t-test) compared to the control microRNA mimic. C. HCT116 cells (hatched bars) or HCT116 p53-/- cells (black bars) were transfected and subjected to FACs analysis as in (A). The percentage cell death, as quantified by Sub-G1 content, is graphed. Asterisks represent a significant difference (p<0.05, two-sided paired student’s t-test) in the level of cell death between HCT116 cells and HCT116 p53-/- cells.
Barsotti et al Figure 1
A. **p53 consensus binding site:** RRRCWWGGYY, RRRCWWGGYY
   - Human Chr 8q24
   - TSS
   - miR-1204
   - p53 RE
   - 1238 bp
   - 172 bp
   - TGACAAGTTT, GGCTTT
   - Mouse Chr 15
   - TSS
   - miR-1204
   - p53 RE
   - 1221 bp
   - 11 bp

B. **HCT116 p53 ChIP:**
   - PVT1 locus
   - p21 locus
   - Relative Binding
   - Untreated
   - Daunorubicin
   - No Antibody

C. **EMSA: p53-curve**
   - Anti-Flag:
   - Flag-p53:
   - Supershifted probe
   - Upshifted probe
   - Barsotti et al Figure 2

D. **Competition EMSA**
   - Cold competitor probe
   - Wild-type
   - Mutated
   - PVT1 p53 RE
A. Ectopic p53

H1299: Luciferase Assay

B. Endogenous p53

HCT116: Luciferase Assay

Barsotti et al Figure 3
A. Chr 8q24: PVT1 locus

B. qPCR: Primary microRNA transcripts of PVT1

C. qPCR: HCT116 mature microRNA transcripts

D. qPCR: primIR-1204

E. qPCR: EW36 mature microRNA transcripts

Barsotti et al Figure 4
A. HCT116: Cell death

B. Ctrl 1204 34a

C. Cell-death

Barsotti et al Figure 5
