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Identification and characterization of *RTVP1/GLIPR1*-like genes, a novel p53 target gene cluster

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Abstract

Our previous finding of *RTVP1* (*GLIPR1*) as a p53 target gene with tumor suppressor functions prompted us to initiate a genome-wide sequence homology search for *RTVP1/GLIPR1*-like (GLIPR1L) genes. In this study we report the identification and characterization of a novel p53 target gene cluster that includes human *RTVP1* (*hRTVP-1*) together with two *GLIPR1L* genes (*GLIPR1L1* and *GLIPR1L2*) on human chromosome 12q21 and mouse *Rtvp1* (*mRTVP-1* or *Glipr1*) together with three *Glipr1*-like (Glipr1l) genes on mouse chromosome 10D1. *GLIPR1L1* has two and *GLIPR1L2* has five differentially spliced isoforms. Protein homology search revealed that *hRTVP-1* gene cluster members share a high degree of identity and homology. *GLIPR1L1* is testis-specific, whereas *GLIPR1L2* is expressed in different types of tissues, including prostate and bladder. Like *hRTVP-1*, *GLIPR1L1* and *GLIPR1L2* are p53 target genes. The similarities of these novel p53 target gene cluster members in protein structure and their association with p53 suggest that these genes may have similar biological functions.

Keywords: *GLIPR1*; *RTVP1*; *GLIPR1L1*; *GLIPR1L2*; p53 target genes; RTVP1/GLIPR1 gene cluster; Prostate cancer; Bladder cancer

The tumor suppressor gene *p53* is the most commonly mutated gene in human cancer. The protein that it encodes is a key regulator of cell cycle, DNA repair, senescence, cell differentiation, and apoptosis [1–5]. Mutations in the *p53* gene can lead to critical cellular malfunctions that contribute to malignant growth and metastasis [6–9]. In prostate cancer, *p53* mutations are found in relatively low frequencies in early, localized tumors, but occur in significantly higher frequencies in advanced, hormone-refractory metastatic tumors [7,8,10,11]. The established association between loss of *p53* function and prostate cancer metastasis prompted us to pursue the identification, characterization, and functional analysis of p53 target gene in prostate cancer.

Human *RTVP1/GLIPR1* (*hRTVP-1*, HGNC approved gene symbol: *GLIPR1*) was initially identified in human glioblastoma and was referred as *GLIPR1* (glioma pathogenesis-related protein 1) [12] or *RTVP1* (related to testes-specific, vespid, and pathogenesis protein 1) [13]. It was also identified as a marker of myelomocytic differentiation in macrophage [14]. The RTVP-1 protein shares significant sequence homology with members of the pathogenesis-related protein (PR) superfamily that is characterized by a PR domain harboring six blocks of consensus sequences [15,16] and the cysteine-rich secretory protein (CRISP) family that is characterized by a PR domain together with a distinct cysteine-rich domain at the carboxyl terminus [17,18]. PR/CRISP proteins are involved in a variety of physiological processes, including innate immunity, male fertility, fertilization, inhibition of calcium/potassium channels and proteases, and interaction with immunoglobulin proteins [17–23].

Mouse *Rtvp1/Glipr1* (*mRTVP-1*, approved gene symbol: *Glipr1*) was one of the p53 target genes identified in our initial screening using differential display PCR [24]. Functional analysis demonstrated that *mRTVP-1* and *hRTVP-1* have proapoptotic activities in prostate and bladder cancer cells [24,25]. Analysis of *hRTVP-1* expression showed that the gene is down-regulated through gene methylation in prostate cancer tissues [25]. Adenoviral vector-mediated gene delivery of *mRTVP-1* in an orthotopic, metastatic mouse model of prostate

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cancer significantly reduced primary tumor wet weight and spontaneous metastasis to lung [26].

The identification of *RTVP1* as a p53 target gene with tumor suppressor functions encouraged us to initiate a genome-wide sequence homology search for *RTVP1/GLIPR1*-like (GLIPR1L) genes. In this study we report the identification and characterization of a novel p53 target gene cluster that includes *hRTVP-1* together with two human GLIPR1L (*GLIPR1L1* and *GLIPR1L2*) genes on human chromosome 12q21 and *mRTVP-1* together with three Glipr1l genes on mouse chromosome 10D1. Our analysis of this novel p53 target gene cluster provides new and important information for the future functional studies of this gene cluster for their roles in the development, progression, and potentially treatment of prostate cancer and other malignancies.

Results

Identification of GLIPR1L and Glipr1l genes

A genome-wide sequence homology search using *hRTVP-1* and *mRTVP-1* sequences led us to identify two human DNA sequences (hypothetical proteins MGC26856 (*GLIPR1L1*) and MGC39497 (*GLIPR1L2*) and three mouse DNA sequences (1700011E04Rik (*Glipr1l1*), 4921508O11Rik (*Glipr1l2*), and LOC544736 (*Glipr1l3*)) that share significant DNA sequence homology with *RTVP1*. Interestingly, these *GLIPR1L* and *Glipr1l* genes are located very near *RTVP1* (within a 170 kb range) and constitute gene clusters on human chromosome 12q21 and mouse chromosome 10D1, respectively (Figs. 1A and 1B). Full-length *GLIPR1L1* and *GLIPR1L2* cDNAs were cloned from human testis RNA using RT-PCR together with primers derived from sequences of hypothetical proteins MGC26856 and MGC39497. Two transcripts were obtained from *GLIPR1L1* RT-PCR and subsequently identified as two differentially spliced mRNAs from the same gene. These two *GLIPR1L1* isoforms were designated as *GLIPR1L1α* and *GLIPR1L1β*. The only transcript obtained from *GLIPR1L2* RT-PCR was designated as *GLIPR1L2α*. To isolate potential isoforms of *GLIPR1L1* and *GLIPR1L2*, cDNAs of *GLIPR1Lα* and *GLIPR1L2α* were used as probes to screen a human testis cDNA library. After three rounds of screening, 30 positive clones were isolated for *GLIPR1L1* and 37 positive clones were isolated for *GLIPR1L2*. The 30 *GLIPR1L1* clones were sorted into two groups by *Pst*I and *Sfi*I restriction mapping and the 37 *GLIPR1L2* clones were sorted into five groups by *Nde*I and *Sfi*I restriction mapping. Three clones from each group were sequenced and the DNA sequences were mapped using available genomic sequence. The results confirmed the two isoforms of *GLIPR1L1* (*GLIPR1L1α* and *GLIPR1L1β*) and revealed four additional isoforms of *GLIPR1L2* (*GLIPR1L2β, γ, δ*, and ε). The structures and alternative transcripts of *GLIPR1L1* and *GLIPR1L2* are summarized in Figs. 2A and 2B.

Sequence comparison of RTVP1 gene cluster proteins

A sequence homology comparison with known protein sequences revealed that hRTVP-1 shares significant homology with PR/CRISP proteins within the PR domain characterized by an N-terminal signal peptide, an N-terminal sequence immediately following the signal peptides, six blocks of consensus sequences (including two extracellular protein signature motifs), and distinct C-terminal sequences (Fig. 3).

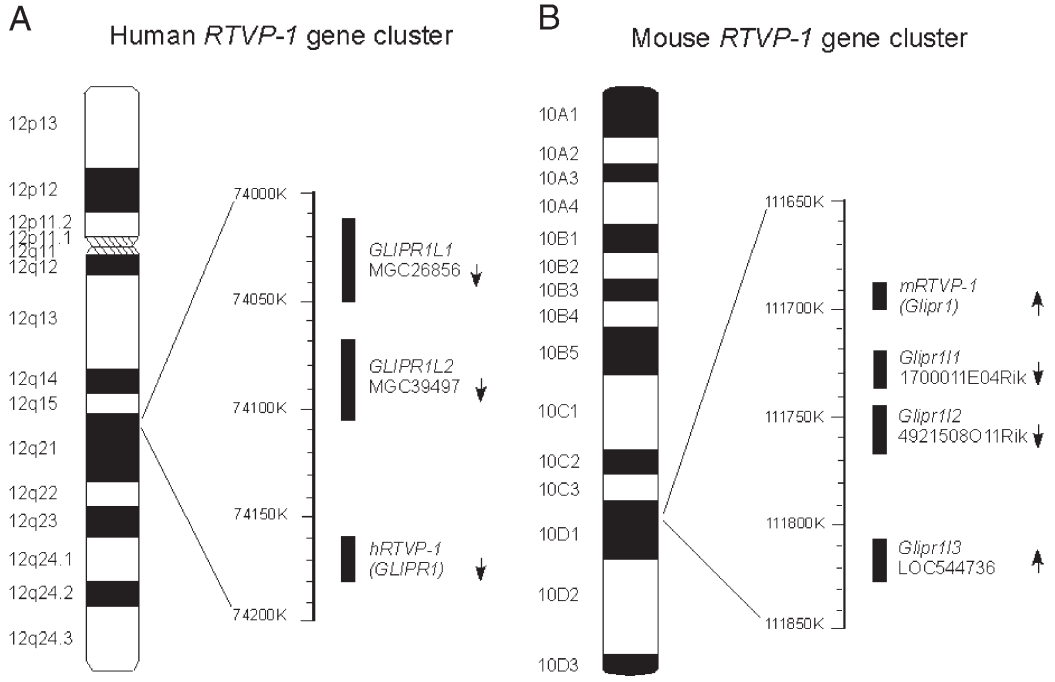

Fig. 1. Chromosome localization of *RTVP1* gene cluster. (A) Human *RTVP1* gene cluster on human chromosome 12q21, which includes *hRTVP-1* (*GLIPR1*), *GLIPR1L1*, and *GLIPR1L2*. (B) Mouse *RTVP1* gene cluster on mouse chromosome 10D1, which includes *mRTVP-1* (*Glipr1*), *Glipr1l1*, *Glipril2*, and *Glipr1l3*. The arrows indicate the direction of gene transcription.

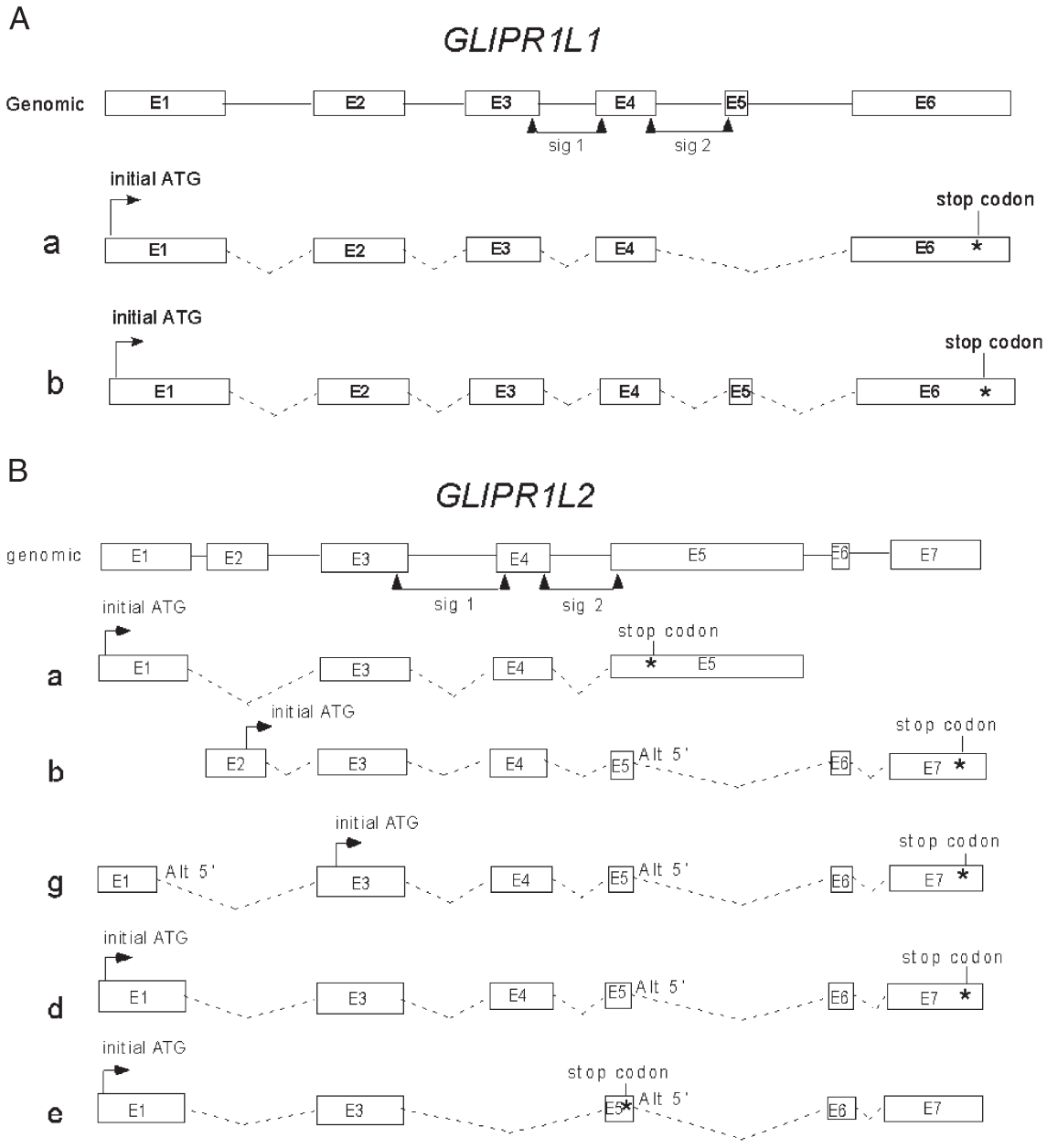

Fig. 2. Alternative transcripts of *GLIPR1L1* and *GLIPR1L2*. (A) *GLIPR1L1* isoforms; (B) *GLIPR1L2* isoforms. E stands for exon. Sig stands for extracellular protein signature motif. Alt 5′ represents alternative splicing site.

A comparison of deduced protein sequences among *RTVP1* gene cluster members showed that member proteins share 35–95% identity and 44–98% homology (Fig. 4 and Table 1). Most members have a putative N-terminus signal peptide and all members, except for GLIPR1L2ε, contain two extracellular protein signature motifs (Figs. 4 and 5). These two characteristics (signal peptide and extracellular protein signature motifs) suggest that most of the gene cluster proteins are located on the surface of the cell membrane or secreted. hRTVP-1; GLIPR1L2β, γ, δ; mRTVP-1; and Glipr1l2 contain a transmembrane domain, whereas GLIPR1L1α and β, GLIPR1L2α and ε, Glipr1l1, and Glipr1l3 do not have this domain (Figs. 4 and 5), suggesting different capacities for secretion. Our experience in recombinant protein production has shown that GLIPR1L1α and GLIPR1L1β have significantly higher secretory activities compared to hRTVP-1 (data not shown). Interestingly, GLIPR1L2β, γ, and δ and Glipr1l2 have an unusual glutamate-rich domain (ERD) that contains 32 glutamate residues within a sequence of 53 amino acid residues (Figs. 4 and 5). The function of the ERD is unclear.

In our data search, we also found a human sequence that is located at chromosome 9p12–p13 and shares slightly lower identity (32%) and homology (52%) with hRTVP-1. This gene was identified and cloned in 2002 and was termed as C9orf19 [40] or Golgi-associated PR-1 protein [27]. Although the crystal structure of this protein was resolved recently [28], little functional information is available at this point. We did not include this gene in the present study since our focus was limited to the gene clusters on human chromosome 12 and mouse chromosome 10.

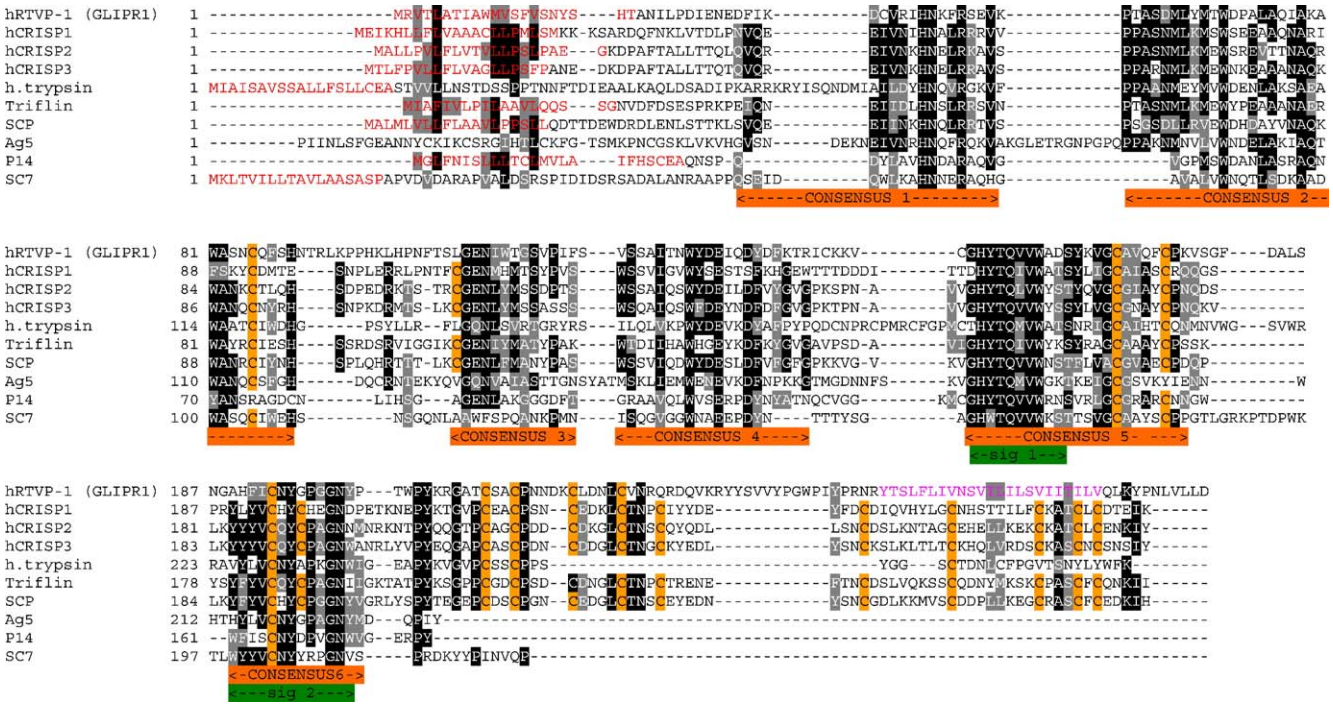

Fig. 3. Homology comparison of hRTVP-1 with PR/CRISP proteins. Identity of amino acid residues is indicated with black-shaded columns, homology of amino acid residues is indicated with gray-shaded columns, cysteines are indicated with orange-shaded columns. Six consensus blocks and extracellular protein signature motifs within PR domain are indicated under the corresponding sequences. Signal peptides are indicated using orange letters and the transmembrane domains are shown using pink letters.

Expression of hRTVP1 cluster genes in different human tissues

To determine expression levels for *hRTVP-1* and *GLIPR1Ls* in different human tissues, we analyzed mRNA levels of these genes in human prostate, kidney, bladder, testis, lung, and bone marrow using QRT-PCR. The results showed that *hRTVP-1* is expressed in all six tissues examined, with relatively higher mRNA levels in prostate, testis, lung, and bone marrow and relatively lower levels in kidney and bladder (Fig. 6). In contrast to the widespread expression of *hRTVP-1*, the expression of *GLIPR1L1* is highly tissue-specific, with very high mRNA levels in testis, a trace amount in bladder, and undetectable expression in prostate, kidney, lung, and bone marrow. Similar to *GLIPR1L1, GLIPR1L2* is highly expressed in testis; however, relatively low level *GLIPR1L2* expression was detected in the other five tissues (Fig. 6). Our data demonstrating a unique *hRTVP-1* expression pattern and tissue-specific expression of *GLIPR1L1* are consistent with a recently published DNA microarray survey of gene expression in normal human tissues [29].

RTVP1 gene cluster members are p53 target genes

Our initial studies demonstrated that mouse and human *RTVP1* are p53 direct target genes and are up-regulated by p53 expression and induction of DNA damage [24,25]. To extend these observations to *RTVP1* gene cluster members, we analyzed the published genomic sequences/region (AC121761) of *hRTVP-1, GLIPR1L1*, and *GLIPR1L2* on human chromosome 12q21 in the National Center for Biotechnology Information genome database for potential p53-binding sites. For the *hRTVP-1* genomic sequence, in addition to the p53-binding site (p53-bs) in the promoter region (site A) that was identified and characterized in our initial studies [25], we identified two additional p53-bs, site B in intron 2 and site C in intron 3. Both sites contain a single base alteration in each half-site compared with the p53 consensus binding site (cbs) (Fig. 7A and [30]). Four potential p53 cbs were identified in intron 2 and intron 4 of *GLIPR1L1* sequences. Sites C1 and C2 share a common half-site, that is, the second half-site of C1 serves as the first half-site of C2 (Fig. 7A). Three potential p53-bs were identified in *GLIPR1L2* sequences, with site A in the promoter region, site B in intron 2, and site C in intron 4 (Fig. 7A).

To test the functionality of these potential p53-bs, we constructed luciferase reporters using these p53-bs in a luciferase reporter vector containing a minimum SV40 promoter (pGL3-pro). Cotransfection with wild-type p53 strongly activated *hRTVP-1* p53-bs-mediated luciferase activity (~12-fold for site A, ~10-fold for site B, and ~52-fold for site C). Similarly, wild-type p53 also strongly activated *GLIPR1L1* or *GLIPR1L2* p53-bs-mediated luciferase activity, with ~25-fold for *GLIPR1L1* site B, ~12-fold for *GLIPR1L2* site B. Mutant p53 and empty vector failed to activate luciferase activity mediated by these p53-bs (Fig. 7B). These data suggest that like *hRTVP-1, GLIPRIL1* and *GLIPR1L2* are p53 target genes and that *GLIPR1L1 and GLIPR1L2* may have specific biological functions that overlap with that of *hRTVP-1*.

To analyze the effects of p53 overexpression on endogenous *hRTVP-1, GLIPR1L1*, or *GLIPRI12* gene expression we used

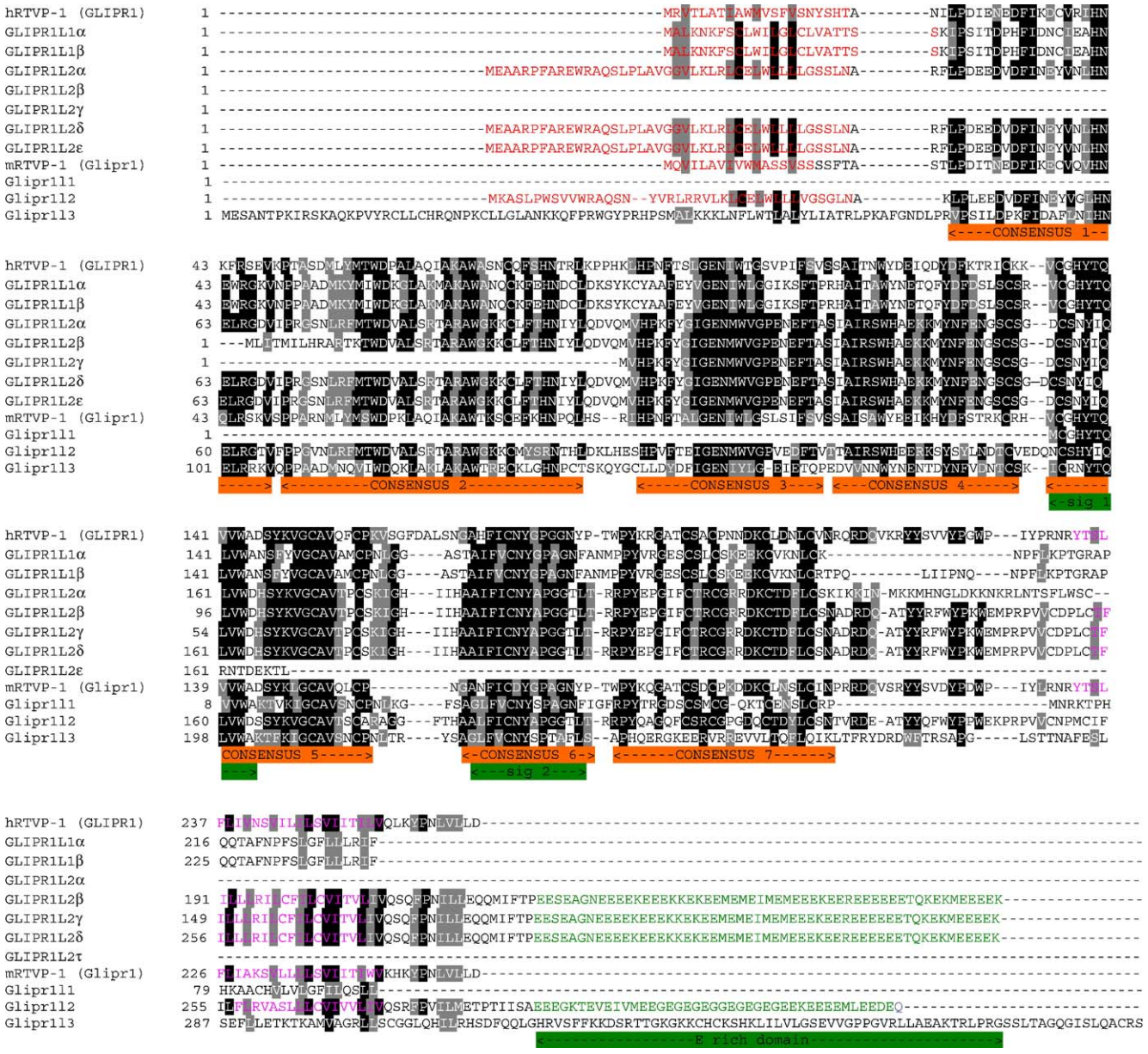

Fig. 4. Protein homology comparison within *RTVP1* gene cluster members. Domains/motifs are shown as described in the legend to Fig. 3. The glutamate (E)-rich domain is indicated with green letters.

two different systems to induce p53 expression and then quantitated mRNA levels (QRT-PCR) for *hRTVP-1, GLIPR1L1*, and *GLIPR1L2*. In 293 PE cells, adenoviral vector-mediated p53 gene delivery led to up-regulation of all three genes (3.3-fold for *hRTVP-1*, 4.1-fold for *GLIPR1L1*, and 2.1-fold for *GLIPR1L2*) compared to control virus Ad-lacZ (Fig. 8A). In the TSU-Pr1 p53 stable clone, induction of p53 expression by doxycycline also stimulated the expression of *hRTVP-1, GLIPR1L1*, and *GLIPR1L2* in a similar pattern (Fig. 8B). These data confirm *hRTVP-1, GLIPR1L1*, and *GLIPR1L2* as p53 target genes.

Discussion

Loss of p53 function is the most frequently observed phenomenon in human cancer [6–9,31]. There are multiple mechanisms that lead to loss of p53 functions. Normal p53 function can be lost when one allele of the gene or the chromosome 17p13 region is deleted and a subtle mutation occurs in the other allele [32]. In many cases a missense mutation occurring in one allele is sufficient to inactivate p53 function [33]. Mutation or aberrant regulation of p53 target genes can be another important mechanism. For example, amplification of p53 target gene and negative regulator MDM2 can result in a similar loss of functional p53 through binding and subsequent degradation of p53 [34–37]. p53-responsive cell cycle regulator p21 and apoptosis regulator Bax are found mutated or lost in multiple types of human cancer [38,39]. However, the relationship between mutation/loss of function for p53 target genes and p53 mutation/loss of function per se within the context of tumor progression has not been fully clarified.

Table 1
Homology comparison of *RTVP1* gene cluster proteins (identities/positives %)

		Human								Mouse		
		hRTVP-1 (GLIPR1)	GLIPR1L1α	GLIPR1L1β	GLIPR1L2α	GLIPR1L2β	GLIPR1L2γ	GLIPR1L2δ	GLIPR1L2ε	mRTVP-1 (Glipr1)	Glipr1l1	Glipr1l2	Glipr1l3
Human	hRTVP-1 (GLIPR1)	100/100											
	GLIPR1L1α	48/65	100/100										
	GLIPR1L1β	48/65	95/95	100/100									
	GLIPR1L2α	43/59	38/54	38/54	100/100								
	GLIPR1L2β	39/52	40/54	37/50	98/98	100/100							
	GLIPR1L2γ	44/55	38/50	38/50	44/44	91/91	100/100						
	GLIPR1L2δ	43/57	36/51	35/50	90/90	91/91	91/91	100/100					
	GLIPR1L2ε	40/58	35/54	35/54	86/86	83/85	53/53	86/86	100/100				
Mouse	mRTVP-1 (Glipr1)	62/73	49/67	48/67	42/57	36/51	35/50	40/52	41/57	100/100			
	Glipr1l1	44/58	48/64	57/72	40/60	38/58	38/58	38/58	–	51/65	100/100		
	Glipr1l2	44/58	36/53	35/52	58/69	60/71	61/71	59/69	53/64	40/52	47/61	100/100	
	Glipr1l3	37/56	46/59	48/61	35/54	35/53	39/57	35/54	38/51	39/55	80/90	31/56	100/100

In light of the critical role of p53 in human cancer and its association with prostate cancer metastasis, we identified novel p53 target genes and analyzed the molecular mechanisms by which these p53 target genes are regulated. In our initial studies we identified *mRTVP-1* and *hRTVP-1* as p53 target genes and reported that *hRTVP-1* is inactivated by methylation in prostate cancer [24,25]. We also demonstrated that *RTVP1* had proapoptotic activities in vitro and that adenoviral vector-mediated delivery of *mRTVP-1* led to suppression of primary tumor growth and metastasis in a mouse prostate cancer model [26]. In this study we identified *GLIPR1L1, GLIPR1L2, Glipr1l1, Glipr1l2*, and *Glipr1l3* as members of an *RTVP1*-related gene cluster. We demonstrated that all three human genes in this cluster are direct targets of p53. In our extended, broader search for p53-bs in the *hRTVP-1* molecule we identified two p53-bs, site B and site C, in addition to site A, which was identified in our initial studies (Fig. 7A). These two new sites have comparable (site B) or increased (site C) efficiency compared to site A in mediating wild-type p53-activated reporter activities (Fig. 7B). We also identified four p53-bs in *GLIPR1L1* with 4- to 25-fold inducibility and three p53-bs in *GLIPR1L2* with 2- to 12-fold inducibility (Fig. 7). Up-regulation of endogenous *hRTVP-1, GLIPR1L1*, or *GLIPR1L2* gene expression by p53 was demonstrated through adenoviral vector-mediated p53 gene delivery in 293 PE and through induction of p53 expression in a TSU-Pr1 p53-stable clone. The results of these studies showed that *hRTVP-1, GLIPR1L1*, and *GLIPR1L2* are p53 target genes. The extent of up-regulation of *hRTVP-1, GLIPR1L1*, or *GLIPR1L2* expression by p53 is moderate (1- to 8-fold) compared to that in binding site reporter assays (12- to 52-fold), reflecting the complexity of gene regulation within the cellular context. We also show that *GLIPR1L1* and *GLIPR1L2* are highly tissue-specific, with high expression levels in testis but very low or undetectable expression levels in other tissues (see Fig. 6), suggesting important regulators other than p53, such as positive or negative tissue-specific cofactors are involved in regulation of GLIPR1L genes. In addition to the pattern of tissue-specific *GLIPR1L* gene expression described in this paper, our previous studies [25] and our unpublished data indicate that methylation in the regulatory regions of this group of genes leads to down-regulation of these genes in prostate cancer. Further studies are necessary to detail the complex regulation of this unique gene cluster.

Another interesting point is that RTVP-1 cluster proteins share significant sequence homology with PR/CRISP proteins. They all contain a distinct PR domain that was initially identified in plants as pathogenesis-related protein and is implicated in innate immunity [20]. Most of them possess a putative signal peptide, two extracellular protein signature motifs (Figs. 4 and 5), indicating that most of proteins in this category are potentially secreted. Given the tissue-specific expression of *GLIPR1L1* and relatively low level of *GLIPR1L2* expression in tissues other than testis, it is important to know whether the capacity of secreted *GLIPR1L1* or *GLIPR1L2* includes tumor suppression functions in addition to testis-related physiological functions. For example, local paracrine or potentially endocrine functions for these proteins may expand their roles as growth

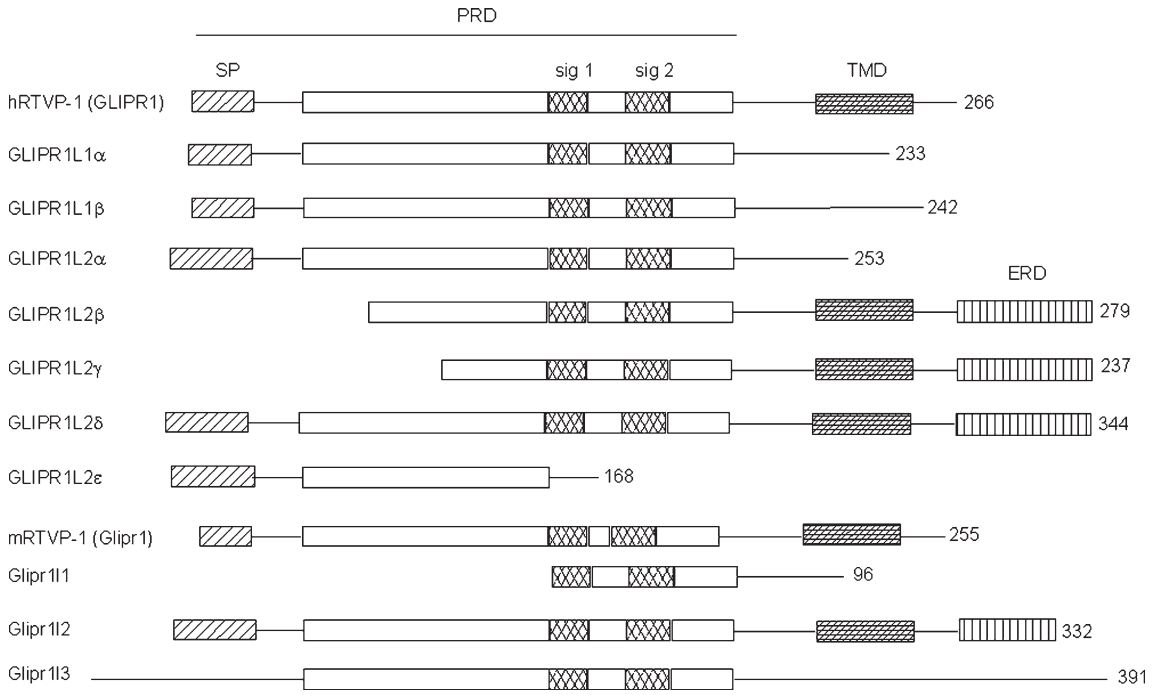

Fig. 5. Summary of domain/motif structures of RTVP-1 gene cluster proteins. PRD, pathogenesis-related protein domain; SP, signal peptide; sig, extracellular protein signature motif; TMD, transmembrane domain; ERD, glutamate-rich domain. The number at the end of each protein indicates the number of amino acids.

regulators for cancer control and potentially within other biological contexts. Although *RTVP1* gene cluster proteins share many similarities, unlike RTVP1, GLIPR1L1α and β, GLIPR1L2α and ε do not contain transmembrane domains (Figs. 4 and 5), suggesting these proteins/isoforms may have a higher capacity for secretion. Our initial studies demonstrated that those isoforms with signal peptides but not transmembrane domains, such as GLIPR1L1α, GLIPR1L1β, and GLIPR1L2α, are predominately secreted, whereas those with both signal peptides and transmembrane domains, such as hRTVP-1, are only partially secreted (unpublished data). GLIPR1L2β and GLIPR1L2γ, which lack signal peptides and contain transmembrane domains, are not expected to be secreted. Overall our data suggest that a signal peptide is required for protein secretion, and the transmembrane domain functions as a negative factor for protein secretion. A unique ERD was found in the C-terminus of GLIPR1L2β, γ, and δ and Glipr1l2. Homology search using the ERD sequence did not match to any known sequence in the database. The function of ERD in these isoforms is unclear. Further characterization of these distinct isoforms will be completed in our future studies.

In summary, we identified a novel p53 target gene cluster on human chromosome 12 and mouse chromosome 10. These gene cluster proteins share significant identity and homology. The results of our studies not only contribute this gene cluster to the list of p53 targets, but also provide important information for the future functional studies of this gene cluster. On the basis of the similarities in protein structure and the association with p53

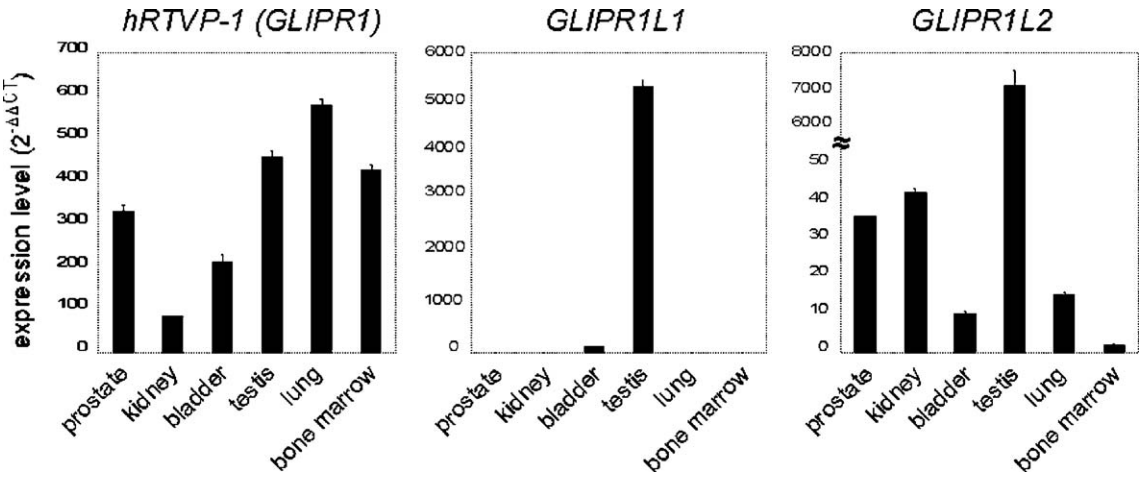

Fig. 6. The mRNA expression levels of *hRTVP-1, GLIPR1L1*, and *GLIPR1L2* in six human tissues were determined by QRT-PCR and were normalized by expression levels of 18S rRNA in the same samples. Bars stand for standard errors.

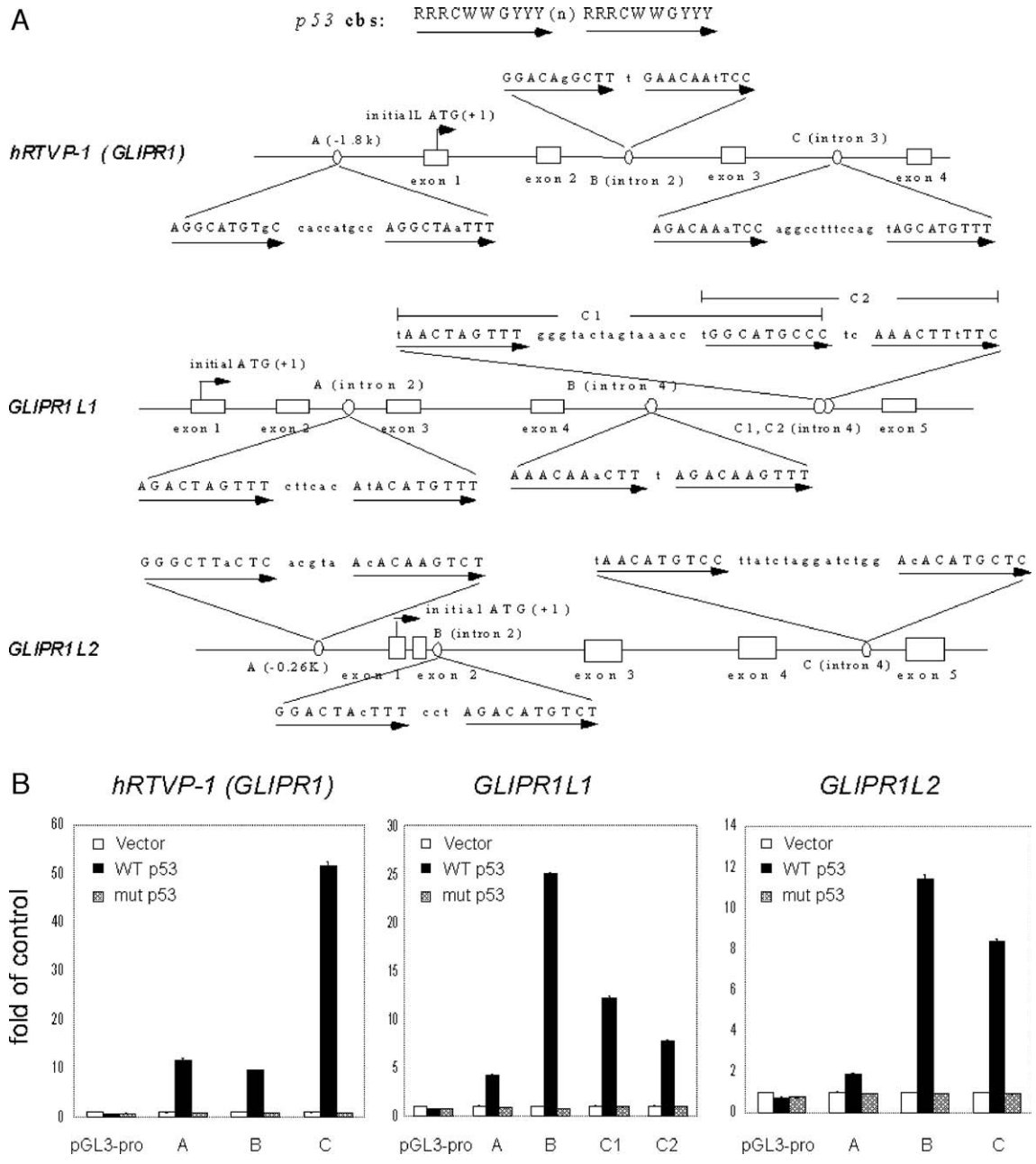

Fig. 7. Identification of p53 binding sites in *hRTVP-1, GLIPR1L1*, and *GLIPR1L2*. (A) Potential p53 binding sites in *hRTVP-1, GLIPR1L1*, and *GLIPR1L2*. (B) Luciferase reporter assays. Luciferase activities were determined 24 h after transfection and were expressed as fold of control (control = luciferase reporter vector + pCDNA empty vector). In the p53 cbs, R is purine, Y is pyrimidine, W is A or T, and n is 0 to 13 of any base. Bars stand for standard errors.

tumor suppressor, we speculate that this group of genes may have similar biological functions and may have important roles in the development, progression, and potentially treatment of prostate cancer and other malignancies.

Materials and methods

Cloning and vector construction

Full-length cDNA of *hRTVP-1* was cloned from human prostate RNA by RT-PCR using the following primer pair: 5′-ACTCAGGCAATCACACTCTC-3′ and 5′-GGTTGGTTTTTGGGTTTT-3′. *GLIPR1L1α* and *GLIPR1L2α* were cloned from human testis RNA by RT-PCR using the following primer pairs: 5′-CATCCTCCGCATCCTCCA-3′ and 5′-TAGGTATCAGGCAAGAGTGTA-3′ for *GLIPR1L1α*; 5′-GGTGAACCATGGAGGCCG-3′ and 5′-GGGGAAG-GAATAGGGTTAAGAT-3′ for *GLIPR1L2α*. The PCRs were performed using Qiagen's HotstarTaq PCR Kit (Qiagen, Valencia, CA, USA). The resulting PCR products were constructed into the pGEM-T Easy vector (Promega, Madison, WI, USA). The DNA sequences were confirmed by sequencing using an automated sequencer, i.e., the ABI Prism310 (Applied Biosystems, Foster City, CA, USA).

Identification of GLIPR1L isoforms

A human testis cDNA library generated from human testis total RNA (Clontech, Palo Alto, CA, USA) using the Creator SMART cDNA Library Construction kit (Clontech) was used. To identify and isolate potential isoforms of *GLIPR1L1* and *GLIPR1L2*, cDNA fragments of *GLIPR1L1α* (nuclear acids 259–688 of NM_152779) and *GLIPR1L2α* (nuclear acids 104–613 of

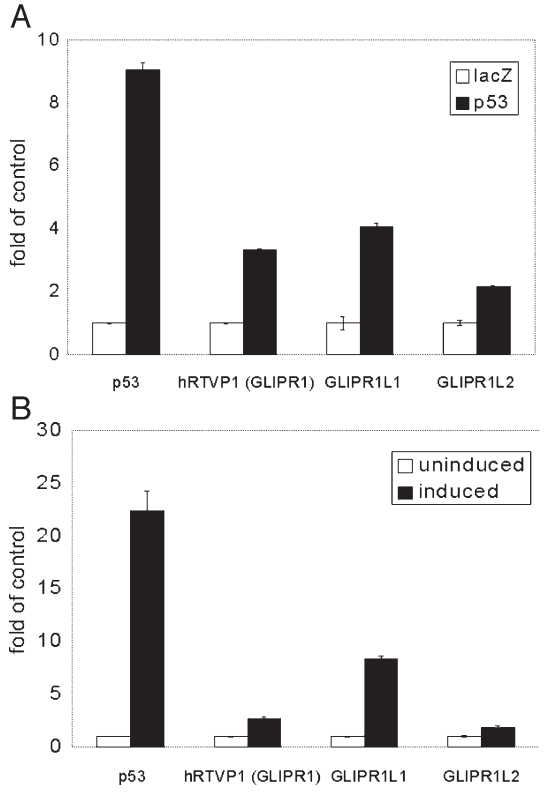

Fig. 8. Analysis of p53 regulation of endogenous *hRTVP-1, GLIPR1L1*, or *GLIPR1L2* gene expression. (A) QRT-PCR analysis for mRNA levels of *hRTVP-1, GLIPR1L1*, and *GLIPR1L2* in 293PE cells 24 h following gene transduction with Ad-p53 or Ad-lacZ. (B) QRT-PCR analysis for mRNA levels of *hRTVP-1, GLIPR1L1*, and *GLIPR1L2* in a TSU-Pr1 p53 stable clone 24 h following p53 induction with doxycycline. The effects of p53 expression on *hRTVP-1, GLIPR1L1*, and *GLIPR1L2* gene expression are expressed as fold of control. Bars stand for standard errors.

NM_152436) were used as probes to screen the human testis cDNA library according to the manufacturer's protocol (Creator SMART cDNA libraries user manual; Clontech). Briefly, *Escherichia coli* cells transformed with the cDNA library were plated on LB plates at low density. Membrane lifting and hybridization were performed. Positive colonies were picked and subjected to a second and third round of screening. Final positive clones were sorted by restriction mapping. Three clones from each group were sequenced and mapped using available genomic sequence.

DNA and protein homology search

DNA and protein homology search was performed using programs provided on the Web site http://www.nlm.nih.gov/BLAST/. Multiple sequence alignments were carried out using the BCM search launcher: http://searchlauncher.bcm.tmc.edu/multi-align/multi-alig.html. Signal peptide and possible cleavage site prediction was defined using CBS SignalP 3.0: http://www.cbs.dtu.dk/services/SignalP/. Transmembrane domains were predicted according to PSORT II Prediction: http://www.psort.ims.u-tokyo.ac.jp/form2.html.

Analysis of gene expression

Human tissues were purchased from BD Biosciences Clontech. Total RNA from tissues or cell lines was extracted with the RiboPure RNA extraction kit (Ambion, Austin, TX, USA). The RT reactions were carried out using the High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's protocol. PCRs were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems). The PCR conditions were 80 ng cDNA/well, 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Duplicate samples were used for PCR. The following TaqMan probe and primer mixes were used for PCR: Hs00199268_m1(GLIPR1) for *hRTVP-1*, Hs00385348_m1 for *GLIPR1L1*, Hs00380808_m1 for *GLIPR1L2*, and 4319413E for 18S rRNA (Applied Biosystems). PCRs were performed using an ABI Prism 7000 Sequence Detection System according to the manufacturer's instructions. The fold difference in gene expression of *hRTVP-1, GLIPR1L1*, and *GLIPR1L2* mRNA was determined by the comparative C_T method for relative quantification. The level of target gene mRNA, normalized by 18S rRNA or GAPDH, is expressed as $2^{-\Delta\Delta CT}$.

p53 binding site analysis

The search ranges for potential p53-bs cover from 2 kb promoter sequence to intron 4 of *hRTVP-1, GLIPR1L1*, and *GLIPR1L2*. The criterion for a p53-bs candidate is ≥90% identity compared to the p53 cbs: RRRCWWGYYY(n) RRRCWWGYYY, where R is purine, Y is pyrimidine, W is A or T, and (n) is 0 to 13 of any bases [30]. Each pair of oligonucleotides that were derived from a putative p53-bs (see Fig. 6 for p53-bs sequences) were annealed and subcloned into the *Sma*I site of the pGL3 luciferase reporter vector with a minimal SV40 promoter (Promega). All constructs were confirmed by DNA sequencing. One microgram of luciferase reporter construct, 0.1 μg of wild-type or mutant pCMVp53-expressing vectors [41] or control empty vector pcDNA3.1(+), and 0.2 μg pCMV-gal were cotransfected into 148-1PA cells using LipofectAmine (Invitrogen, Carlsbad, CA, USA). Luciferase activity assay was performed 24 h after transfection. Luciferase activity was standardized to the cotransfected β-galactosidase as previously described [24,25] and expressed as fold of control (control was pcDNA3.1(+) with pGL3 promoter–luciferase vector).

Analysis of p53 regulation of endogenous hRTVP1, GLIPR1L1, or GLIPR1L2 gene expression

Adenoviral vectors Ad-lacZ and Ad-p53 were generated using standard recombination protocols. 293PE cells were transduced with Ad-lacZ and Ad-p53 at an m.o.i. of 50 in serum-free medium for 3 h, at which time complete medium (high glucose DMEM) was restored. Cell growth continued until extraction of total RNA at 24 and 48 h following gene transduction.

Human *p53* cDNA was inserted into pTRE2hyg to form pTRE2hyg-p53. pTRE2hyg-p53 was cotransfected together with a Tet-On vector (BD Biosciences Clontech) into TSU-Pr1 cells using LipofectAmine Plus (Invitrogen). The stable clones were selected using 100 μg/ml hygromycin and 500 μg/ml G418. The induction of p53 expression in these stable clones was analyzed by QRT-PCR. A representative TSU-PR1 p53 stable clone was used for this study. *p53* expression was induced by incubation of cells with 2 μg/ml doxycycline in the medium for 24 and 48 h. A parallel control experiment was conducted by incubation of cells under the same conditions without doxycycline in the medium. Total RNA from cells was extracted and QRT-PCR was performed as described above.

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