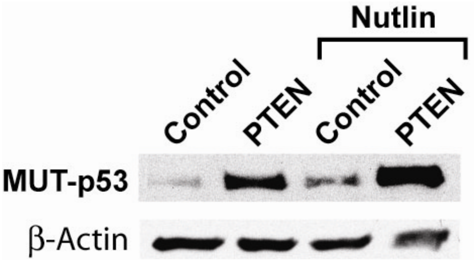
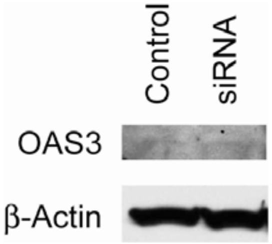
Effects of Mdm2 inhibition on PTEN-induced mut-p53 protein levels

We used Nutlin-3 to assess the effects of PTEN on mut-p53 in the setting of inhibited Mdm2 binding to p53. Mut-p53 U373 cells were treated with 50 µM Nutlin-3 one hour before infection with Ad-PTEN or Ad-control. Cell extracts were isolated after 24 hrs and immunoblotted for mut-p53 as described in the methods. The immunoblots shown below show that Nutlin-3 and PTEN additively induce mut-p53 protein levels. The additive effects of Nutlin-3 and PTEN can be explained by either an incomplete inhibition of Mdm2 binding by Nutlin-3 and/or by only partial dependence of PTEN-induced mut-p53 on Mdm2 inhibition.



Exclusion of non-specific siRNA-induced anti-viral effects:

To exclude siRNA/shRNA-induced non-specific antiviral effects, lysates from all experiments using siRNA/shRNA and control untreated lysates were immnublotted for 2'-5'-oligoadenylate synthetase 3 (OAS3). OAS3 is an interferon-induced protein that is used to assess general antiviral responses to short nucleic acids. siRNA/shRNA did not induce the expression of OAS3 in any of the lysates tested and OAS3 expression levels were generally low in siRNA and control cells. A representative immunoblot from these experiments is shown below:



Determination of the mutational status of the cell lines used in the study:

The mutational status of all cell lines used in the study was determined as described below:

SNP Genotyping of p53-175 and p53-272

The genotype for the alleles in U87, A172, U373, SNB19 and U1242 cell lines was determined by Taqman Allelic Discrimination method. The assay used two differently labeled fluorescent minor groove-binding (MGB) probes that match the sequence of the two alleles with a common pair of primers. The assay was designed with the aid of the Primer Express software (Applied Biosystems, Forster City, CA). The details of the assay are shown in the following table:

For p53-r175h

5'-CTACAAGCAGTCACAGCACATGAC-3'	Forward
5'-TCTGAGCAGCGCTCATGGT-3'	Reverse

5’6-FAM-TGTGAGGCaCTGCC-MGB-BHQ3’	Probe1
5’VIC-TTGTGAGGCgCTGC-MGB-BHQ3’	Probe2

For p53-r273h

5’-GTAATCTACTGGGACGGAACAGCT-3’	Forward
5’-CTCTGTGCGCCGGTCTCT-3’	Reverse
5’6-FAM-TGAGGTGCaTGTTT-MGB-BHQ3’	Probe1-mbg-fam
5’VIC-TGAGGTGCgTGTTT-MGB-BHQ3’	Probe2-mbg-vic

The geneotyping was performed on an Applied Biosystems 7900-HT Sequence Detection systems instrument with the following cycle profile:

		(3) CYCLE (45 cycles)	
(1) HOLD	(2) HOLD	Denature	Anneal/Extend
Temp=50°C	Temp=95°C	Temp=95°C	Temp=60°C
Time=2 min	Time=10 min	Time=15 sec	Time=45 sec

The autocall function with a 95% confidence setting was used to make the preliminary determination of the genotypes, which were then further examined by the displacement of a particular data point in the clusters.

Regular Taqman Gene Quantification

Regular Taqman gene expression analysis was performed to further confirm the site mutation in the p53 amino acid positions #175 and #273. The primers and probes used were identical to the list above but only one probe was used in a certain reaction. The PCR conditions were the same as used for the allelic discrimination.

Results:

U87: 175(G/G), 273(G/G)
CTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCT
GCTCAGATAGCGATGGTGAGCAGCTGGGGCTGGAGAGACGACAGGGCTGGTTGCCCAGG
GTCCCCAGGCCTCTGATTCCTCACTGATTGCTCTTAGGTCTGGCCCCTCCTCAGCATCTTA
TCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAG
TGTGGTGGTGCCCTATGAGCCGCCTGAGGTCTGGTTTGCAACTGGGGTCTCTGGGAGGA
GGGGTTAAGGGTGGTTGTCAGTGGCCCTCCAGGTGAGCAGTAGGGGGGCTTTCTCCTGC
TGCTTATTTGACCTCCCTATAACCCCATGAGATGTGCAAAGTAAATGGGTTTAACTATTGCA
CAGTTGAAAAAACTGAAGCTTACAGAGGCTAAGGGCCTCCCCTGCTTGGCTGGGCGCAGT
GGCTCATGCCTGTAATCCCAGCACTTTGGGAGGCCAAGGCAGGCGGATCACGAGGTTGG
GAGATCGAGACCATCCTGGCTAACGGTGAAACCCCGTCTCTACTGAAAAATACAAAAAAAA
ATTAGCCGGGCGTGGTGCTGGGCACCTGTAGTCCCAGCTACTCGGGAGGCTGAGGAAGG
AGAATGGCGTGAACCTGGGCGGTGGAGCTTGCAGTGAGCTGAGATCACGCCACTGCACT
CCAGCCTGGGCGACAGAGCGAGATTCCATCTCAAAAAAAAAAAAAAAAAGGCCTCCCCTGC
TTGCCACAGGTCTCCCCAAGGCGCACTGGCCTCATCTTGGGCCTGTGTTATCTCCTAGGTT
GGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGCGGCA
TGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACTT
GCCACCCTGCACACTGGCCTGCTGTGCCCCAGCCTCTGCTTGCCTCTGACCCCTGGGCCC
ACCTCTTACCGATTTCTTCCATACTACTACCCATCCACCTCTCATCACATCCCCGGCGGGG
AATCTCCTTACTGCTCCCACTCAGTTTTCTTTTCTCTGGCTTTGGGACCTCTTAACCTGTGG
CTTCTCCTCCACCTACCTGGAGCTGGAGCTTAGGCTCCAGAAAGGACAAGGGTGGTTGGG
AGTAGATGGAGCCTGGTTTTTTAAATGGGACAGGTAGGACCTGATTTCCTTACTGCCTCTT
GCTTCTCTTTTCCTATCCTGAGTAGTGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCG
TGTTTGTGCCTGTCCTGGGAGAGACCGGCGCACAGAGGAA

A172: 175(G/G), 273(G/G)
CTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCT
GCTCAGATAGCGATGGTGAGCAGCTGGGGCTGGAGAGACGACAGGGCTGGTTGCCCAGG
GTCCCCAGGCCTCTGATTCCTCACTGATTGCTCTTAGGTCTGGCCCCTCCTCAGCATCTTA
TCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAG
TGTGGTGGTGCCCTATGAGCCGCCTGAGGTCTGGTTTGCAACTGGGGTCTCTGGGAGGA
GGGGTTAAGGGTGGTTGTCAGTGGCCCTCCAGGTGAGCAGTAGGGGGGCTTTCTCCTGC
TGCTTATTTGACCTCCCTATAACCCCATGAGATGTGCAAAGTAAATGGGTTTAACTATTGCA
CAGTTGAAAAAACTGAAGCTTACAGAGGCTAAGGGCCTCCCCTGCTTGGCTGGGCGCAGT
GGCTCATGCCTGTAATCCCAGCACTTTGGGAGGCCAAGGCAGGCGGATCACGAGGTTGG
GAGATCGAGACCATCCTGGCTAACGGTGAAACCCCGTCTCTACTGAAAAATACAAAAAAAA
ATTAGCCGGGCGTGGTGCTGGGCACCTGTAGTCCCAGCTACTCGGGAGGCTGAGGAAGG
AGAATGGCGTGAACCTGGGCGGTGGAGCTTGCAGTGAGCTGAGATCACGCCACTGCACT
CCAGCCTGGGCGACAGAGCGAGATTCCATCTCAAAAAAAAAAAAAAAAGGCCTCCCCTGC
TTGCCACAGGTCTCCCCAAGGCGCACTGGCCTCATCTTGGGCCTGTGTTATCTCCTAGGTT
GGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGCGGCA
TGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACTT
GCCACCCTGCACACTGGCCTGCTGTGCCCCAGCCTCTGCTTGCCTCTGACCCCTGGGCCC
ACCTCTTACCGATTTCTTCCATACTACTACCCATCCACCTCTCATCACATCCCCGGCGGGG
AATCTCCTTACTGCTCCCACTCAGTTTTCTTTTCTCTGGCTTTGGGACCTCTTAACCTGTGG
CTTCTCCTCCACCTACCTGGAGCTGGAGCTTAGGCTCCAGAAAGGACAAGGGTGGTTGGG
AGTAGATGGAGCCTGGTTTTTTAAATGGGACAGGTAGGACCTGATTTCCTTACTGCCTCTT
GCTTCTCTTTTCCTATCCTGAGTAGTGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCG
TGTTTGTGCCTGTCCTGGGAGAGACCGGCGCACAGAGGAA

U373: 175(G/G), 273(A/A)
CTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCT
GCTCAGATAGCGATGGTGAGCAGCTGGGGCTGGAGAGACGACAGGGCTGGTTGCCCAGG
GTCCCCAGGCCTCTGATTCCTCACTGATTGCTCTTAGGTCTGGCCCCTCCTCAGCATCTTA
TCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAG
TGTGGTGGTGCCCTATGAGCCGCCTGAGGTCTGGTTTGCAACTGGGGTCTCTGGGAGGA
GGGGTTAAGGGTGGTTGTCAGTGGCCCTCCAGGTGAGCAGTAGGGGGGCTTTCTCCTGC
TGCTTATTTGACCTCCCTATAACCCCATGAGATGTGCAAAGTAAATGGGTTTAACTATTGCA
CAGTTGAAAAAACTGAAGCTTACAGAGGCTAAGGGCCTCCCCTGCTTGGCTGGGCGCAGT
GGCTCATGCCTGTAATCCCAGCACTTTGGGAGGCCAAGGCAGGCGGATCACGAGGTTGG
GAGATCGAGACCATCCTGGCTAACGGTGAAACCCCGTCTCTACTGAAAAATACAAAAAAAA
ATTAGCCGGGCGTGGTGCTGGGCACCTGTAGTCCCAGCTACTCGGGAGGCTGAGGAAGG
AGAATGGCGTGAACCTGGGCGGTGGAGCTTGCAGTGAGCTGAGATCACGCCACTGCACT
CCAGCCTGGGCGACAGAGCGAGATTCCATCTCAAAAAAAAAAAAAAAAGGCCTCCCCTGC
TTGCCACAGGTCTCCCCAAGGCGCACTGGCCTCATCTTGGGCCTGTGTTATCTCCTAGGTT
GGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGCGGCA
TGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACTT
GCCACCCTGCACACTGGCCTGCTGTGCCCCAGCCTCTGCTTGCCTCTGACCCCTGGGCCC
ACCTCTTACCGATTTCTTCCATACTACTACCCATCCACCTCTCATCACATCCCCGGCGGGG
AATCTCCTTACTGCTCCCACTCAGTTTTCTTTTCTCTGGCTTTGGGACCTCTTAACCTGTGG
CTTCTCCTCCACCTACCTGGAGCTGGAGCTTAGGCTCCAGAAAGGACAAGGGTGGTTGGG
AGTAGATGGAGCCTGGTTTTTTAAATGGGACAGGTAGGACCTGATTTCCTTACTGCCTCTT
GCTTCTCTTTTCCTATCCTGAGTAGTGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCA
TGTTTGTGCCTGTCCTGGGAGAGACCGGCGCACAGAGGAA

SnB19: 175(G/G), 273(A/A)
CTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCT
GCTCAGATAGCGATGGTGAGCAGCTGGGGCTGGAGAGACGACAGGGCTGGTTGCCCAGG
GTCCCCAGGCCTCTGATTCCTCACTGATTGCTCTTAGGTCTGGCCCCTCCTCAGCATCTTA
TCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAG

TGTGGTGGTGCCCTATGAGCCGCCTGAGGTCTGGTTTGCAACTGGGGTCTCTGGGAGGA
GGGGTTAAGGGTGGTTGTCAGTGGCCCTCCAGGTGAGCAGTAGGGGGGCTTTCTCCTGC
TGCTTATTTGACCTCCCTATAACCCCATGAGATGTGCAAAGTAAATGGGTTTAACTATTGCA
CAGTTGAAAAAACTGAAGCTTACAGAGGCTAAGGGCCTCCCCTGCTTGGCTGGGCGCAGT
GGCTCATGCCTGTAATCCCAGCACTTTGGGAGGCCAAGGCAGGCGGATCACGAGGTTGG
GAGATCGAGACCATCCTGGCTAACGGTGAAACCCCGTCTCTACTGAAAAATACAAAAAAAA
ATTAGCCGGGCGTGGTGCTGGGCACCTGTAGTCCCAGCTACTCGGGAGGCTGAGGAAGG
AGAATGGCGTGAACCTGGGCGGTGGAGCTTGCAGTGAGCTGAGATCACGCCACTGCACT
CCAGCCTGGGCGACAGAGCGAGATTCCATCTCAAAAAAAAAAAAAAAAAGGCCTCCCCTGC
TTGCCACAGGTCTCCCCAAGGCGCACTGGCCTCATCTTGGGCCTGTGTTATCTCCTAGGTT
GGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGCGGCA
TGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACTT
GCCACCCTGCACACTGGCCTGCTGTGCCCCAGCCTCTGCTTGCCTCTGACCCCTGGGCCC
ACCTCTTACCGATTTCTTCCATACTACTACCCATCCACCTCTCATCACATCCCCGGCGGGG
AATCTCCTTACTGCTCCCACTCAGTTTTCTTTTCTCTGGCTTTGGGACCTCTTAACCTGTGG
CTTCTCCTCCACCTACCTGGAGCTGGAGCTTAGGCTCCAGAAAGGACAAGGGTGGTTGGG
AGTAGATGGAGCCTGGTTTTTTAAATGGGACAGGTAGGACCTGATTTCCTTACTGCCTCTT
GCTTCTCTTTTCCTATCCTGAGTAGTGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCA
TGTTTGTGCCTGTCCTGGGAGAGACCGGCGCACAGAGGAA

U1242: 175(A/A), 273(G/G)
CTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCACTGCCCCCACCATGAGCGCTG
CTCAGATAGCGATGGTGAGCAGCTGGGGCTGGAGAGACGACAGGGCTGGTTGCCCAGGG
TCCCCAGGCCTCTGATTCCTCACTGATTGCTCTTAGGTCTGGCCCCTCCTCAGCATCTTAT
CCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAGT
GTGGTGGTGCCCTATGAGCCGCCTGAGGTCTGGTTTGCAACTGGGGTCTCTGGGAGGAG
GGGTTAAGGGTGGTTGTCAGTGGCCCTCCAGGTGAGCAGTAGGGGGGCTTTCTCCTGCT
GCTTATTTGACCTCCCTATAACCCCATGAGATGTGCAAAGTAAATGGGTTTAACTATTGCAC
AGTTGAAAAAACTGAAGCTTACAGAGGCTAAGGGCCTCCCCTGCTTGGCTGGGCGCAGTG
GCTCATGCCTGTAATCCCAGCACTTTGGGAGGCCAAGGCAGGCGGATCACGAGGTTGGG
AGATCGAGACCATCCTGGCTAACGGTGAAACCCCGTCTCTACTGAAAAATACAAAAAAAAA
TTAGCCGGGCGTGGTGCTGGGCACCTGTAGTCCCAGCTACTCGGGAGGCTGAGGAAGGA
GAATGGCGTGAACCTGGGCGGTGGAGCTTGCAGTGAGCTGAGATCACGCCACTGCACTC
CAGCCTGGGCGACAGAGCGAGATTCCATCTCAAAAAAAAAAAAAAAAAGGCCTCCCCTGCTT
GCCACAGGTCTCCCCAAGGCGCACTGGCCTCATCTTGGGCCTGTGTTATCTCCTAGGTTG
GCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGCGGCAT
GAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACTTG
CCACCCTGCACACTGGCCTGCTGTGCCCCAGCCTCTGCTTGCCTCTGACCCCTGGGCCCA
CCTCTTACCGATTTCTTCCATACTACTACCCATCCACCTCTCATCACATCCCCGGCGGGGA
ATCTCCTTACTGCTCCCACTCAGTTTTCTTTTCTCTGGCTTTGGGACCTCTTAACCTGTGGC
TTCTCCTCCACCTACCTGGAGCTGGAGCTTAGGCTCCAGAAAGGACAAGGGTGGTTGGGA
GTAGATGGAGCCTGGTTTTTTAAATGGGACAGGTAGGACCTGATTTCCTTACTGCCTCTTG
CTTCTCTTTTCCTATCCTGAGTAGTGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCGT
GTTTGTGCCTGTCCTGGGAGAGACCGGCGCACAGAGGAA