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Targeting negative regulation of p53 by MDM2 and WIP1 as a therapeutic strategy in cutaneous melanoma

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Abstract

Background: Cutaneous melanoma is the most serious skin malignancy and new therapeutic strategies are needed for advanced melanoma. *TP53* mutations are rare in cutaneous melanoma so activation of wild type p53 is a potential therapeutic strategy in cutaneous melanoma. Here, we investigated the WIP1 inhibitor, GSK2830371, and MDM2-p53 binding antagonists (nutlin-3, RG7388 and HDM201) alone and in combination treatment in cutaneous melanoma cell lines and explored the mechanistic basis of these responses in relation to the genotype and induced gene expression profile of the cells.

Methods: A panel of three $p53^{WT}$ (A375, WM35, C8161) and three $p53^{MUT}$ (WM164, WM35-R, CHL-1) melanoma cell lines were used. The effects of MDM2 and WIP1 inhibition were evaluated by growth-inhibition and clonogenic assays, immunoblotting, qRT-PCR gene expression profiling and flow cytometry.

Results: GSK2830371, at doses (≤10 μM) which alone had no growth-inhibitory or cytotoxic effects on the cells, nevertheless significantly potentiated the growth-inhibitory and clonogenic cell killing effects of MDM2 inhibitors in $p53^{WT}$ but not $p53^{MUT}$ melanoma cells, indicating the potentiation worked in a p53-dependent manner. SiRNA-mediated knockdown of p53 provided further evidence to support the p53-dependence. GSK2830371 increased p53 stabilization through Ser15 phosphorylation, consequent Lys382 acetylation, decreased ubiquitination and proteasome-dependent degradation when it was combined with MDM2 inhibitors. These changes were at least partly ATM-mediated, shown by reversal with the ATM inhibitor (KU55933). GSK2830371 enhanced the induction of p53 transcriptional target genes, cell cycle arrest and apoptosis.

Conclusions: GSK2830371, a WIP1 inhibitor, at doses with no growth-inhibitory activity alone, potentiated the growth-inhibitory and cytotoxic activity of MDM2 inhibitors, by increasing phosphorylation, acetylation and stabilization of p53 in cutaneous melanoma cells in a functional p53-dependent manner.

Introduction

Cutaneous melanoma is the most serious skin malignancy(Miller & Mihm, 2006) and one of the most common cancers in developed countries.(Torre *et al*, 2015) The prognosis of melanoma patients depends on the stage at presentation and the survival outcomes of patients with advanced stage were extremely poor.(Balch *et al*, 2009) Both the MAPK (RAS–RAF–ERK)(Davies *et al*, 2002; Demunter *et al*, 2001) and ARF–MDM2–p53(Chin, 2003) pathways are critical in melanoma tumorigenesis and genetic alterations associated with them provide important druggable targets. The combination of BRAF and MEK inhibitors has become the standard treatment for unresectable or metastatic melanoma harboring a $BRAF^{V600}$ mutation.(Larkin *et al*, 2014; Robert *et al*, 2015) However, a therapeutic strategy targeting the p53 network has not been established in melanoma and could be a good option for advanced melanoma patients harboring no $BRAF^{V600}$ mutation.(Lu *et al*, 2014)

The tumor suppressor p53 is a transcription factor that regulates a number of genes with a broad range of functions, including deoxyribonucleic acid (DNA) repair, metabolism, cell cycle arrest, apoptosis and senescence.(Gannon & Jones, 2012; Levine *et al*, 1991; Wade *et al*, 2010) Mutation of *TP53* results in loss of wild-type (WT) p53 tumor suppressor function and can also have dominant oncogenic functions (gain-of-function mutations) that are entirely independent of WT p53, causing cancer cell development, survival, and proliferation.(Muller & Vousden, 2013) However, melanomas have p53 mutations only in a minority of cases (~20%). Furthermore, p53 mutations and loss of *CKDN2A* appear to be mutually exclusive, which possibly reflects p53 dependence (Hodis *et al*, 2012; Zhang *et al*, 2016). Therefore, a therapeutic strategy to rescue and reactivate p53 function can be envisioned in melanomas that retain WT p53.

MDM2-p53 binding antagonists block the p53-binding pocket of MDM2 and stabilize p53 by preventing MDM2-mediated ubiquitylation and degradation. This results in activation of the p53 pathway in $p53^{WT}$ (p53-wild type) rather than $p53^{MUT}$ (p53-mutated) cancer cells, causing cell cycle arrest, apoptosis, and growth inhibition of human cancer cells.(Brown *et al*, 2009) Nutlin-3 was the first MDM2-p53 binding antagonist to be developed and shown to have efficacy *in vitro* and *in vivo*.(Polanski *et al*, 2014; Sachweh *et al*, 2013; Vassilev *et al*, 2004) RG7388 (Idasanutlin), an orally available second generation MDM2-p53 binding antagonist, efficiently suppressed tumor growth *in vivo*; (Ding *et al*, 2013) clinical trials of RG7388 are currently ongoing to investigate the clinical efficacy of MDM2 inhibitors in relapsed multiple myeloma (NCT02633059), relapsed or refractory acute myeloid leukemia (NCT02670044, NCT02545283) and relapsed or refractory follicular lymphoma or diffuse large B-cell lymphoma (NCT02624986). HDM201, a new-generation and highly potent and selective MDM2 inhibitor, is also under investigation in early clinical trials (Hyman *et al*, 2016).

PPM1D encodes wild-type p53-induced phosphatase 1 (WIP1) which is involved in homeostatic regulation of p53 function and stability by directly dephosphorylating p53 after cellular stress. *PPM1D* is also a direct transcriptional target of p53, thus forming a negative auto-regulatory loop with the p53 network by dephosphorylating p53 (Ser15) and other signaling components (such as ATM (*ataxia-telangiectasia mutated)*, ATR (ataxia telangiectasia and Rad3-related) and MDM2) involved in p53 post-translational regulation (Lowe *et al*, 2012; Lu *et al*, 2007). GSK2830371 allosterically inhibits the enzymatic activity of WIP1 protein and also enhances ubiquitin-mediated degradation of WIP1(Gilmartin *et al*, 2014). Pre-clinical studies have shown that GSK2830371 can enhance p53-mediated tumor suppression by MDM2 inhibitors, nutlin-3 (Esfandiari *et al*, 2016; Pechackova *et al*, 2016), nutlin-3a (Sriraman *et al*,

2016), and RG7388 (Esfandiari *et al*, 2016) or by chemotherapy (Gilmartin *et al*, 2014; Pechackova *et al*, 2016).

The aim of the current study was to investigate WIP1 inhibition (WIP1i), GSK2830371, and MDM2-p53 inhibitors, nutlin-3, RG7388 and HDM201, alone and in combination treatment specifically in cutaneous melanoma cell lines and to explore the mechanistic basis of these responses in relation to the genotype and induced gene expression of the cells.

Materials and Methods

Cell lines and reagents

Cutaneous melanoma cell lines were routinely cultured using Dulbecco's modified Eagle's medium (DMEM) medium containing 4.5g/L glucose (Sigma, Dorset, UK) and supplemented with 10% (v/v) fetal calf serum. All the cell lines were authenticated by serial tandem repeat (STR) profiling (NewGene, Newcastle, UK) and tested to confirm lack of mycoplasma infection. Nutlin-3 was purchased from NewChem (Newcastle, UK), RG7388 and HDM201 were obtained from Astex Pharmaceuticals, and GSK2830371 was purchase from Sigma-Aldrich. All compounds were initially dissolved in DMSO (Sigma-Aldrich) and used to dose cells at a final concentration of 0.5% DMSO with minimal cytotoxic effects on cells.

Growth inhibition assay

Melanoma cells were seeded in 96-well plates 24 hours before 72-hour treatment with nutlin-3 RG7388, HDM201, GSK2830371 or combinations. The cells were fixed using Carnoy's fixative followed by Sulforhodamine B (SRB) assay.(Skehan *et al*, 1990) The GI_{50} value, the concentration of a compound which can reduce the growth of the cell population by 50% compared to solvent control, was determined. The details of the calculation for growth inhibition are described in supplementary information.

Clonogenic assay

Melanoma cell lines were seeded in 6-well plates and left for 24 hours before treatment with MDM2 inhibitors for 72 hours, combined with or without WIPi. Fresh medium was replaced and the cells were fixed after 10 – 21 days depending on the growth rates of the cells. The LC_{50} value,

the concentration of a compound which can reduce the number of colonies by 50% compared to solvent control, was determined.

Immunoblotting

Cell lysates were harvested by 2% SDS lysis buffer, heated and sonicated. Equal quantities of protein were loaded onto and separated by SDS-polyacrylamide gels (4–20% Mini-PROTEAN® TGX™ Gel, BioRad). The separated proteins were transferred and immobilized onto Amersham™ nitrocellulose membranes (GE Healthcare Life Science). Primary antibodies against p53 (DO-7) (#M7001, Dako), MDM2 (Ab-1) (#OP46, Merck Millipore), $p21^{WAF1}$ (EA10) (#OP64, Calbiochem), WIP1 (F-10) (#sc-376257, Santa Cruz Biotechnology), phospho-p53(Ser-15) (#9284, Cell Signaling Technology), acetyl-p53(Lys382) (#2525, Cell Signaling Technology), BAX (#2772, Cell Signaling Technology; ab1431, Abcam), GAPDH (14C10) (#2118, Cell Signaling Technology) and secondary goat anti-mouse/rabbit horseradish peroxidase–conjugated antibodies (#P0447/P0448, Dako) were used . All antibodies were diluted in 5% (w/v) non-fat milk or BSA in TBS-tween. Proteins were visualized using enhanced chemiluminescence (GE Healthcare Life Sciences) and x-ray film (Fujifilm). Densitometry was carried out using ImageJ software.

Site-directed mutagenesis and ectopic expression of mutant p53

The details of site-directed mutagenesis were described in previous study.(Esfandiari *et al*, 2016) WM35 were transfected with plasmid cDNA constructs encoding either wild-type or mutated p53 (S15A or S15D) using Lipofectamine 2000 (Thermo Fisher Scientific) and incubated for 18 hours to allow protein expression before collecting lysates for immunoblotting.

Denaturing immunoprecipitation

Cell lysates were collected by 2% SDS-lysis buffer and aliquots were used as input. Non-SDS lysis buffer was used to dilute to the rest of lysates. A total of 2 μg of anti-p53 (#sc-126, Santa Cruz Biotechnology), anti-Ub (#sc-8017, Santa Cruz Biotechnology) or normal IgG (#sc-2025, Santa Cruz Biotechnology) was incubated with lysates. Dynabeads (ThermFisher SCIENTIFIC) were used for immunoprecipitation according to the manufacturer's protocol. Immunoblotting was performed as above and anti-mouse IgG VeriBlot for IP secondary antibody (HRP) (ab131368, abcam) was used.

RNA extraction and qRT-PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Germany). RNA purity and concentration was estimated with an ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific, UK). Complementary DNA was generated using the High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific) as described by the manufacturer. qRT-PCR was carried out using SYBR green RT-PCR master mix (Life Technologies) as per the manufacturer's guidelines and the following primers:

MDM2: F-AGTAGCAGTGAATCTACAGGGA, R-CTGATCCAACCAATCACCTGAAT

CDKN1A: F-TGTCCGTCAGAACCCATGC, R-AAAGTCGAAGTTCCATCGCTC

PUMA: F-ACCTCAACGCACAGTACGA, R-CTGGGTAAGGGCAGGAGTC

TP53INP1: F-TCTTGAGTGCTTGGCTGATACA, R-GGTGGGGTGATAAACCAGCTC

FAS: F-AGATTGTGTGATGAAGGACATGG, R-TGTTGCTGGTGAGTGTGCATT

TNFRSF10B: F-ATGGAACAACGGGGACAGAAC, R-CTGCTGGGGAGCTAGGTCT

BAX: F-CCCGAGAGGTCTTTTTCCGAG, R-CCAGCCCATGATGGTTCTGAT

GAPDH: F-CAATGACCCCTTCATTGACC, R-GATCTCGCTCCTGGAAGAT

qRT-PCR reactions using a total of 20ng of the cDNA samples per 10 µL final reaction volume, with the standard cycling parameters were performed and products detected in real time on an ABI 7900HT system.(Esfandiari *et al*, 2016) GAPDH was used as endogenous control and samples of cells exposed to DMSO solvent control were used as the calibrator for each independent repeat, with the formula $2^{\Delta\Delta Ct}$ used to calculate fold-changes. Analysis was carried out using SDS 2.2 software (Applied Biosystems).

Reverse Phase Protein Arrays (RPPA)

RPPA detection of proteins was carried out by ArrayGen UK on a collaboration basis. The detailed experimental procedure and technical background are described in supplementary information (Voshol *et al*, 2009).

Fluorescence-activated cell sorting (FACS)

After treatment, floating and adhered cells were pooled and fixed using 70% cold ethanol. Samples were incubated in 250 µl PBS with 40 µg/mL propidium iodide (Sigma-Aldrich), 20 µg/mL RNAse A (Sigma-Aldrich) for 20 minutes in the dark at room temperature, then were analyzed on a FACSCalibur™ flow cytometer using CellQuest Pro software (Becton Dickinson, Oxford, UK). Cell cycle distribution based on DNA content was determined using Cyflogic (CyFlo Ltd, Turku, Finland).

Caspase 3/7 activity assay

Melanoma cells were seeded in white 96-well plates and treated after 24 hours. Caspase-3/7 enzymatic activities were measured using a FLUOstar Omega plate reader (BMG Labtech) after adding a 1:1 ratio of CaspaseGlo 3/7 reagent (Promega) to growth media and incubating for 30 minutes. All values were expressed as a ratio of signal relative to solvent control.

Transfection of siRNAs

A total of 40nM siRNA duplex (Eurogentec) against *TP53* and control noncoding sequence was transfected by Lipofectamine 2000 (Thermo Fisher Scientific) in OptiMEM-glutamax (Optimem) serum free media (Invitrogen). The sequences were designed as follows: Control SiRNA (SiControl), sense: 5'-GCGCGCUUUGUAGGAUUCGdTdT-3', antisense: 5'-CGAAUCCUACAAAGCGCGCdTdT-3'; two alternative *TP53* targeted SiRNA (SiP53), SiP53 #1, sense: 5'-CCACCAUCCACUACAACUAdTdT-3', antisense: 5'-UAGUUGUAGUGGAUGGUGGdTdT-3', SiP53 #2, sense: 5'-CUGGAUGGAGAAUAUUUCA-3', antisense: 5'-UGAAAUAUUCUCCAUCCAG-3'.

Statistical analysis

Data were presented as mean $\pm$ standard error of mean (SEM) unless otherwise stated. Statistical tests were carried out using GraphPad Prism 6 software and all p-values represent paired t-tests of at least three independent repeats. A p-value less than 0.05 was considered as statistically significant.

Results

GSK2830371, at doses showing no growth-inhibition alone, potentiated the effects of MDM2 inhibitors on A375 and WM35 cells

The expression of WIP1 protein after p53 activation was evaluated by immunoblotting, showing induction of WIP1 was associated with p53 stabilization after RG7388 (0.2 μM) treatment for 6 hours in $p53^{WT}$ but not $p53^{MUT}$ melanoma cell lines (Figure 1A). WIP1i, GSK2830371, was use as a single agent in growth inhibitory experiments. Growth inhibition measured by SRB assay showed GSK2830371 at concentrations ≤ 10 μM had no or minimal growth-inhibitory effect on $p53^{WT}$ (A375, WM35, C8161) and $p53^{MUT}$ (WM164, WM35-R, CHL-1) melanoma cells (Figure 1B). Immunoblotting of WM35 cells treated by MDM2 inhibitor (either 5 μM Nutlin-3 or 1 μM RG7388), combining with different concentrations of GSK2830371 (Figure 1C), illustrated that WIP1 protein decreased gradually with increased dosages of GSK2830371. Phospho-p53 (Ser15) and acetyl-p53 (Lys382) were probed because phospho-p53 (Ser15) is a direct substrate of WIP1 phosphatase and p53 can be acetylated by CBP/P300 which has been reported to be recruited after phosphorylation of p53 (Ser15) (Meek, 2015). Marked increases in phospho-p53 (Ser15) and acetyl-p53 (Lys382) as a consequences of WIP1 inhibition were observed. The transcriptional targets of p53, MDM2 and p21, increased modestly after combination treatment with GSK2830371.

To choose an optimal concentration of GSK2830371, growth-inhibition assays were performed for A375 and WM35 treated with either MDM2 inhibitor (5 μM Nutlin-3 or 1 μM RG7388) combined with different concentrations of GSK2830371 (Figure 1D-G). Dose-dependent grow-inhibition by GSK2830371 was only found after p53 activation with nutlin-3 or RG7388. A GSK2830371 concentration of 2.5 μM displayed the best potentiation when

combined with either MDM2 inhibitor and was therefore used for subsequent experiments to evaluate the role of WIP1 in p53 network responses.

Supplementary Figure S1A shows immunoblotting of A375, WM35 and C8161 cells treated with MDM2 inhibitor (5 μM Nutlin-3 or 1 μM RG7388) combined with 2.5 μM GSK2830371 for 6 hours. Supplementary Figure S1B shows immunoblotting of A375 and C8161 cells treated with HDM201 combined with 2.5 μM GSK2830371 for 6 and 24 hours. The protein changes were similar to the findings in Figure 1C. The same treatment was performed on A375 and phospho-p53 (Ser15) was detected by reverse phase protein array (RPPA). Significantly increased phospho-p53 (Ser15) was found after combination treatment with either Nutlin-3 or RG7388 and GSK2830371 (Supplementary Figure S1C). The signals were supressed by alkaline phosphatase, validating the antibody used in the RPPA to be specific for phosphorylated-protein.

GSK2830371 potentiated the growth-inhibitory and cytotoxic activity of MDM2 inhibitors in a p53-dependent manner

Growth inhibition and clonogenic survival following treatment with MDM2 inhibitor (either nutlin-3, RG7388 or HDM201), with or without GSK2830371 (2.5μM) combination was evaluated for a panel of $p53^{WT}$ (A375, WM35 and C8161) and $p53^{MUT}$ (WM164, WM35-R, CHL-1) melanoma cell lines (Figure 2 and 3, Supplementary Figure S2). GSK2830371 enhanced the growth-inhibitory effects of MDM2 inhibitors in $p53^{WT}$ rather than $p53^{MUT}$ melanoma cells, showing that potentiation by GSK2830371 worked in a p53-dependent manner (Figure 2A, Supplementary Figure S2A). GSK2830371 significantly decreased the GI_{50} for nutlin-3 or

RG7388 comparing to the GI_{50} for nutlin-3 or RG7388 alone in $p53^{WT}$ melanoma cells (Figure 2B, Supplementary Figure S2B).

A clonogenic assay was performed to see if GSK2830371 can enhance the cytotoxic activity of MDM2 inhibitors. GSK2830371 further inhibited colony formation when it was added to either MDM2 inhibitor in $p53^{WT}$ melanoma cells. (Figure 3A, Supplementary Figure S2C) GSK2830371 significantly decreased the LC_{50} for nutlin-3 or RG7388 compared to the LC_{50} for MDM2 inhibitor alone for A375 or C8161 cells ($p < 0.05$) and with a similar trend for WM35 (Figure 3B, Supplementary Figure S2D). Compared with control (DMSO), there was no significant reduction of colony formation on treatment by GSK2830371 (2.5μM) as a single agent. (Figure 3C, D) The potentiation by GSK2830371 in clonogenic assays was limited to $p53^{WT}$ and was not seen with $p53^{MUT}$ melanoma cells. (Figure 3C)

Increased p53 stabilization by GSK2830371 through increased phosphorylation and acetylation when combined with MDM2 inhibitors could be reversed by ATM inhibition

Ubiquitylation and acetylation happen on the same lysine residues of the C-terminal region of p53 and both are mutually exclusive. Therefore, it was hypothesized that inhibition of WIP1 protein by GSK2830371 could enhance the stabilization of p53 through increasing acetylation and decreased ubiquitin-mediated degradation. The lack of obvious change in total p53 protein in the presence of WIP1 inhibition in figure 1C was postulated to reflect saturation of p53 stabilization following treated by 5μM nutlin-3 and 1μM RG7388. Therefore, lower concentrations of nutlin-3 and RG7388 were tested in combination with GSK2830371. The resulting immunoblots confirmed that combination of lower MDM2 inhibitor doses and WIP1i

increased p53 stabilization compared with the effect of MDM2 inhibitor as a single agent (Figure 4A, Supplementary Figure S1B, S3). Furthermore, this increase was associated with higher levels of phosphorylation (Ser15) and acetylation (Lys382) of p53, and higher expressions of MDM2 and p21.

To examine whether the phosphorylation of p53 (Ser15) is a key residue for subsequent C-terminal acetylation of p53, WM35 cells were transfected with different p53 mutant plasmid constructs, including WT, S15A and S15D mutations (Figure 4B). After 18 hours transfection with 1μg plasmid, increased p53 expression was detected for all three constructs. However, phospho-p53 (Ser15) could only be detected in cells after WT p53 cDNA transfection and not p53 mutant transfections, showing the antibody used was specific against WT p53 (Ser15) phosphorylation. Acetyl-p53 (Lys382) was detected in the cells after p53 (WT) and the S15D phosphomimetic mutant transfection but not when ser15 was mutated to the non-phosphorylatable alanine, indicating that Ser15 and its phosphorylation is an essential and sufficient residue for p53 acetylation.

To evaluate whether WIP1 inhibition by GSK2830371 stabilizes p53 by slowing down its degradation, cycloheximide (CHX) was used to block *de novo* protein synthesis (Figure. 4C). C8161 was treated with 0.2 μM RG7388 for 4 hours to make p53 accumulate. The cells were then treated with CHX (100 μl/ml) to block protein synthesis, in the presence or absence of GSK2830371 for 2 and 4 hours. Protein lysates were collected for immunoblotting at the indicated time points. During the 4-hour CHX treatment, GSK2830371 slowed down the p53 degradation, evidenced by significantly higher levels of p53 protein with concurrent GSK2830371 treatment than without GSK2830371 treatment. ($p=0.02$) (Fig. 4D and E) To test whether the degradation is proteasome-dependent, A375 cells were treated with RG7388 ±

WIP1i and MG132 for 6 hours. MG132 completely prevented the degradation of p53 and masked any difference in stabilization of p53 by RG7388 ± WIP1i. (Figure 4F) Co-immunoprecipitation showed GSK2830371 treatment decreased ub-p53 by pulling down either p53 or ubiqutin. (Figure 4G)

We next examined whether the p53 (Ser15) phosphorylation seen following combined treatment with MDM2 inhibitors and WIP1i is ATM-mediated. A375 cells were treated with 0.2 μM RG7388 ± WIP1i and ± ATM inhibitor (KU55933 10 μM) concurrently for 6 hours. The phosphorylation, acetylation and stabilization of p53 were reversed by ATM inhibitor, which indicated that these changes are at least in part mediated by basal ATM activity (Figure 4H). KU55933 also decreased the growth-inhibitory effects of MDM2 inhibitors on A375 cells (Supplementary Figure S4).

Paired WM35 and WM35-R cells showed GSK2830371 increased p53 stabilization in a p53-dependent manner

WM35-R is an MDM2 inhibitor resistant cell line which we have selected from parental WM35 cell by culturing in medium containing 5μM RG7388. Sanger sequencing revealed that WM35-R has a homozygous *TP53* point mutation (1001G>T) resulting in a Gly334Val amino acid substitution in the p53 protein (Supplementary Figure S5), which is the hinge between β-strand (residues 326–333) and α-helix (residues 335–354) regions in the oligomerization domain.(Kawaguchi *et al*, 2005) Therefore, this pair of melanoma cells is useful for exploring p53-dependent pathway mechanisms in cutaneous melanoma.

Compared with MDM2 inhibitors alone, the combination of MDM2 inhibitor and GSK2830371 increased p53 phosphorylation (Ser15), acetylation (Lys382), and stabilization in WM35 but did not increase p53 acetylation (Lys382) and stabilization in WM35-R (Supplementary Figure S6A). A CHX experiment was performed in WM35 and WM35-R cells to investigate whether GSK2830371 inhibition slowed down the degradation of p53 only in WM35, because acetylation was not found in WM35-R (Supplementary Figure S6). Interestingly, the mutant p53 in WM35-R cells was still subject to some degradation, however after the 4-hour CHX treatment, GSK2830371 decreased the wt-p53 degradation in WM35 more than the mut-p53 in WM35-R (Supplementary Figure S6).

GSK2830371 increased the mRNA expression of p53 transcriptionally regulated genes when combined with MDM2 inhibitors

To test the hypothesis that GSK2830371 enhances the transcriptional activity of p53 in cutaneous melanoma cells treated by MDM2 inhibitors, mRNA expression of candidate genes related to cell cycle arrest and apoptosis were evaluated by quantitative real time polymerase chain reaction (qRT-PCR). The $p53^{WT}$ cells (A375, C8161, WM35) and one $p53^{MUT}$ cell line, WM35-R, were treated with either 1μM nutlin-3 or 0.2μM RG7388, combined with or without GSK2830371 (2.5μM) for 6 hours.

Overall, nutlin-3 or RG7388 induced expression of candidate genes and GSK2830371 potentiated the induced genes in $p53^{WT}$ cells rather than WM35-R. The fold changes of mRNA in response to MDM2 inhibitors with or without WIP1i were less in WM35 than A375 and C8161 (Figure 5 and Supplementary Figure S7). Interestingly, WIP1i alone could significantly induce some of the p53 transcriptional target genes. However, the effects of GSK2830371 on

transcript levels were modest, consistent with GSK2830371 alone having no or minimal growth-inhibitory effect in SRB and clonogenic assays (Figure 1B, 3C-D) and with the lack of obvious changes in p21 and MDM2 proteins in immunoblotting (Figure 1C, Supplementary Figure S1).

Genes for cell cycle arrest, *CDKN1A*, and autoregulatory negative feedback, *MDM2,* were generally induced to a higher level by combination treatment with GSK2830371 and MDM2 inhibitors in $p53^{WT}$ cells, except for the RG7388 plus GSK2830371 combination treatment in WM35. Pro-apoptotic *BAX, FAS, PUMA, TNFBSF10B and TP53INP1* genes were also evaluated. For *BAX*, the induction was modest even though some of the differences were statistically significant. This finding was consistent with another study, which showed no significant increase of *BAX* expression in ovarian cancer cells treated by 5 μM Nutlin-3 or 0.5 μM RG7388 for 6 hours.(Zanjirband *et al*, 2016) For *FAS, PUMA, TNFBSF10B and TP53INP1* genes in A375 and C8161, GSK2830371 significantly potentiated the effect of RG7388 on the expression levels of these genes ($p < 0.05$). GSK2830371 also showed the same trend for enhancing the effect of nutlin-3 on the expression of these genes, but the fold changes were mostly not statistically significant ($p > 0.05$).

WM35 had less fold changes of pro-apoptotic genes than in A375 and C8161. GSK2830371 appeared to increase the effect of nutlin-3 treatment in WM35 on the expression of pro-apoptotic genes although some of these trends were not statistically significant ($p > 0.05$). The poor potentiation of RG7388 by GSK2830371 found in WM35 was possibly because the transcripts of those genes were saturated by RG7388 treatment alone; WM35 treatment with 1 μM RG7388 did not increase more mRNA expression of *MDM2, CDKN1A, PUMA, TNFBSF10B, TP53INP1* compared with 0.2 RG7388, either with or without WIP1i (Supplementary Figure S8).

The effect of combined MDM2 and WIP1 inhibition on cell cycle distribution and apoptosis

Given that GSK2830371 potentiated the growth inhibition and clonogenic reduction by MDM2 inhibitors, FACS analysis was carried out to investigate changes in cell cycle distribution. In addition, Sub-G1 events detected by FACS analysis and caspase 3/7 catalytic activity were used as indicators of apoptosis. The responses to MDM2 and WIP1 inhibitors were cell line dependent.

In all the cell lines, 2.5μM GSK2830371 alone did not affect the cell cycle distribution through 72-hours of treatment. (Figure 6, Supplementary Figure S9, S10) In general, RG7388 (0.2μM) or HDM201 (0.2μM) treatment of $p53^{WT}$ cells (A375, WM35, C8161) decreased the proportion of cells in S-phase, which was accompanied by G1-phase increases, with or without G2-phase increases. GSK2830371 treatment further enhanced the changes in cell cycle distribution markedly when it was combined with MDM2 inhibitors.

Apoptotic responses, shown by increased FACS Sub-G1 signals and caspase 3/7 activity after RG7388 or HDM201 treatment, was evident for A375 cells but not for WM35 or C8161 cells. The combination of MDM2 and WIP1 inhibitors significantly increased the apoptotic response of A375 cells. Cell cycle distribution was not affected in *TP53* mutant WM35-R cells regardless of the treatment conditions, demonstrating that the changes in cell cycle distribution observed are p53-dependent.

SiRNA-mediated p53 knockdown

To further investigate the p53 dependence of MDM2 and WIP1 inhibition in cutaneous melanoma, siRNA mediated p53 knockdown was performed in two $p53^{WT}$ melanoma cells, A375 and WM35. The cells were treated with siRNA for 24 hours followed by RG7388 or

HDM201 ± GSK2830371 treatment. The potentiation of growth inhibition by GSK2830371 on RG7388 or HDM201 was suppressed by the p53 knockdown (Figure 7).

Discussion

In the current study, we demonstrated that doses of the GSK2830371, WIP1 inhibitor, with no growth-inhibitory activity, nevertheless potentiated the growth inhibition and cell killing of wild-type p53 cutaneous melanoma cells by MDM2 inhibitors, nutlin-3, RG7388 and HDM201. Mechanistic studies linked this potentiation to increased phosphorylation, acetylation and decreased ubiquitylation. This has a two-fold effect, resulting in not only increased stabilization of p53, but also increased functional activation by the increased post-translational phosphorylation and acetylation (Figure 8).

Among the three $p53^{WT}$ melanomas investigated in the current study, we found A375 to be particularly primed to undergo apoptosis in response to p53 activation rather than the other two cell lines. This was evidenced by significant increases in sub-G1 signals on FACS cell cycle analysis and caspase 3/7 activity after combination treatment. The p53-dependent transcription profiles possibly explain some differences between the A375 and WM35 responses to MDM2 and WIP1 inhibition (Figure 5 and Supplementary Figure S7). Although p53 expression has generally been associated with the levels of transcriptional target genes responsible for cell cycle arrest and apoptosis, apoptosis has been reported only to proceed in certain conditions, such as achieving a transcriptional threshold by p53 reactivation or suppression of its negative regulators (Khoo *et al*, 2014; Kracikova *et al*, 2013; Lu *et al*, 2013). Therefore, two distinct subgroups of response are identifiable, depending on whether cells undergo apoptosis or not after p53 reactivation by MDM2 inhibitors. Irreversible apoptosis only occurs with some cell types and reversible growth suppression or senescence without apoptosis is observed with other cell types. (Lu *et al*, 2013; Tseng *et al*, 2010) In the current study, inhibition of WIP1, a negative regulator of p53, potentiated the activation of p53 in all three $p53^{WT}$ melanoma cell lines through post-

translation modification, but only enhanced apoptosis in A375 cells and did not shift cell cycle arrest to apoptosis in the cells not primed for apoptosis (WM35 and C8161 in the current study).

Post-translational modification of p53 has critical effects on its stability and function (Kruse & Gu, 2009). A key post-translational modification is the phosphorylation of p53 at Ser15, which is mediated by the ATM and ATR protein kinases, and has been considered as an initiating event in p53 activation(Saito *et al*, 2002) by promoting the acetylation of lysine residues of the p53 C-terminal region, through recruitment of p300/CBP histone acetyltransferase.(Dumaz & Meek, 1999; Gu & Roeder, 1997; Lambert *et al*, 1998; Reed & Quelle, 2014) MDM2 can ubiquitinate the same lysine residues of p53 (Brooks & Gu, 2011; Reed & Quelle, 2014), and acetylation and ubiquitination are mutually exclusive. Therefore, p53 acetylation increases its stability by inhibiting MDM2-mediated ubiquitination of p53 and can stimulate its sequence–specific DNA binding activity (Gu & Roeder, 1997; Li *et al*, 2002).

WIP1 phosphatase and ATM kinase work antagonistically on the same p53-related substrates, ATM (Ser1981), MDM2 (Ser395) and p53 (Ser15), to maintain a fine balance of these proteins in order to regulate the function and stability of p53. For example, autophosphorylation of ATM (Ser1981) after irradiation has been shown to stabilize ATM at DNA damage sites and phosphorylate p53 (Ser15).(So *et al*, 2009) Ser1981 of ATM is also a substrate of WIP1 phosphatase (Shreeram *et al*, 2006) and WIP1 inhibition by GSK2830371 can increase phospho-ATM (Ser1981) in a non-genotoxic manner.(Gilmartin *et al*, 2014) Following DNA damage stress, ATM is able to phosphorylate MDM2 at Ser395 which attenuates degradation of p53.(Khosravi *et al*, 1999; Maya *et al*, 2001) WIP1 can stabilize MDM2 in an ATM-dependent manner and also inhibit ubiquitination of MDM2.(Lu *et al*, 2007) Therefore, GSK2830371 is an effective non-genotoxic way of increasing activation of the ATM-mediated

network by inhibition of WIP1 to maintain the phosphorylated state of key proteins. This is particularly useful when the p53-dependent WIP phosphatase is induced by non-genotoxic MDM2 inhibitors.

GSK2830371 as a single agent selectively inhibits the growth of MCF-7 and MX-1 cells, *PPM1D* amplified breast cancer cells, and a subset of $p53^{WT}$ hematological cancer cell lines.(Gilmartin *et al*, 2014) In one previous report using cells with different *PPM1D* gene alterations (gain of function mutations, amplification,or copy number gain), all cells except MCF-7 cell were resistant to GSK2830371 (GI_{50} > 10 μM) as a single agent treatment.(Esfandiari *et al*, 2016) Consistent with this report, GSK2830371 alone had no obvious growth-inhibitory activity (GI_{50} > 10 μM) in all of the melanoma cell lines we investigated in the current study (Figure 1A). Actually, modest phosphorylation of p53 (Ser15) following treatment with single agent GSK2830371 was detected by immunoblotting (Figure 4A) and RPPA (Supplementary Figure S1B), which resulted in a statistically significant but small increase in transcript level of some genes (Figure 6) and slight induction of p21 protein (Figure 1C, 4A). However, the transactivation of genes by this small level of p53 phosphorylation was insufficient to have a significant effect on the growth of the cutaneous melanoma cells.

Previous studies with genotoxic modalities such as ionizing or UV irradiation indicated a model in which resultant phosphorylation of p53 on Ser15 recruits histone acetyl transferases (HATs) to acetylate lysine residues in the p53 C-terminal domain.(Saito *et al*, 2002) In the current study, this model was more-specifically tested and shown to be applicable to non-genotoxic treatment with MDM2-p53 binding antagonists and WIP1 inhibitor, providing a mechanistic basis for the potentiation. Consistent with a recent report (Sriraman *et al*, 2016) using nutlin-3a and GSK2830371, our results showed that enhanced phosphorylation (Ser15) and

acetylation (Lys382) of p53 were found after non-genotoxic nutlin-3 and GSK2830371 treatment. Here we extended these observations to more specific and potent MDM2 inhibitors, which are progressing through clinical trials. In addition, we transfected p53(Ser15) mutants to prove the Ser15 is the essential and sufficient residue for p53 acetylation. Furthermore, we investigated the p53 stability after combination treatment in current study.

CHX treatment was used to investigate the effect of acetylation of p53 after GSK2830371 treatment on p53 half-life and showed p53 levels were increased by slowing down degradation. MG132 reversed the change in p53 expression, indicating that GSK2830371 decreased the proteasomal degradation of p53. Both the p53 acetylation leading to inhibition of its ubiquitination by MDM2 (Li *et al*, 2002) and WIP1 inhibition by siRNA leading to decrease in p53 ubiquitination (Lu *et al*, 2007) are consistent with our finding that WIP1 inhibition by GSK2830371 stabilized p53 as a result of the decreased MDM2-mediated ubiquitination shown by co-immunoprecipitation. When the cells were treated by the lower concentrations of MDM2 inhibitors, the MDM2 was only partially inhibited by MDM2 inhibitors, so p53 acetylation then played a critical role in p53 stability by antagonizing ubiquitiylation (Figure 4).

GSK2830371 potentiated MDM2 inhibitors to amplify the expression of p53 transcriptionally targeted genes which are responsible for cell cycle arrest and apoptosis in $p53^{WT}$ melanoma cells, however the degree of potentiation was cell line dependent. Greater potentiation by GSK2830371 was found with A375 and C8161 cells than for WM35, by qRT-PCR, immunoblotting, growth inhibition, and clonogenic assay. Although combination treatment stabilized p53 by decreasing degradation in WM35 cells (Figure 5), the increases in p53-target gene mRNAs and proteins in this cell line were modest. Consequently, the fold-changes in GI_{50} and LC_{50} values were more limited. We also checked basal expressions of MDMX and WIP1

(Supplementary Figure S11) and showed there was no clear correlation between basal expression levels and responses to MDM2 and WIP1 inhibition, as MDM4 was overexpressed in ~65% melanoma cases.(Gembarska *et al*, 2012) The results of the current study indicate that the responsiveness of melanoma cell lines to MDM2 inhibitors and WIP1i depends to a large part on the fold differences between the basal and maximal relative transcript levels of downstream p53 transcriptional target genes, but is also likely to be modulated by the downstream status of pro-survival and anti-apoptotic pathways. (Supplementary Figure S8)

In summary, we report the non-genotoxic potentiation of nutlin-3 and clinically relevant second generation MDM2-p53 binding antagonists, RG7388 and HDM201, by the WIP1 phosphatase inhibitor GSK280371, through p53 phosphorylation, acetylation, stabilization and increased transcription of p53 regulated genes, in a panel of $p53^{WT}$ cutaneous melanoma cell lines. This combination treatment activated the ATM-mediated response without DNA damage, highlighting a novel non-genotoxic therapeutic strategy to further explore for melanoma.

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Figure Legends

Figure 1. WIP1 phosphatase induction by p53 activation with either nutlin-3 or RG7388 and inhibition by GSK2830371(WIP1i). (A) Increase in WIP1 expression in $p53^{WT}$ but not $p53^{MT}$ melanoma cell lines, by p53 activation with RG7388 (0.2 μM) for six hours (D, DMSO, R, RG7388). (B) Growth inhibition by SRB assay showed WIP1i at $\leq$10 μM has no growth-inhibitory effect on $p53^{WT}$ or $p53^{MT}$ melanoma cells. (C) Immunoblotting of A375 and WM35 cells treated by MDM2 antagonists (5μM Nutlin-3 or 1μM RG7388), combined with different concentrations of WIP1i for 6 hours. (D-G) Growth-inhibition of A375 and WM35 by either 5μM Nutlin-3 or 1μM RG7388, combined with different concentrations of WIP1i for 72 hours. The % growth is shown either normalized to DMSO solvent control treatment (D, E) or to the fixed doses of MDM2 inhibitors (F, G).

Figure 2. GSK2830371(WIP1i) potentiated the growth inhibitory effect of MDM2 antagonists in a p53 dependent manner. (A) Growth inhibition measured by SRB assay for $p53^{WT}$ (A375, WM35 and C8161) and $p53^{MT}$ (WM164, WM35-R, CHL-1) melanoma cell lines treated with different concentrations of MDM2 antagonists (either nutlin-3 or RG7388), combined with or without WIP1i (2.5μM) for 72 hours. All % of growth was normalized to DMSO treatment. (B) Summary of GI_{50} (mean $\pm$ SEM) values for nutlin-3 or RG7388 with or without WIP1i in $p53^{WT}$ melanoma cells. SEM, standard error of the mean.

Figure 3. The reduction in melanoma clonogenic cell survival by MDM2 antagonists was potentiated by GSK2830371(WIP1i). (A) P53-wild type (WT) melanoma cell lines (A375, WM35 and C8161) were treated by different concentrations of MDM2 antagonists (nutlin-3 or RG7388), combined with or without WIP1i (2.5μM) for 72 hours. (B) Summary of the effect of WIP1 inhibition on LC_{50} values for nutlin-3 or RG7388 from at least three independent repeats. (C) Clonogenic survival of $p53^{WT}$ and $p53^{MT}$ cells treated with either DMSO, WIP1i, nutlin-3, RG7388 or combination. (D) Clonogenic formation of C8161 cells following treatment with nutlin-3 (200nM), RG7388 (20nM), WIP1i (2.5μM) or combination. *, $p < 0.05$; **, $p < 0.005$., *** $p<0.0005$; SEM, standard error of the mean.

Figure 4. p53 increase by GSK2830371(WIP1i) and MDM2 inhibitors was reversed by ATM inhibition (ATMi, KU55933) (A) Immunoblotting of C8161 cells treated with nutlin-3, WIP1i or combination of both for 6 hours. (B) Effect of WM35 transfection with different p53 (Ser15) mutant plasmid constructs on Acetyl-p53(Lys 382). (C) Design of a cycloheximide (CHX) experiment to measure protein turnover. (D) Immunoblotting for C8161 cells treated by RG7388 (0.2μM) followed by CHX with or without 2.5μM WIP1i. (E) The relative expression of p53 by densitometry, normalized to GAPDH; p-value shown for 3 independent repeats. (F) Immunoblotting for A375 cells treated by MG132 $\pm$ RG7388 $\pm$ WIP1i for 6 hours. (G) A375 was treated by MG132 $\pm$ WIP1i for 6 hours. Ub-p53 was immunoprecipitated with anti-p53 antibody and immunoblot with anti-ub antibody and by reverse way. The last lane is the IgG pulldown negative control which was incubated with lysates after MG132 treatment. (H) Immunoblotting of A375 and C8161 cells treated with R7388, WIP1i, ATMi, and combinations for 6 hours.

Figure 5 mRNA expression of p53 transcriptional target genes by qRT-PCR. mRNA expression of p53 transcriptionally regulated genes in response to either 2.5μM WIP1i, 1 μM Nutlin-3, 0.2 μM RG7388 or combinations for 6 hours relative to DMSO solvent and GAPDH control in A375 (A), C8161 (B), WM35 (C) and WM35-R (D) melanoma cells. Statistical significance of differences (*, $p \leq 0.05$, ** $p \leq 0.005$, ***, $p \leq 0.0005$) is shown above each bar for each treatment compared with DMSO control. The significance of differences with or without WIP1i are indicated above the horizontal bars. Only the p-values less than 0.05 are shown. Data are presented as mean ± standard error of mean (SEM) for three independent repeats.

Figure 6. The effect of 24 hour treatment with combinations of MDM2 and WIP1 inhibitors on cell cycle distribution and apoptosis. Melanoma cells were treated by either 2.5μM WIP1i, 0.2 μM RG7388, 0.2 μM HDM201 or combinations for 24 hours. (A) Cell cycle distribution changes by FACS analysis. (B) Sub-G1 events by FACS and (C) caspase 3/7 activity, were used as indicators of apoptosis. Statistically significant p-values (*, $p \leq 0.05$) are indicated. Data are presented as mean ± standard error of mean (SEM) for three independent repeats.

Figure 7. The combination of WIP1 and MDM2 inhibitions is p53-dependent. A375 and WM35 were subjected to siRNA-mediated knockdown of p53 for 24 hours followed by RG7388 and HDM201 treatments. (A-D) Growth inhibition of A375 (A, C) and WM35 (B, D) treated with siRNA followed by 72-hour RG7388 (A, B) / HDM201 (C, D) with or without GSK2830371(WIP1i). (E, F) Immunoblotting of A375, WM35 treated with siRNA followed by HDM201 $\pm$ WIP1i. (G) Caspase 3/7 activity of A375 treated with siRNA followed by RG7388 /

HDM201 ± WIP1i for 24 hours. Statistically significant p-values (*, $p \leq 0.05$) are indicated. Data are presented as mean ± standard error of mean (SEM) for at least three independent repeats.

Figure 8. Proposed model for GSK2830371 (WIP1i) potentiation of the growth-inhibitory and cytotoxic effects of MDM2 antagonists. After activation of p53 by MDM2 antagonists (Nutlin-3/RG7388/HDM201), WIP1 inhibition (WIP1i) increases phospho-p53 (Ser 15) through allosteric inhibition of WIP1 phosphatase. Phospho-p53 (Ser 15) recruits histone acetyl transferases (HATs, e.g. CBP/P300) which acetylate lysine residues in the C-terminal region of p53. Acetylation of p53 increases the transcriptional activity of p53 directly, and also indirectly by stabilizing p53. Dashed lines indicate p53 transcriptional upregulation of the corresponding genes for MDM2 and WIP1. TAD, transcriptional activation domain; DBD, DNA binding domain; REG, C-terminal regulatory region.

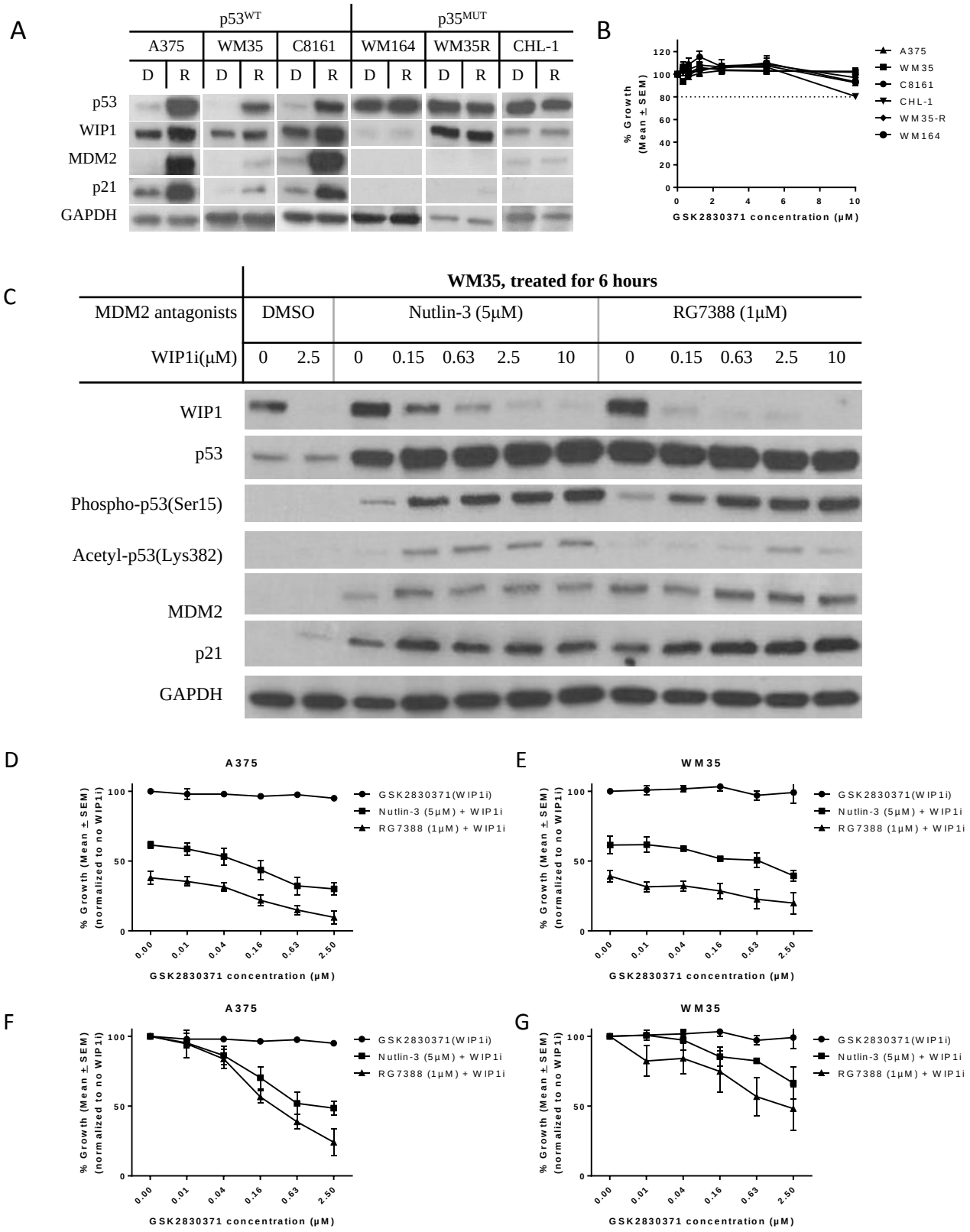
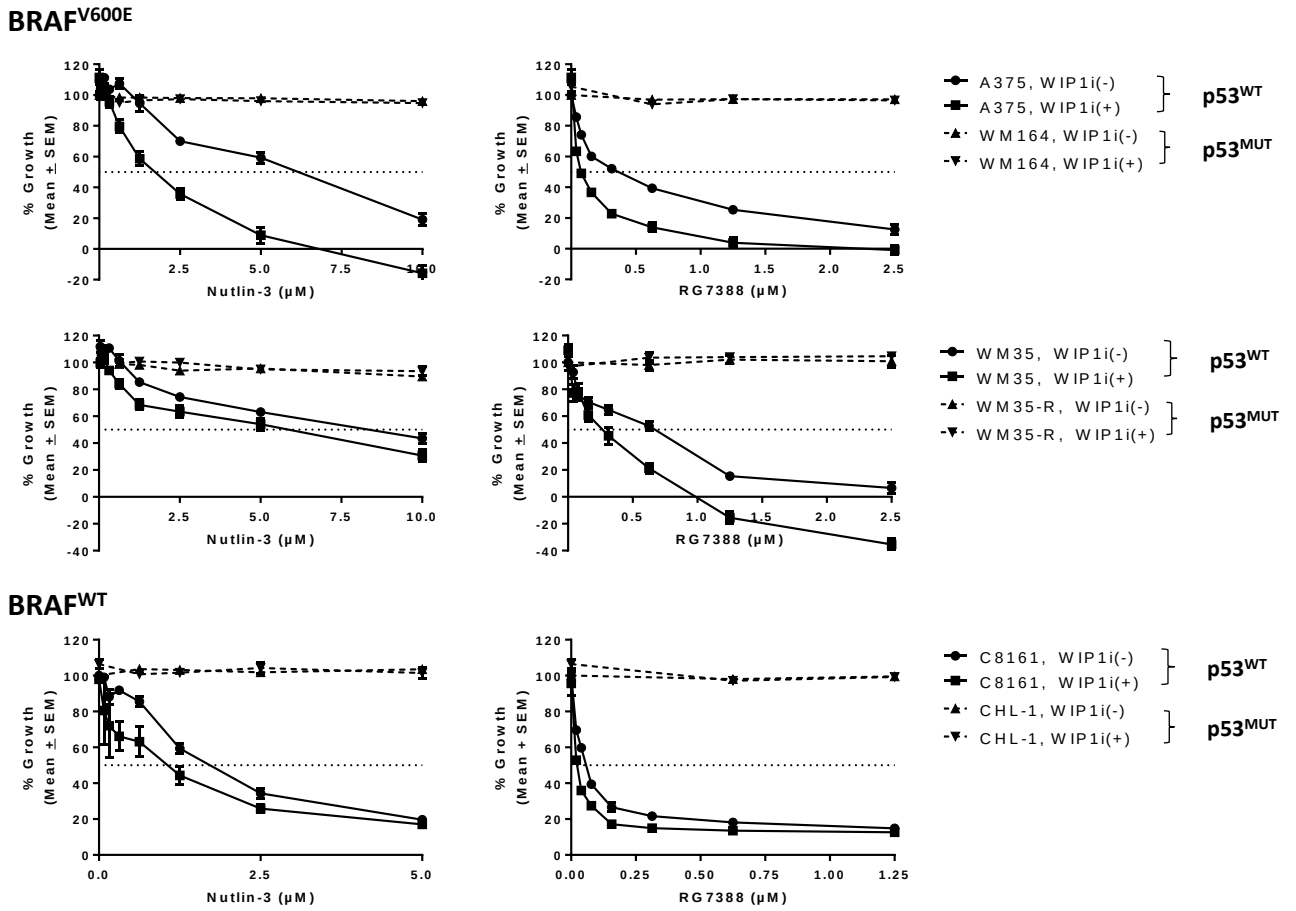
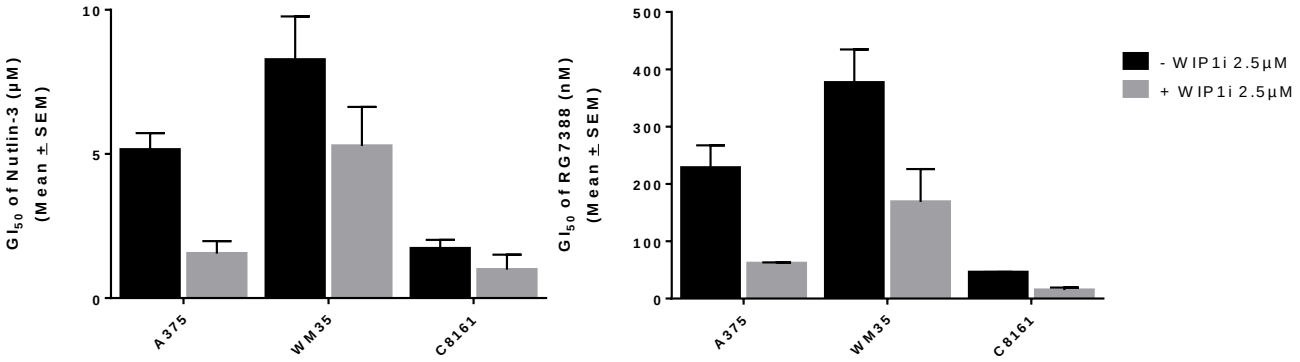


Figure 1. Figure 1. WIP1 phosphatase induction by p53 activation with either nutlin-3 or RG7388 and inhibition by GSK2830371(WIP1i). (A) Increase in WIP1 expression in $p53^{WT}$ but not $p53^{MT}$ melanoma cell lines, by p53 activation with RG7388 (0.2 μM) for six hours (D, DMSO, R, RG7388). (B) Growth inhibition by SRB assay showed WIP1i at $\leq$10 μM has no growth-inhibitory effect on $p53^{WT}$ or $p53^{MT}$ melanoma cells. (C) Immunoblotting of A375 and WM35 cells treated by MDM2 antagonists (5μM Nutlin-3 or 1μM RG7388), combined with different concentrations of WIP1i for 6 hours. (D-G) Growth-inhibition of A375 and WM35 by either 5μM Nutlin-3 or 1μM RG7388, combined with different concentrations of WIP1i for 72 hours. The % growth is shown either normalized to DMSO solvent control treatment (D, E) or to the fixed doses of MDM2 inhibitors (F, G).

A



B



GI50, μM	A375	WM35	C8161
WIP1i(-)	5.1 ± 0.6	8.3 ± 1.5	1.7 ± 0.3
WIP1(+)	1.5 ± 0.4	5.3 ± 1.4	1.0 ± 0.5
p value	0.012	0.010	0.049

GI50, nM	A375	WM35	C8161
WIP1i(-)	228.1 ± 39.1	376.6 ± 58.0	46.0 ± 0.4
WIP1(+)	61.6 ± 1.5	168.8 ± 57.3	14.8 ± 4.5
p value	0.025	0.028	0.009

Figure 2. GSK2830371(WIP1i) potentiated the growth inhibitory effect of MDM2 antagonists in a p53 dependent manner. (A) Growth inhibition measured by SRB assay for $p53^{WT}$ (A375, WM35 and C8161) and $p53^{MT}$ (WM164, WM35-R, CHL-1) melanoma cell lines treated with different concentrations of MDM2 antagonists (either nutlin-3 or RG7388), combined with or without WIP1i (2.5μM) for 72 hours. All % of growth was normalized to DMSO treatment. (B) Summary of GI_{50} (mean ± SEM) values for nutlin-3 or RG7388 with or without WIP1i in $p53^{WT}$ melanoma cells. SEM, standard error of the mean.

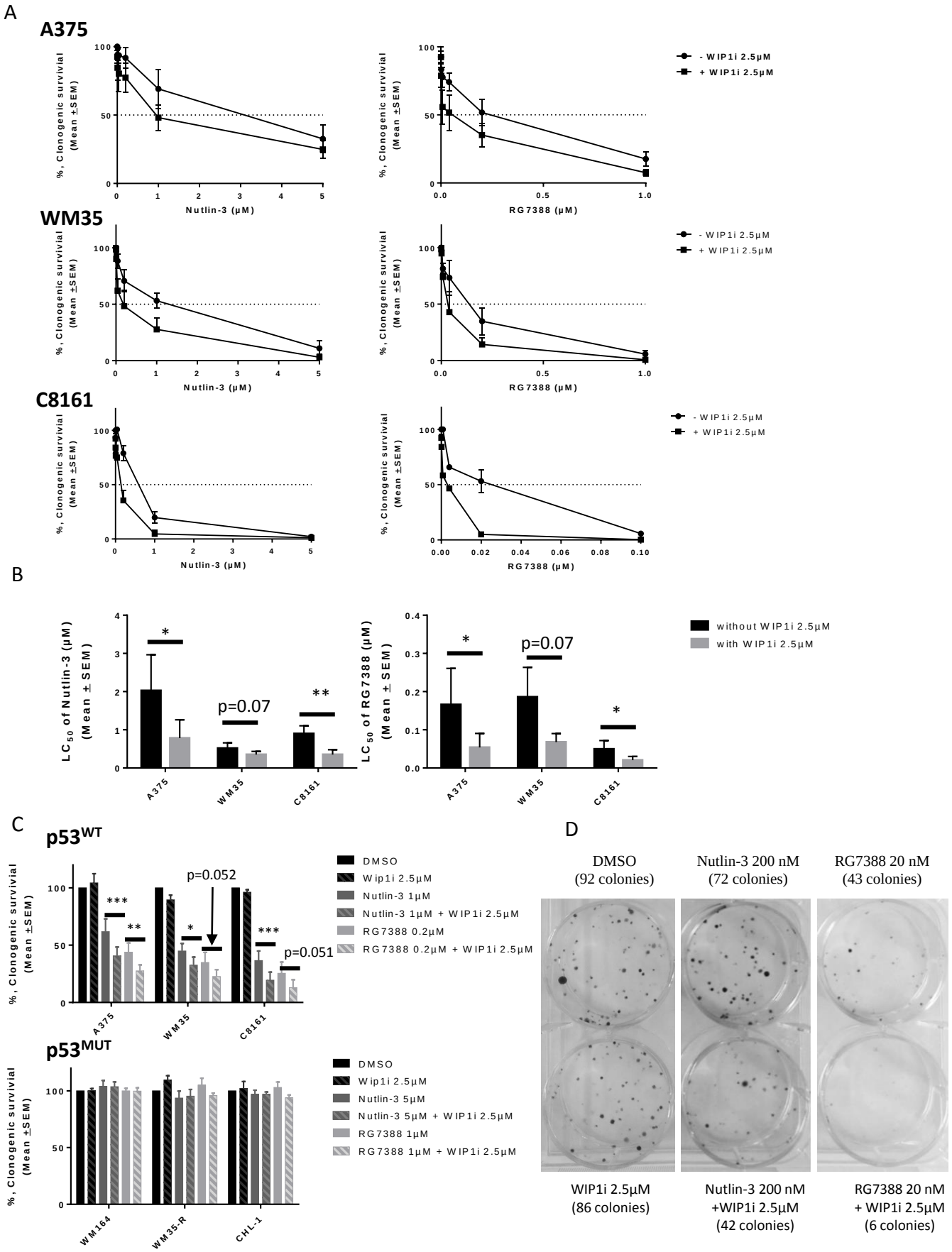


Figure 3. The reduction in melanoma clonogenic cell survival by MDM2 antagonists was potentiated by GSK2830371(WIP1i). (A) P53-wild type (WT) melanoma cell lines (A375, WM35 and C8161) were treated by different concentrations of MDM2 antagonists (nutlin-3 or RG7388), combined with or without WIP1i (2.5μM) for 72 hours. (B) Summary of the effect of WIP1 inhibition on LC_{50} values for nutlin-3 or RG7388 from at least three independent repeats. (C) Clonogenic survival of $p53^{WT}$ and $p53^{MT}$ cells treated with either DMSO, WIP1i, nutlin-3, RG7388 or combination. (D) Clonogenic formation of C8161 cells following treatment with nutlin-3 (200nM), RG7388 (20nM), WIP1i (2.5μM) or combination. *, $p < 0.05$; **, $p < 0.005$., *** $p < 0.0005$; SEM, standard error of the mean.

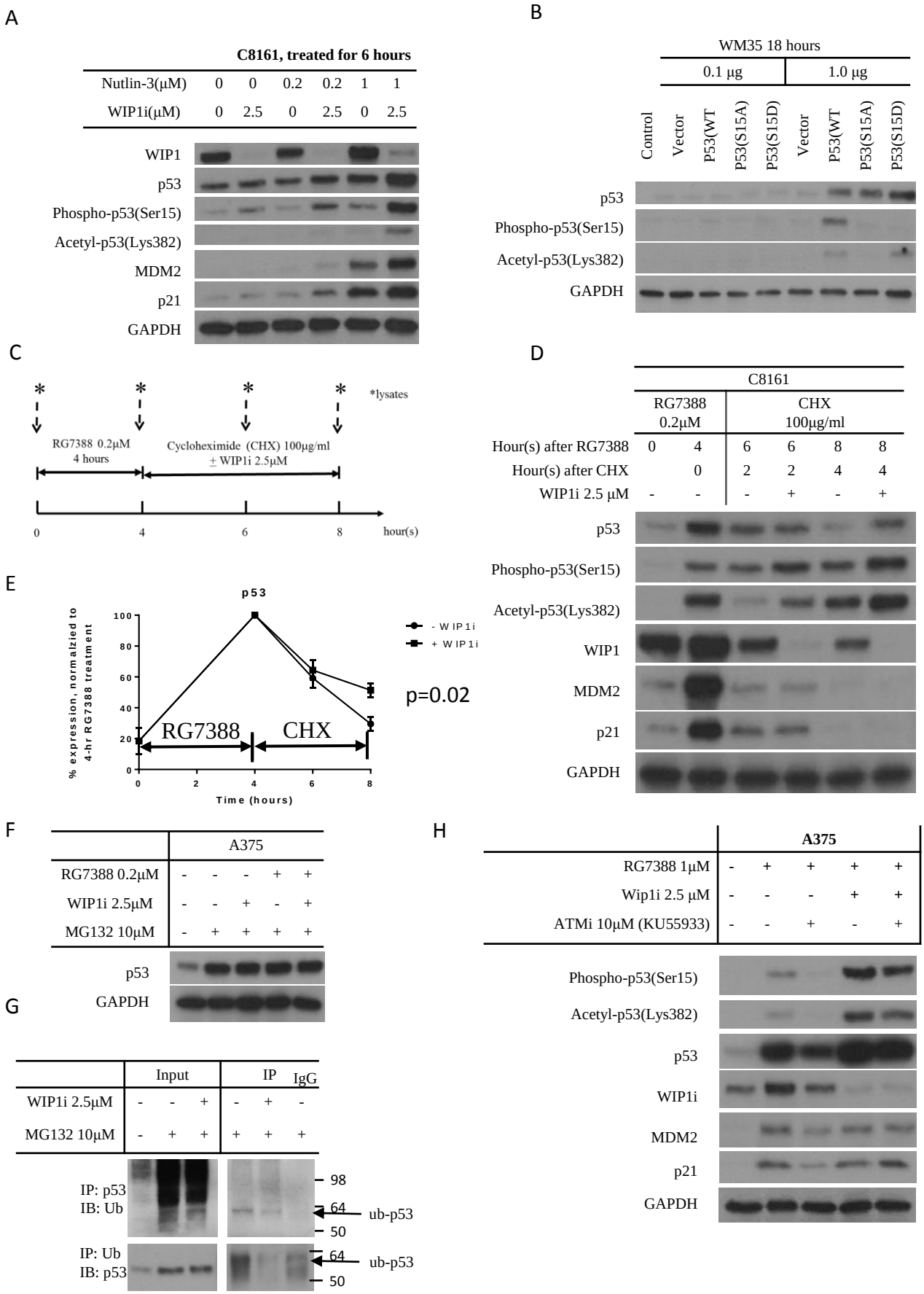


Figure 4. p53 increase by GSK2830371(WIP1i) and MDM2 inhibitors was reversed by ATM inhibition (ATMi, KU55933) (A) Immunoblotting of C8161 cells treated with nutlin-3, WIP1i or combination of both for 6 hours. (B) Effect of WM35 transfection with different p53 (Ser15) mutant plasmid constructs on Acetyl-p53(Lys 382). (C) Design of cycloheximide (CHX) experiment to measure protein turnover. (D) Immunoblotting for C8161 cells treated by RG7388 (0.2μM) followed by CHX with or without 2.5μM WIP1i. (E) Relative expression of p53 by densitometry, normalized to GAPDH; p-value for 3 independent repeats. (F) Immunoblotting for A375 cells treated by MG132 ± RG7388 ± WIP1i for 6 hours. (G) A375 was treated by MG132 ± WIP1i for 6 hours. Ub-p53 was immunoprecipitated with anti-p53 antibody and immunoblotted with anti-ub antibody and vice-versa. The last lane is the IgG pulldown negative control which was incubated with lysates after MG132 treatment. (H) Immunoblotting of A375 and C8161 cells treated with R7388, WIP1i, ATMi, and combinations for 6 hours.

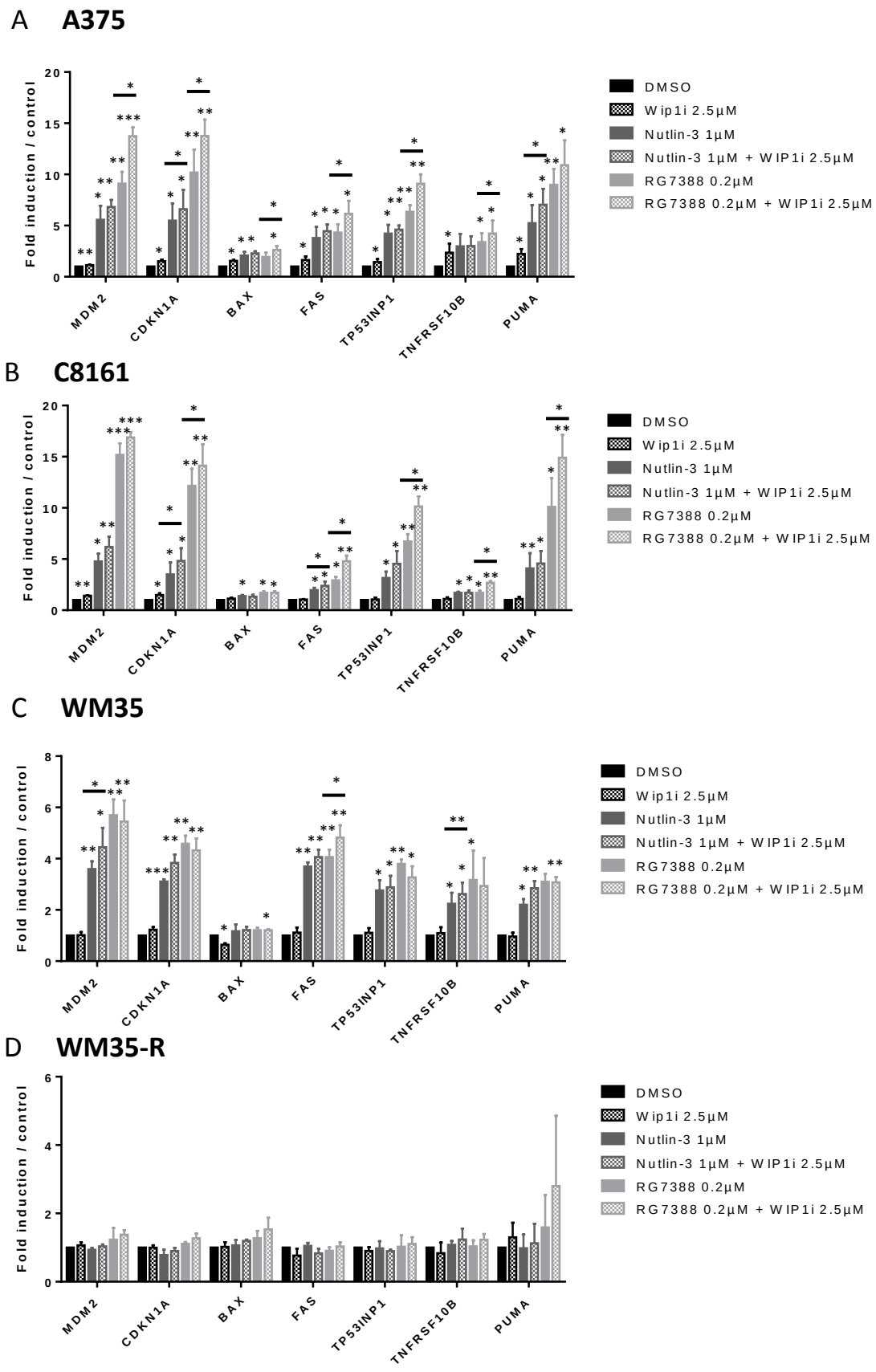
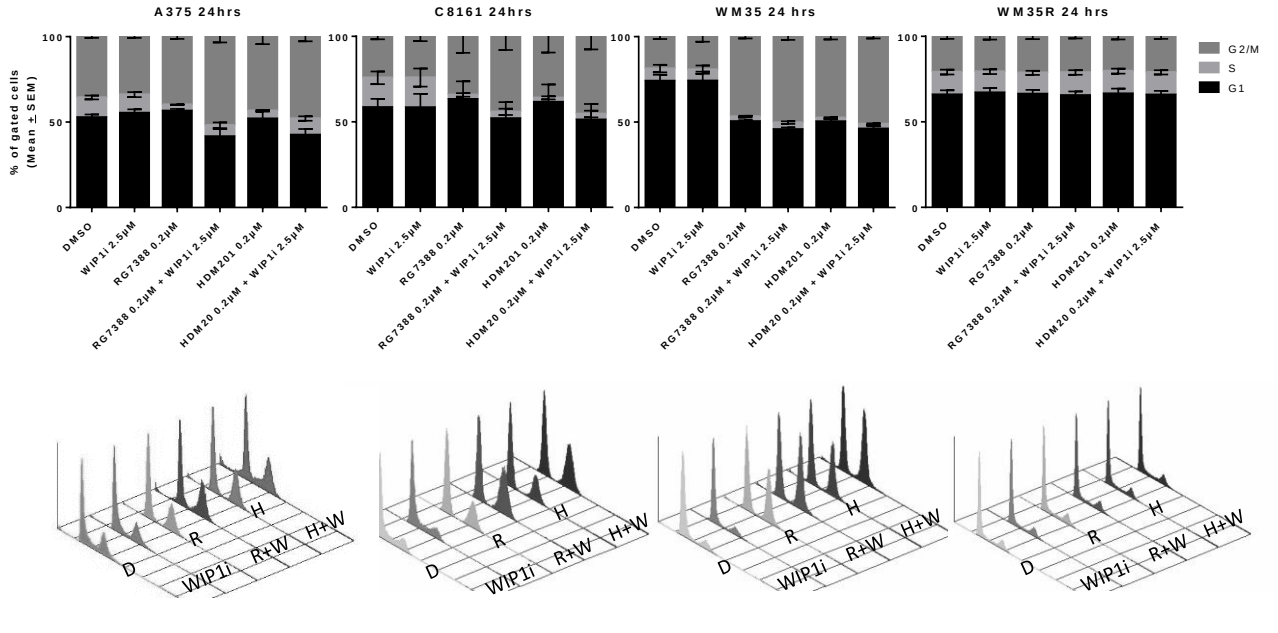


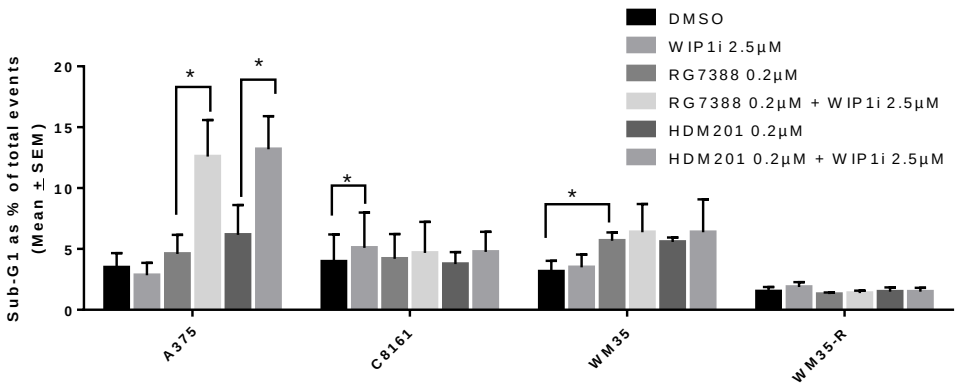
Figure 5 mRNA expression of p53 transcriptional target genes by qRT-PCR. mRNA expression of p53 transcriptionally regulated genes in response to either 2.5µM WIP1i, 1 µM Nutlin-3, 0.2 µM RG7388 or combinations for 6 hours relative to DMSO solvent and GAPDH control in A375 (A), C8161 (B), WM35 (C) and WM35-R (D) melanoma cells. Statistical significance of differences (*, $p \leq 0.05$, ** $p \leq 0.005$, ***, $p \leq 0.0005$) is shown above each bar for each treatment compared with DMSO control. The significance of differences with or without WIP1i are indicated above the horizontal bars. Only the p-values less than 0.05 are shown. Data are presented as mean ± standard error of mean (SEM) for three independent repeats.

A



D, DMSO; W, WIP1i; R, RG7388; R+W, RG7388 + WIP1i; H, HDM201, H+D, HDM201 + WIP1i

B



C

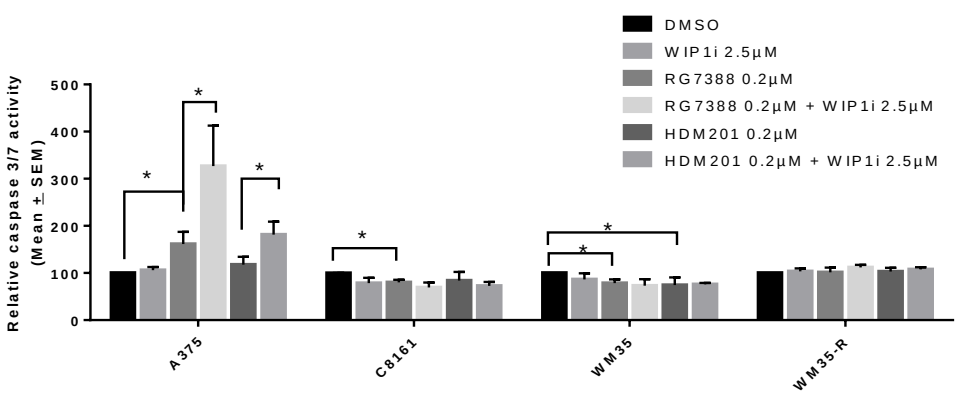


Figure 6. The effect of 24 hour treatment with combinations of MDM2 and WIP1 inhibitors on cell cycle distribution and apoptosis. Melanoma cells were treated by either 2.5μM WIP1i, 0.2 μM RG7388, 0.2 μM HDM201 or combinations for 24 hours. (A) Cell cycle distribution changes by FACS analysis. (B) Sub-G1 events by FACS and (C) caspase 3/7 activity, were used as indicators of apoptosis. Statistically significant p-values (*, $p \leq 0.05$) are indicated. Data are presented as mean ± standard error of mean (SEM) for three independent repeats.

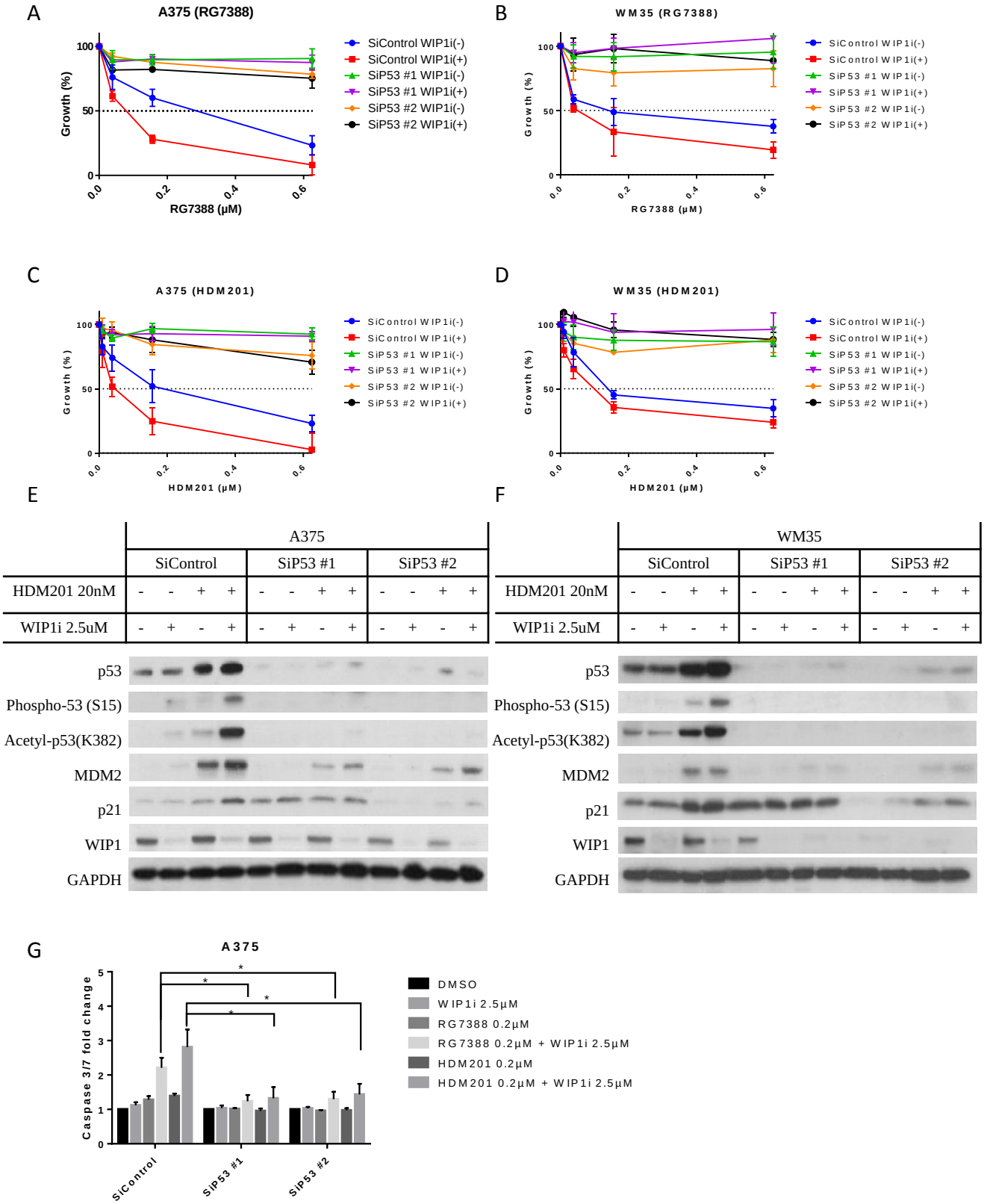


Figure 7. The combination of WIP1 and MDM2 inhibitions is p53-dependent. A375 and WM35 were treated with siRNA-mediated knockdown of p53 for 24 hours followed by RG7388 and HDM201 treatments. (A-D) Growth inhibition of A375 (A, C) and WM35 (B, D) treated with siRNA followed by 72-hour RG7388 (A, B) / HDM201 (C, D) with or without GSK2830371(WIP1i). (E, F) Immunoblotting of A375, WM35 treated with siRNA followed by HDM201 ± WIP1i. (G) Caspase 3/7 of A375 treated with siRNA followed by RG7388 / HDM201 ± WIP1i for 24 hours. Statistically significant p-values (*, $p \leq 0.05$) are indicated. Data are presented as mean ± standard error of mean (SEM) for at least three independent repeats.

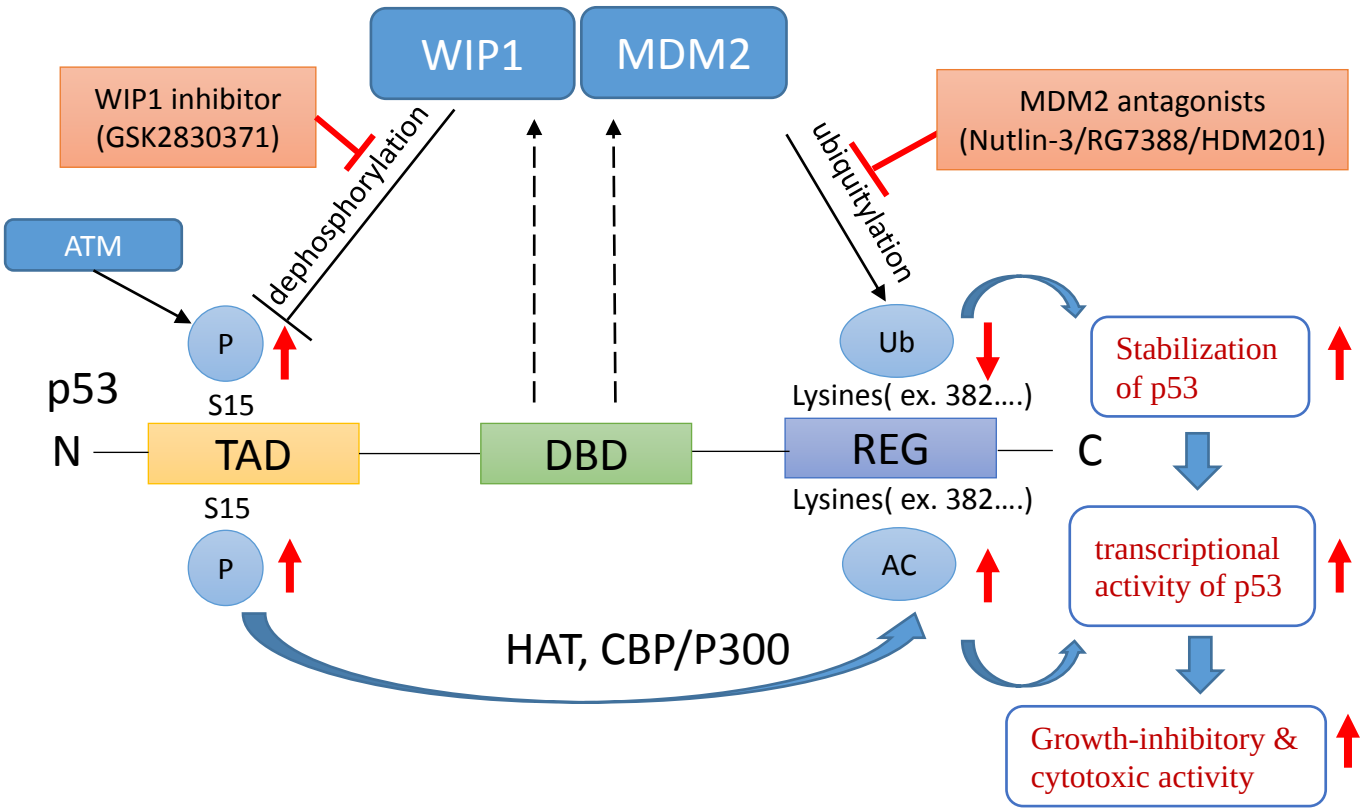


Figure 8. Proposed model for GSK2830371 (WIP1i) potentiation of the growth-inhibitory and cytotoxic effects of MDM2 antagonists. After activation of p53 by MDM2 antagonists (Nutlin-3/RG7388/HDM201), WIP1 inhibition (WIP1i) increases phospho-p53 (Ser 15) through allosteric inhibition of WIP1 phosphatase. Phospho-p53 (Ser 15) recruits histone acetyl transferases (HATs, e.g. CBP/P300) which acetylate lysine residues in the C-terminal region of p53. Acetylation of p53 increases the transcriptional activity of p53 directly, and also indirectly by stabilizing p53. Dashed lines indicate p53 transcriptional upregulation of the corresponding genes for MDM2 and WIP1. TAD, transcriptional activation domain; DBD, DNA binding domain; REG, C-terminal regulatory region.