SUPPLEMENTAL INFORMATION

Studies of p53 mutations, p53 codon 72 polymorphism, and MDM2 SNP309 polymorphism

METHODS

Clinical Samples

Tumor samples used included a total set of 31 adrenocortical cancer specimens from patients with adult sporadic disease. These samples were from 15 men and 16 women with a mean age of 48.9 years (range 23.3-77.7). The average size of the tumors was 10.8 cm (range of 3 to 21.5cm). These samples included flash frozen tumors collected at the Mayo Clinic in Rochester, Minnesota, the University Hospital Essen (Essen, Germany) and the University of Calgary (Alberta, Canada). The study was conducted under Western Institutional Review Board approved protocol (WIRB #20051769) that required study subjects to sign a consent form.

Genomic Analysis

Genomic DNA came from a total of 31 tumors and 2 cell lines and from both bulk tumor and nuclei flow-sorted on the basis of DNA content [1]. Analysis of p53 was done by direct PCR sequencing of exons 1-11 of the TP53 gene. As blood lymphocytes or adjacent normal adrenal tissue from the same patient was not available, we adopted a strategy to use flow-cytometry based sorting of nuclei to separate aneuploid tumor nuclei from the diploid nuclei of supporting normal tissue [1]. We then used the diploid DNA as substitute for normal genomic DNA for each patient. Loss of heterozygosity of TP53 was determined in the 11 samples with DNA from sorted diploid and aneuploid nuclei through analysis of the VNTR1 pentanucleotide repeat [2] in intron 1 of TP53 and/or through the codon 72 polymorphism.

Expression Microarrays

We used previously published data sets from Affymetrix U133 Plus 2 chips that included both normal adrenal and ACC samples annotated with survival data [1, 2] to identify the number of ACC samples over-expressing PLK1. The .CEL files from Affymetrix U133 Plus 2 chips were downloaded from GEO

(GSE10297 and GSE19750). The data were filtered to include only normal adrenal samples without evidence of disease in the contralateral gland and ACC tumor sample from patients over 18 years of age. Using the Expression File Creator in GenePattern [3], the data were normalized by gcRMA[4] with quantile normalization and background subtraction. Batch effects were minimized using COMBAT [5] with the non-parametric option, floored at 0.00001, and log2 transformed. Normalized expression data was extracted for the PLK1 (202240_at) and TP53 (201746_at) probe sets. MDM2 is represented by multiple probes, so we extracted probe sets 211832_s_at, 205386_s_at, 229711_s_at, and 217542_at. The geometric mean of these probe sets was computed for each sample. The relative fold change to the geometric mean of the corresponding normal samples for each study was computed.

RESULTS

Analysis for p53 mutation

Mutations of the coding regions of TP53 are uncommon in adult sporadic ACC. A total of 31 ACC and two ACC cell lines, SW-13 and H295R were analyzed for TP53 mutations through a combination of methods. We sequenced exons 1-11 of TP53 amplified from DNA extracted from bulk tumor (21 of 31) as well as from the aneuploid nuclei from flow sorted tumors (11 of the 31) [6]. Of the 31 ACC tumors, one failed sequencing. Four mutations were detected in 3 tumors (Supplemental Tables 1 and 2). Of note, none of the mutations detected have been reported in ACC previously. Because mutations in small tumor cell populations can be missed when sequencing from bulk material, we examined the 6 tumors that were sequenced from both bulk material and sorted aneuploid material for evidence of missed mutations. No additional mutations were detected. We detected no mutation of TP53 in H295R. SW-13 carried the previously documented H193Y mutation. These results are in line with the sequencing done by the Sanger Institute as part of the COSMIC Cell Lines Project [7].

The 11 tumors subject to flow-sorting were also examined for loss of heterozygosity (LOH) of p53 using the diploid cell population as the “normal” control. LOH occurred in 3 of 11 (27%) of the ACC tumors studied. No tumor with LOH also harbored a mutation. Although the SNP variant with a proline

allele of codon 72 has been associated with an increased risk of ACC [8], we did not observe any difference in the proline allele frequency from that of the expected population frequency in our data.

Analysis of regulator of p53 activity

The low rate of mutations in p53, the lack of overlap of mutations with LOH, and a lack of an increase in the prevalence of a proline allele in codon 72 in our ACC samples suggests that disruption of the p53 pathway may involve regulators of p53. MDM2 is the predominant negative regulator of p53 activity. Previous studies have shown that conversion of T to G at position 309 generates a strong SP1 binding site and increases MDM2 expression [9, 10]. Most our tumors did over-express MDM2 at the RNA level (Supplemental Table 3). Therefore, we genotyped SNP 309 in MDM2 in the ACC tumors. We did not observe a change in allele frequency from the expected population frequency, nor did we observe a significant increase MDM2 expression in tumors with a G allele compared to tumors homozygous for the T allele (Tables 1 and 3).

Supplemental Table 1: Clinical parameters, p53 status and MDM2 SNP 309 status in ACC tumors

Sample*	Gender	Age (years)	Survival (years)	Tumor Size (cm)	Tumor Weight (g)	Tumor Stage	Tumor Grade	p53 status¶	Amino acid change	Codon 72 analysis	p53 LOH	MDM2 SNP309
ACC 1	M	69.5	14.2	5	32	NA	3	WT	NA	R/R	ND	T/G
ACC 2	M	66.5	2.7	7	60	NA	2	WT	NA	R/R	ND	T/G
ACC 3	F	63.8	19/7	14	845	NA	4	NA	NA	ND	ND	ND
ACC 5*	F	23.3	3.0	19	1100	2	3	WT	NA	P/R	ND	T/T
ACC 7	M	38.0	11.9	17	130	NA	NA	NA	NA	ND	ND	T/G
ACC 8*	F	56.5	0.6	9	190	4	4	WT	NA	R/R	ND	T/G
ACC 9*	M	53.2	9.0	8	195	3	2	WT	NA	R/R	ND	T/T
ACC 10*	M	67.8	1.7	7.6	150	2	4	g.7520186 C>A; g.7518969 delGT	p.A76S; Frame shift	R/R	ND	T/T
ACC 11*	F	54.2	18	15	890	2	1	WT	NA	R/R	LOH	T/T
ACC 12*	M	72.1	0.4	9.5	175	4	4	WT	NA	R/P	ND	G/G

ACC 13*	M	46.9	0.1	12	235	4	4	g.7518281 C>T	p.C242Y	P/P	ND	T/T
ACC 14	M	35.7	0.5	21	1160	NA	3	WT	NA	R/R	ND	T/T
ACC 15	M	64.9	0.4	9	120	NA	3	WT	NA	P/R	ND	T/T
ACC 16	F	42.1	10.9	21	885	NA	4	NA	NA	ND	NI	T/T
ACC 17*	F	26.7	16.6	3	NA	Recurrence	4	WT	NA	R/R	ND	T/G
ACC 18	M	77.7	0.2	17	1345	NA	3	WT	NA	R/R	ND	T/G
ACC 19*	M	48.5	3.1	9	195	2	3	WT	NA	R/R	ND	T/T
ACC 20	F	62.3	7.8	15	1115	NA	4	WT	NA	R/R	NI	T/T
ACC 21*	F	36.9	13.8	6.5	NA	Recurrence	4	g.7517848 A>G	p.V272A	R/R	ND	T/T
ACC 22*	F	67.3	6.4	10	230	2	2	WT	NA	R/P	LOH	T/T
ACC 23*	F	27.7	9.8	8	149	2	2	WT	NA	R/P	N	T/G
ACC 24	M	43	5.0	21.5	2880	NA	NA	WT	NA	R/R	ND	T/G

ACC 25	F	35.2	4.2	4	20.5	NA	NA	WT	NA	R/P	N	T/G
ACC 26*	F	51.5	NA	11.7	NA	2	3	WT	NA	R/R	ND	T/G
ACC 27*	M	NA	NA	NA	NA	Recurrence	2	WT	NA	R/R	ND	T/G
ACC 28*	F	58	NA	9	153.8	1	2	WT	NA	R/P	N	T/T
ACC 45	F	32	NA	11	106	2	1	NA	NA	ND	N	T/G
ACC 47	M	43	NA	4	60	Recurrence	1	NA	NA	ND	N	T/G
ACC 51	M	40	NA	12	480	NA	2	NA	NA	ND	N	T/T
ACC 84	F	27	NA	9.5	300	NA	1	NA	NA	ND	N	T/T
H295R	NA	NA	NA	NA	NA	NA	NA	WT	NA	ND	ND	NA
SW-13	NA	NA	NA	NA	NA	NA	NA	g.7518997 C>T	p.H193Y	P/P	ND	NA
MPI3*	F	37.0	NA	NA	NA	3	2	WT	NA	R/P	ND	NA

*Sample previously reported in Stephan, EA *et al* (Stephan, et al. 2008) and Demeure, MJ *et al*, Surgery (in press) 2013 (Demeure 2013)
¶ Referenced against NC_000017.9 (chr.17, build hg18)
ND – not done, NA – not available, NI – Not Informative, P – Proline, R - Arginine

Supplemental Table 2: p53 mutations detected in ACC tumors

Tumor No.	Exon	Chromosomal location	Base change	Amino acid change	Type of mutation	Consequences of the mutation
ACC 10	4	7520186	g.7520186C>A	p.A76S	Missense	Functional transactivation, disrupts splicing
ACC 10	6	7518969	g.7518969delGT	-	Frame shift leading to nonsense mutation [#]	NA
ACC 13	7	7518281	g. 7518281C>T	p.C242Y	Missense	Non-functional transactivation, gain of function and dominant negative activity
ACC 21	8	7517848	g.7517848A>G	p.V272A	Missense	Partial functionality, reduced transactivation with temperature sensitivity, disrupts splicing
SW-13	6	7518997	g. 7518997C>T	p.H193Y	Missense	Non-functional transactivation, temperature sensitivity

* GenBank Acc : NC_000017.9 (chr.17, hg18)

[#] never previously reported in an ACC

Supplemental Table 3: Fold change of PLK-1 relative to normal adrenal for the microarray expression data

	Log2 Fold-change from Normal		
Tumor	PLK1	TP53	MDM2
G-NC069	0.00	0.31	-0.08
G-NC088	0.00	-1.07	0.01
G-NC092	0.00	0.76	0.06
G-ACC001	0.00	1.62	1.33
G-ACC008	25.85	1.20	-0.18
G-ACC011	23.41	2.52	-0.41
G-ACC013	0.00	1.88	0.06
G-ACC014	25.22	2.87	2.51
G-ACC017	24.82	0.93	1.01
G-ACC018	30.41	1.89	0.91
G-ACC019	29.86	3.23	-0.35
G-ACC032	0.00	2.55	0.04
G-ACC034	24.82	1.85	0.12
G-ACC035	0.00	0.68	0.37
G-ACC038	27.68	2.00	-0.02
G-ACC039	0.00	1.10	0.71
G-ACC042	29.11	2.33	0.22
G-ACC046	23.40	1.03	0.53
G-ACC047	30.74	-28.73	-0.51

	Log2 Fold-change from Normal		
Tumor	PLK1	TP53	MDM2
G-ACC048	25.85	3.15	-0.18
G-ACC051	24.98	1.87	-0.25
G-ACC053	0.00	1.91	0.28
G-ACC054	0.00	2.15	-0.10
G-ACC055	25.25	1.86	0.09
G-ACC056	26.33	1.87	0.93
G-ACC057	25.73	1.56	1.04
G-ACC058	28.77	2.54	0.23
G-ACC084	0.00	2.25	0.08
G-ACC089	29.12	0.39	-0.03
G-ACC094	29.46	1.88	2.41
G-ACC098	0.00	1.62	1.58
G-ACC101	25.44	1.75	0.71
G-ACC102	26.28	1.20	0.21
G-ACC103	0.00	2.21	0.17
B-ACC-0520	-0.01	0.07	0.18
B-ACC-0523	0.02	-0.16	0.00
B-ACC-Norm1	0.03	-0.02	-0.12
B-ACC-Norm2	-0.03	0.11	-0.06
B-ACC-5	0.13	0.18	0.32

	Log2 Fold-change from Normal		
Tumor	PLK1	TP53	MDM2
B-ACC-8	4.35	0.97	0.09
B-ACC-10	4.00	-1.29	0.39
B-ACC-12	0.28	0.89	1.28
B-ACC-13	0.02	2.40	-0.07
B-ACC-17	0.03	0.56	0.18
B-ACC-19	0.01	1.41	2.60
B-ACC-21	0.03	1.13	1.57
B-ACC-26	2.48	0.38	0.34
B-ACC-9	0.02	0.88	0.31
B-ACC-MPI3	0.02	-0.59	0.12
B-ACC-11	0.00	0.75	0.30
B-ACC-22	0.05	0.52	0.99
B-ACC-23	-0.01	2.23	0.26
B-ACC-27	-0.01	0.76	0.98
B-ACC-28	0.59	1.93	0.91
B-ACC-4	-0.01	1.21	1.33
B-ACC-MPI1	0.00	0.74	0.02
B-ACC-MPI2	-0.01	1.84	-0.15
B1-ACC-30	0.59	1.01	1.50
B1-ACC-31	0.19	0.91	-0.48

	Log2 Fold-change from Normal		
Tumor	PLK1	TP53	MDM2
B1-ACC-32	1.47	0.51	0.45
B1-ACC-35	0.30	1.23	1.11
B1-ACC-45	0.26	0.29	0.68
B1-ACC-47	0.32	1.69	-0.27
B1-ACC-48	0.87	-0.46	-1.37
B1-ACC-51	0.10	0.65	1.44
B1-ACC-59	0.60	1.06	1.05
B1-ACC-61	3.04	1.48	0.33
B1-ACC-84	0.34	2.06	1.30
B1-ACC-85	0.59	1.09	2.47
B1-ACC-112	0.22	-0.08	-7.88
B1-ACC-114	0.59	0.57	1.05
B1-ACC-115	0.04	1.99	1.60
B1-ACC-117	0.02	2.27	1.73
B1-ACC-118	-0.06	0.38	-1.29
B1-ACC-123	0.11	0.84	-0.80
B1-ACC-129	0.01	1.94	1.68
B1-ACC-132	0.18	0.77	1.31
B1-ACC-134	0.17	0.38	-8.45
B1-ACC-136	-0.05	1.59	1.98

	Log2 Fold-change from Normal		
Tumor	PLK1	TP53	MDM2
B1-ACC-140	4.72	-0.11	-7.59
B1-ACC-149	0.07	0.30	-0.48
B1-ACC-150	2.34	0.17	-0.39

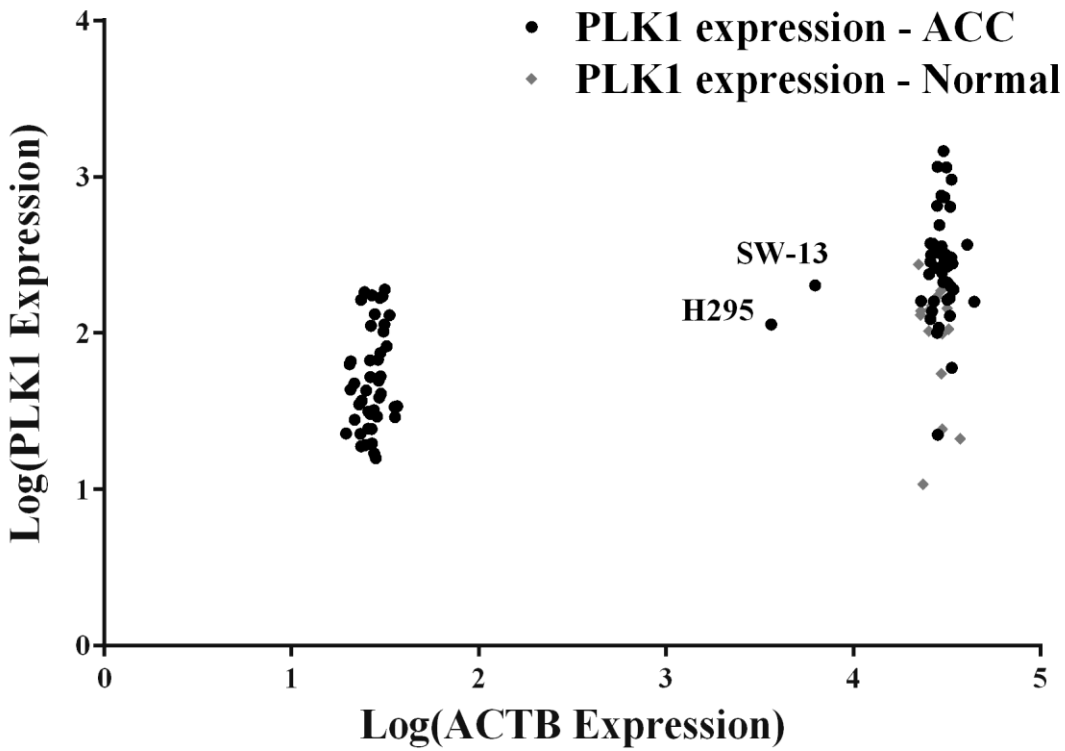
Supplemental Table 4: Fold-Change Relative to DMSO (vehicle) Control of RT-qPCR Results for TP53 and CDKN1A Expression After PLK1 Inhibition with BI-2536 in H295R

	TP53			CDKN1A (p21)		
Cells Only	0.0126	0.155		0.647	3.25	
BI-2536 IC10	0.388	0.0821	0.897	0.806	0.756	0.933
BI-2536 IC25	0.472	0.0999	0.614	1.056	1.12	1.14
BI-2536 IC50	0.758	0.146	1.11	1.21	1.467	1.53

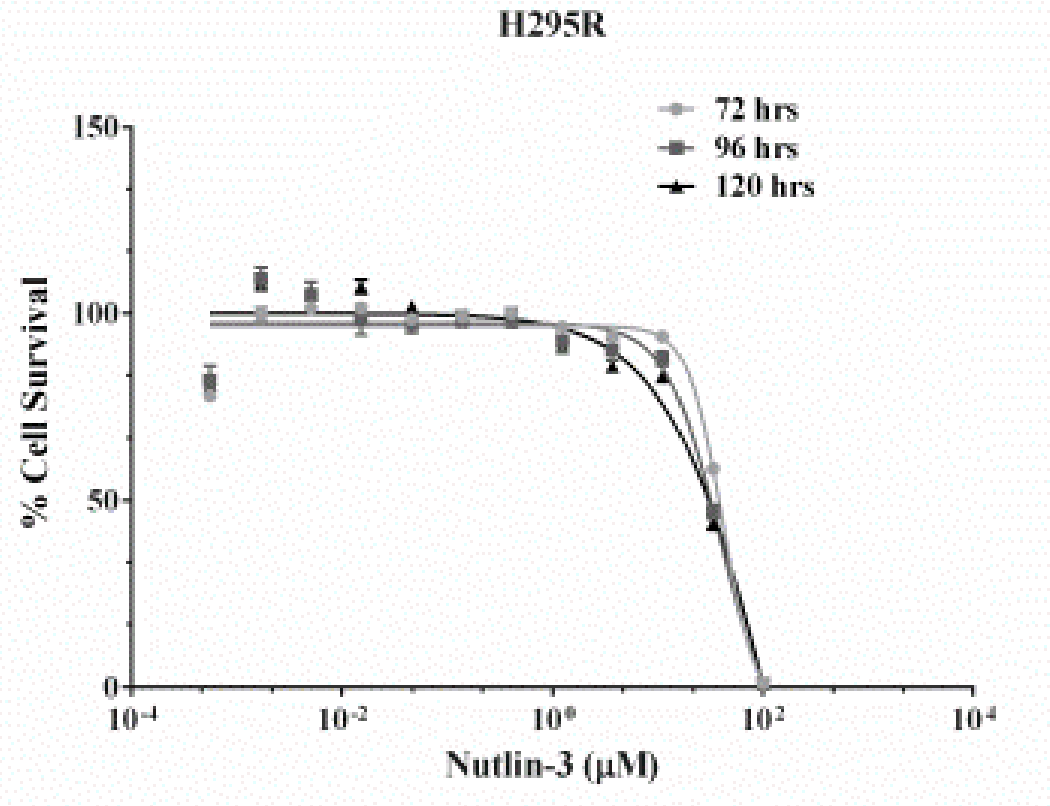
Supplemental Table 5: Fold-Change Relative to DMSO (vehicle) Control of RT-qPCR Results for TP53 and CDNK1A Expression After PLK1 Inhibition with BI-2536 in SW-13

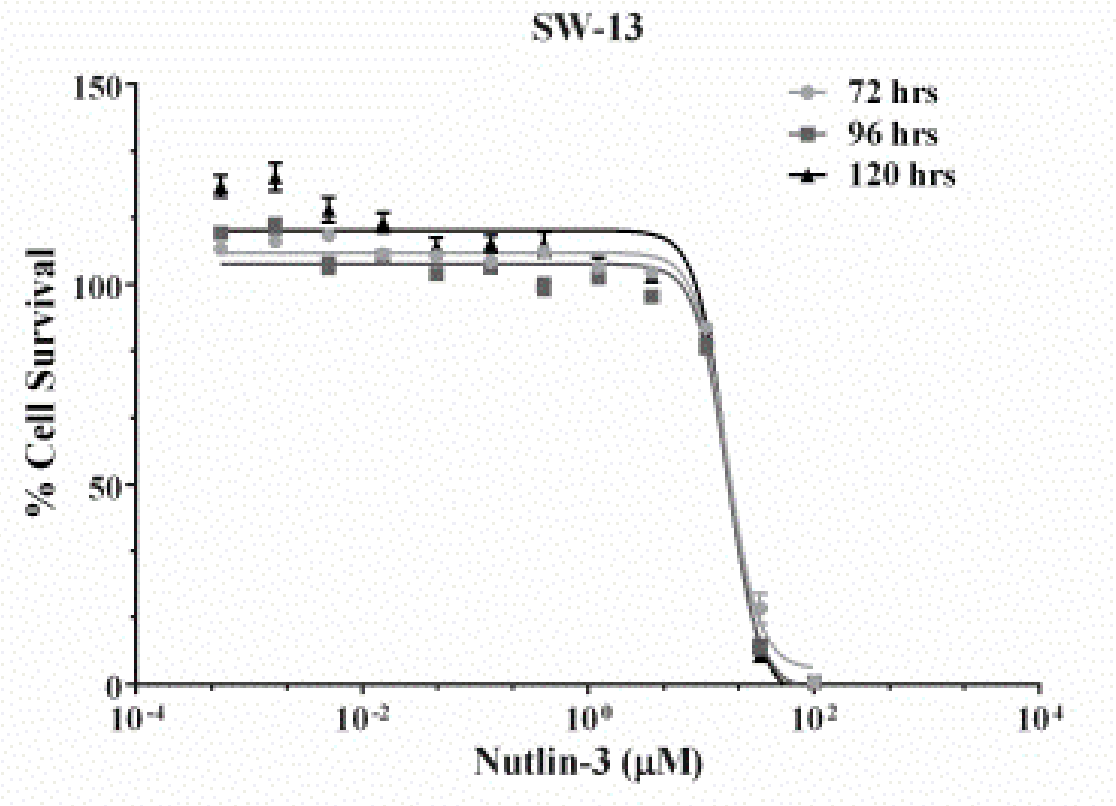
	TP53			CDKN1A (p21)		
Cells Only	1.11	1.32		1.84	1.15	
BI-2536 IC10	1.16	1.05	1.25	0.705	0.862	1.51
BI-2536 IC25	1.35	1.02	1.01	0.835	0.498	1.30
BI-2536 IC50	0.999	0.251	1.04	0.656	0.987	1.24

Supplemental Figure 1: Distribution of the log of PLK1 versus the log of β-actin mRNA expression of normal adrenal glands, ACC tumor samples, and the ACC cell lines SW-13 and H295 (parent line to H295R). The ACC cell lines express comparable amounts of PLK1 mRNA as clinical samples.

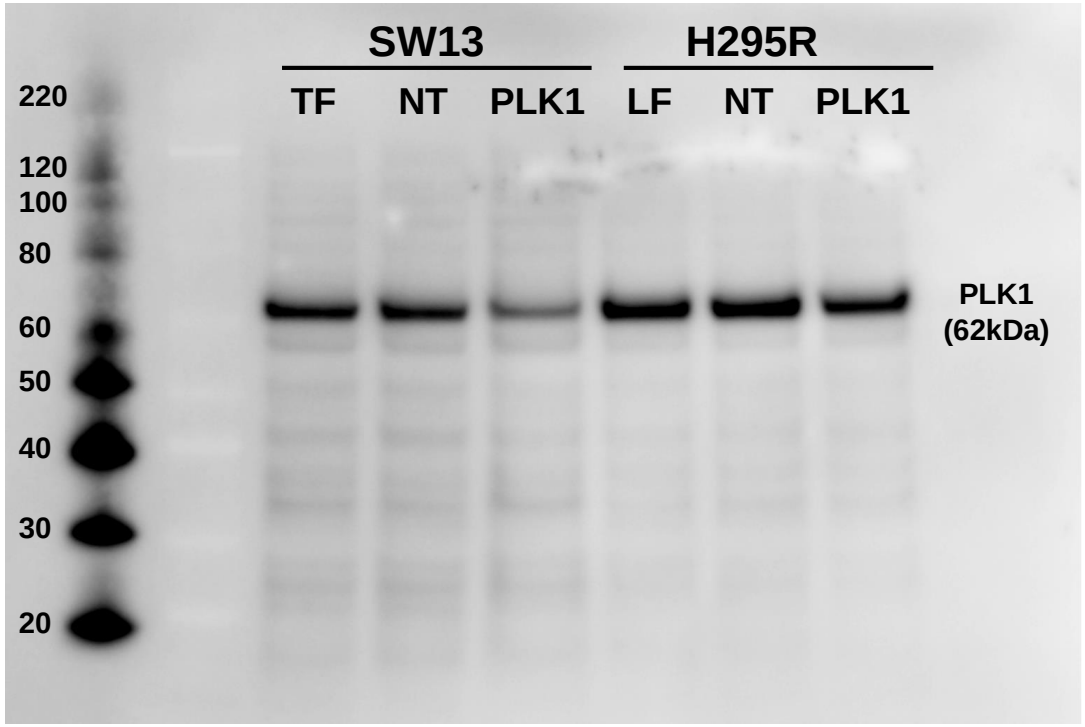


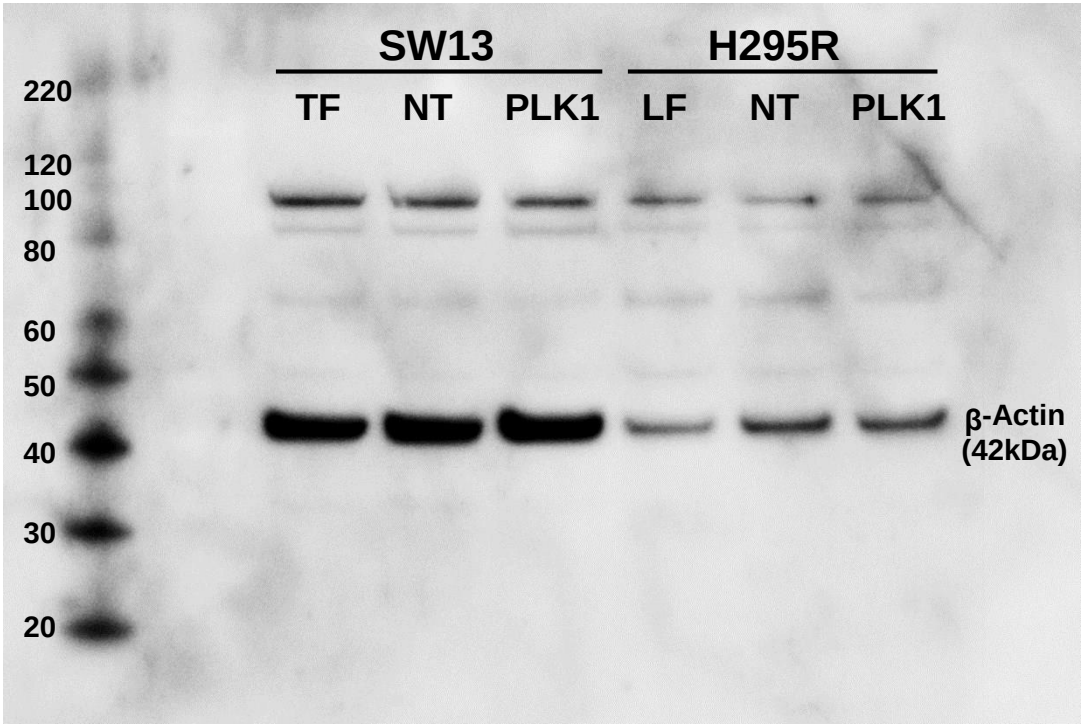
Supplemental Figure 2: Drug dose response curves for H295R and SW13 in response to MDM2 inhibition with Nutlin-3 at 72, 96, and 120 hour post addition of drug. Assays were normalized first to cells alone and then to a vehicle control. This resulted in viability measures of greater than 100%.



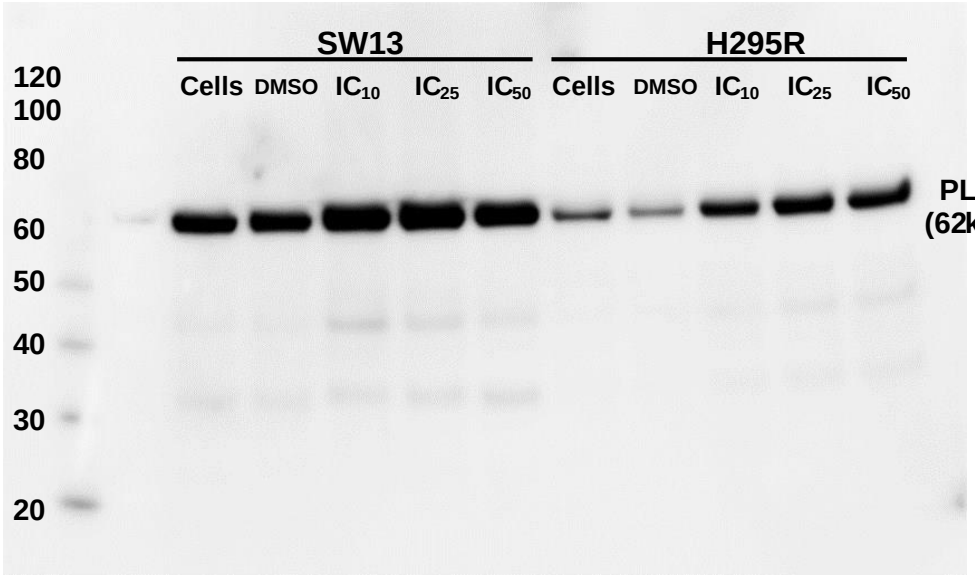


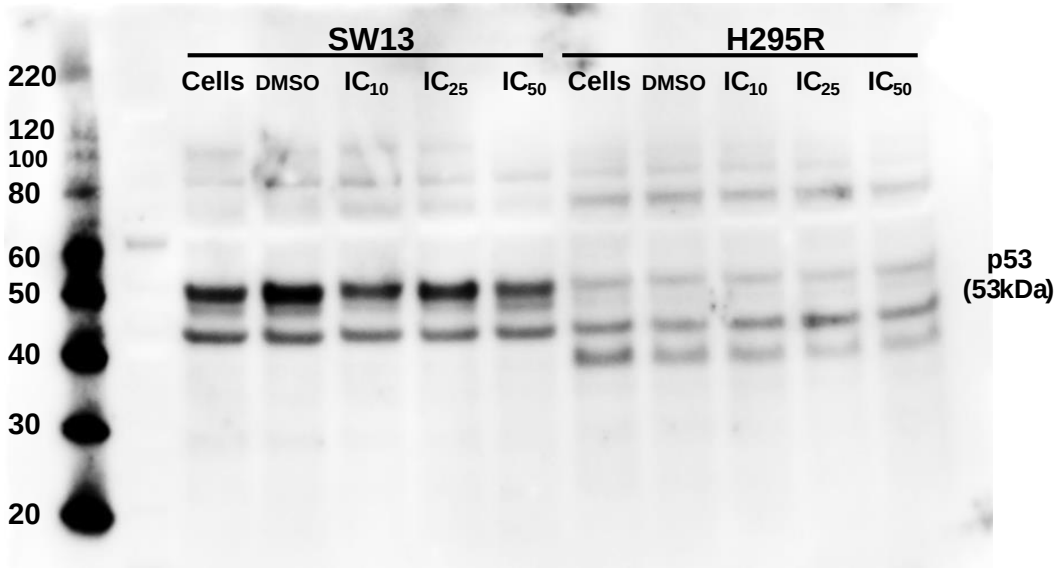
Supplemental Figure 3: Western blots for PLK1 & β-Actin after siRNA knockdown in SW13 & H295R. TF - Transfection reagent only; NT- Non-targeting control siRNA; PLK1- PLK1 siRNA.

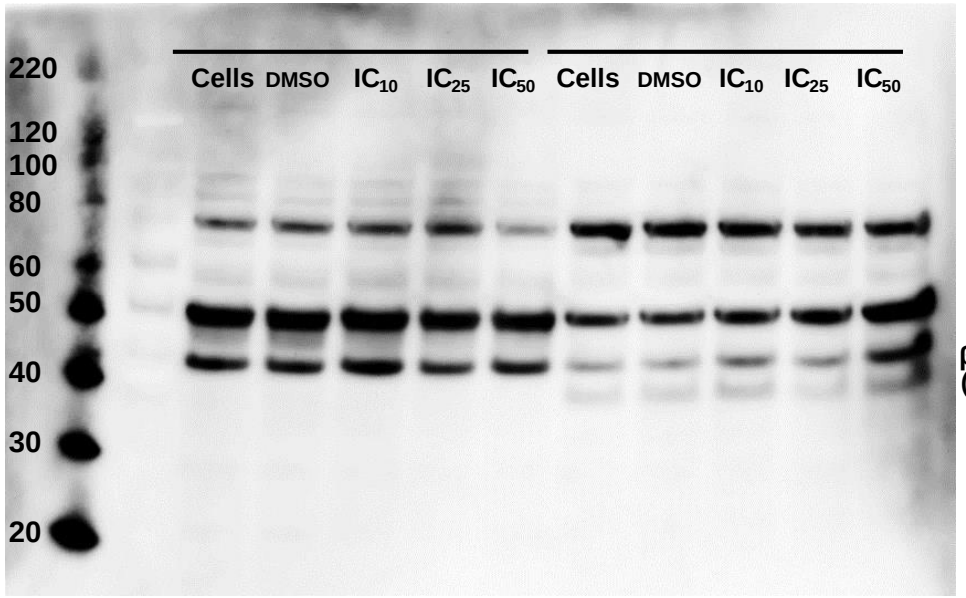




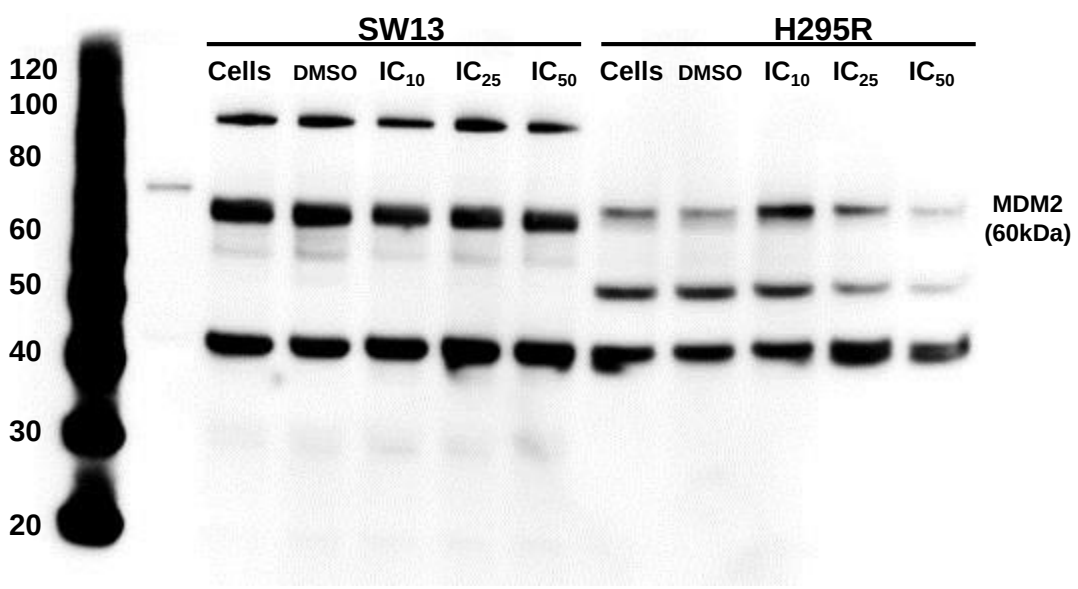
Supplemental Figure 4: Western blots for PLK1 & p53 after BI-2536 treatment in SW13 & H295R. Cells – Cells alone. DMSO – Vehicle Control; IC_{10} – IC_{50} – concentration of BI-2536 to which the cells were exposed.

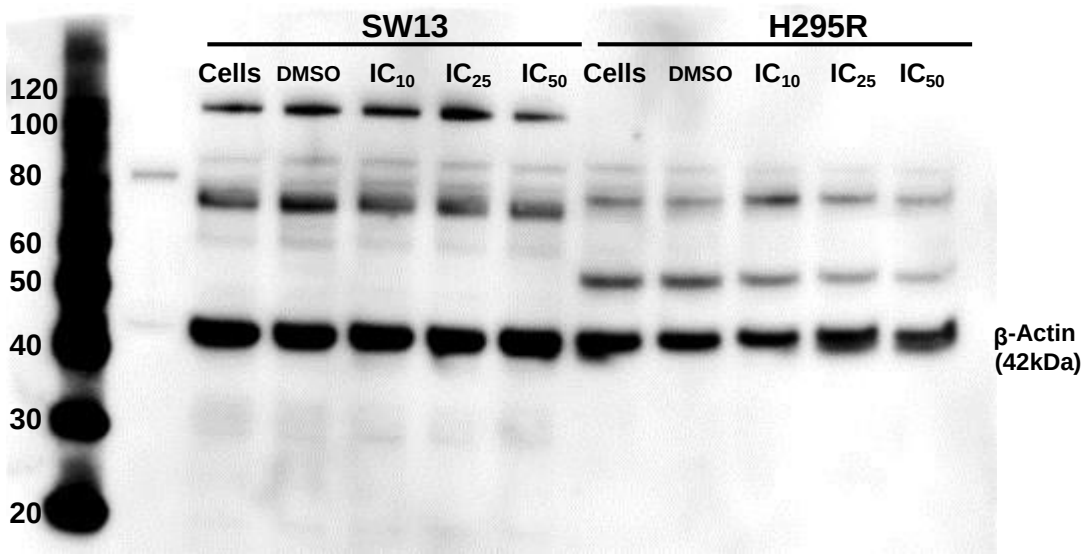






Supplemental Figure 5: Full Western blots showing MDM2 proteins levels do not change with increasing concentrations of BI-2536. Cells – Cells alone. DMSO – Vehicle Control; IC_{10} – IC_{50} – concentration of BI-2536 to which the cells were exposed.





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