p53-responsive TLR8 SNP enhances human innate immune response to respiratory syncytial virus

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Supplemental material.

Sequences and information for primers used in luciferase assay and gene expression evaluation.

Primer sequence / Information	**Assay**
TLR8 p53RE-G allele 5-CGTGTAAGGCAAGATGAAACATGTCACATCCC-3 5-TCGAGGGATGTGACATGTTTCATCTTGCCTTACACGGTAC-3	Luciferase reporter assay
TLR8 p53RE-A allele 5-CGTGTAAGGCAAGATGAAACATATCACATCCC-3 5-TCGAGGGATGTGATATGTTTCATCTTGCCTTACACGGTAC-3	Luciferase reporter assay
RVprimer3 (Promega) 5-CTAGCAAAATAGGCTGTCCC-3	Luciferase reporter sequencing
TLR8 Hs00152972_m1 (Applied Biosystems)	Real-time RT-PCR analysis
GUSB Hs00939627_m1(Applied Biosystems)	Real-time RT-PCR analysis
GAPDH Hs02786624_g1(Applied Biosystems)	Real-time RT-PCR analysis

Supplemental Table 1. Demographics of subjects in this study and genotype information for *TLR8* p53RE SNP rs3761624 of donor population. The *TLR8* SNP rs3761624 is located in the promoter region of the gene and the common allele "A" impairs functionality of a p53RE when compared the mutant allele "G". Since the *TLR8* gene is X-linked, males have one copy and females have two copies of the allele. Initially genotyping was achieved by Illumina sequencing and later confirmed by a TaqMan based assay as described in Methods section. n=27 samples. Once the samples were unblinded for the genotype, we identified two samples (showed in red) for which genotyping approaches did not coincide. Those samples were not considered for the functional analysis

Supplemental Table 2. Demographics of subjects in this study and genotype information for *TLR8* SNPs evaluated in EPR population. The regulatory *TLR8* p53RE SNP rs3761624 is in nearly 100% linkage disequilibrium (*) with the coding *TLR8* SNP rs3764880. Samples labeled as "BSxxx and "CRUxxx" correspond to human PBMC and alveolar macrophages respectively from previous study (4).

Supplemental Table 3. Odds for duration in hospitalization due to RSV severity in males and females with the *TLR8* rs3761624 G allele.

Supplemental Table 4. Odds for severe Lower Respiratory Tract Infections due to RSV in males and females with *TLR8* rs3761624 G allele by age.

Supplemental Figure 1. *TLR8* gene and protein expression in PHA stimulated lymphocytes grouped by TLR8 SNP rs3761624 genotypes. Freshly isolated human peripheral blood mononuclear cells (PBMC) from healthy subjects (n = 25) were incubated with PHA to stimulate T-lymphocyte expansion. After 48 h, cells were exposed to indicated drugs. Cells were harvested following 24 h treatment. Presented are SNP genotypes and the A) mRNA levels of TLR8 assessed by qPCR and B) TLR8 protein expression changes evaluated by cytofluorometry following treatment with DMSO, Nutlin (10 μM), DXR (0.5 μM) and IR (4Gy) relative to the no treated samples. Since the *TLR8* gene is X-linked, males have one copy and females have two copies of the allele.

Supplemental Figure 2. *TLR8* gene expression induced by p53 activating and DNA damaging drugs in immune primary human cells depends on the SNP in p53RE of TLR8 promoter. A) Presented are SNP genotypes and TLR8 mRNA levels in males (squares) and females (circles) from two population studies. Symbols in dark colors represent PHA stimulated lymphocytes samples (n = 25) from the present study. Lighter colored symbols correspond to samples from previous study (4) corresponding to PHA stimulated lymphocytes or alveolar macrophages (light symbols with dots) (n = 24). The mean for each group is represented by the horizontal bar. All samples were treated for 24h with Nutlin (10 μM, n = 48), DXR (0.5 μM, n = 49) or IR (4 Gy, n = 43) followed by 24 hr incubation. B) Induced expression of *TLR8* gene in primary immune cells via DNA stressors and activation of the p53 pathway. In this "boxes-and-whiskers" diagram, the limits of the 2nd and 3rd quartiles of observed values (*i.e.*, the middle 50% of observations) are at the ends of the box; the median is the horizontal line within the box; "+" is the average. * corresponds to $P < 0.0001$

Supplemental Figure 3. *TLR8* SNP impacts TLR8 gene and protein expression as well as p53 binding in response to p53 activating drugs. Association plots based on the rs3761624 SNP genotypes for A) *TLR8* gene vs TLR8 protein expression and B) *TLR8* gene expression vs p53 occupancy of the p53RE-containing *TLR8* SNP rs3761624 following treatment with Nutlin (10 μM), DXR (0.5 μM) and IR (4 Gy).

Supplemental Figure 4. Representative FACS plots for TLR8 protein expression in lymphocytes from people with different rs3761624 SNP genotypes. Representative examples for TLR8 protein expression profiles from donors carrying AA, AG and GG genotypes for *TLR8* rs3761624 SNP. Detection of TLR8 protein levels was assessed by flow cytometry as described in methods section.

Supplemental Figure 5. TLR8 SNP rs3761624 improves p53 binding at the p53RE located in promoter of TLR8 gene. Representative examples of p53 occupancy assessed by ChIP-PCR in PHA stimulated human lymphocytes from donors with different *TLR8* SNP rs3761624 genotypes and treated with p53 activating drugs. p53 occupancy for the p53RE associated with p53 target *CDKN1A/ p21* was used as an internal positive control, while immunoprecipitation with IgG was used as negative control. * $P < 0.001$ when compared to no treatment ("NT") samples.

Supplemental Figure 6. Inhibition of p53 activity by pifithrin-α reduces p53-dependent TLR8 and p21 expression in response to chemotherapeutic drugs. Freshly isolated human peripheral blood mononuclear cells (PBMC) from healthy subjects were incubated with PHA to stimulate T-lymphocyte expansion. After 48 h, cells were pretreated with p53 inhibitor pifithrin-α (PFT-α, 30 μM). Three hours later, cells were treated with Nutlin (10μM) and were harvested 24 h later. On the x-axis are the donors with *TLR8* SNP rs3761624genotypes and the corresponding mRNA levels of A) *TLR8* and B) *CDKN1A/ p21* assessed by qPCR. (M-G/male G-allele; F-GA and F-AG/female heterozygous; F-AA/female homozygous; M-A/ A allele). Presented are the average and SD for each sample run in triplicate.

Supplemental Figure 7. Ability of WT and various p53 mutants to drive *TLR8* expression depends on SNP in p53RE of *TLR8* promoter. A) SaOS2 cells were co-transfected with pGL3P:luc vector containing the TLR8 p53RE with the p53 nonresponsive (A) or responsive (G) allele along with 500 ng of p53 WT or mutant expression vector under control of the CMV promoter. 48h later luciferase activities were determined. Among the p53 mutants, those in blue are considered loss-of-function. Presented are the average and SD from 3 independent transfections. * corresponds to $P<0.001$ relative to vector transfected cells. ^ corresponds to $P<0.001$ when compared with cells transfected with TLR8 p53RE SNP-A reporter vector

Supplemental Table 1

DONOR	AGE	GENDER	RACE	*TLR8* p53RE rs3761624
EPR27	34	Male	Other	G
EPR28	41	Male	White	G
EPR39	40	Male	White	G
EPR40	45	Male	White	G
EPR42	66	Male	Black African American	G
EPR43	74	Male	White	G
EPR48	54	Female	Black African American	GG
EPR49	43	Female	White	GG
EPR50	*53*	*Female*	*White*	*GG*
EPR51	60	Female	White	GG
EPR53	*74*	*Female*	*Black African American*	*GG*
EPR37	66	Female	White	AG
EPR41	23	Female	Other	AG
EPR44	49	Female	Black African American	AG
EPR45	34	Female	Black African American	AG
EPR46	68	Female	Black African American	AG
EPR52	41	Female	White	AG
EPR38	58	Female	Black African American	AA
EPR54	48	Male	White	A
EPR55	46	Male	White	A
EPR56	36	Male	White	A
EPR57	78	Male	White	A
EPR58	73	Male	Black African American	A
EPR59	53	Female	Black African American	AA
EPR60	62	Female	White	AA
EPR62	58	Female	Black African American	AA
EPR63	51	Female	White	AA

Supplemental Table 2.

DONOR	AGE	GENDER	RACE	*TLR8* p53RE rs3761624*	*TLR8* rs3764880*	*TLR8* rs5744077	*TLR8* rs5744082
EPR27	34	Male	Other	G	G	A	G
EPR28	41	Male	White	G	G	A	G
EPR37	66	Female	White	AG	AG	AA	GG
EPR38	58	Female	Black African American	AA	AA	AA	GG
EPR39	40	Male	White	G	G	A	G
EPR40	45	Male	White	G	G	A	G
EPR41	23	Female	Other	AG	AG	AA	GG
EPR42	66	Male	Black African American	G	G	A	G
EPR43	74	Male	White	G	G	A	G
EPR44	49	Female	Black African American	AG	AG	AA	GG
EPR45	34	Female	Black African American	AG	AG	AA	GG
EPR46	68	Female	Black African American	AG	AG	AA	GG
EPR48	54	Female	Black African American	GG	GG	AA	GG
EPR49	43	Female	White	GG	GG	AA	GG
EPR51	60	Female	White	GG	GG	AA	GG
EPR52	41	Female	White	AG	AG	AA	GG
EPR54	48	Male	White	A	A	A	G
EPR55	46	Male	White	A	A	A	G
EPR56	36	Male	White	A	A	A	G
EPR57	78	Male	White	A	A	A	G
EPR58	73	Male	Black African American	A	A	A	G
EPR59	53	Female	Black African American	AA	AA	AG	GG
EPR60	62	Female	White	AA	AA	AA	GG
EPR62	58	Female	Black African American	AA	AA	GG	GG
EPR63	51	Female	White	AA	AA	AA	GG
BS#1	31	Male	White	G			
BS#2	57	Female	White	AA			
BS#4	52	Female	Black African American	GG			
BS#5	25	Female	White	AG			
BS#7	25	Female	White	GG			
BS#8	35	Female	White	AG			
BS#9	42	Female	White	AA			
BS#11	25	Female	White	AA			
BS#12	42	Male	White	A			
BS#13	40	Male	White	A			
BS#15	29	Female	White	AG			
BS#16	38	Male	White	G			
BS#19	22	Male	White	G			
BS#20	23	Male	White	A			
BS#21	26	Female	White	GG			
BS#25	30	Female	Black African American	AA			
BS#26	47	Male	White	A			
BS#27	23	Male	White	G			
CRU 1149	21	Male	White	A			
CRU 1161	21	Female	White	AA			
CRU 1163	22	Male	White	G			
CRU 1164	28	Male	White	G			
CRU 1169	34	Male	Black African American	G			
CRU 1173	19	Male	White	G			

Supplemental Table 3.

Days in hospital	Odds ratio	Std. Error	z	P value	95% Cofidence Interval
Male G/- Female G/G	1.9	0.6	2.03	0.042	1.02-3.52

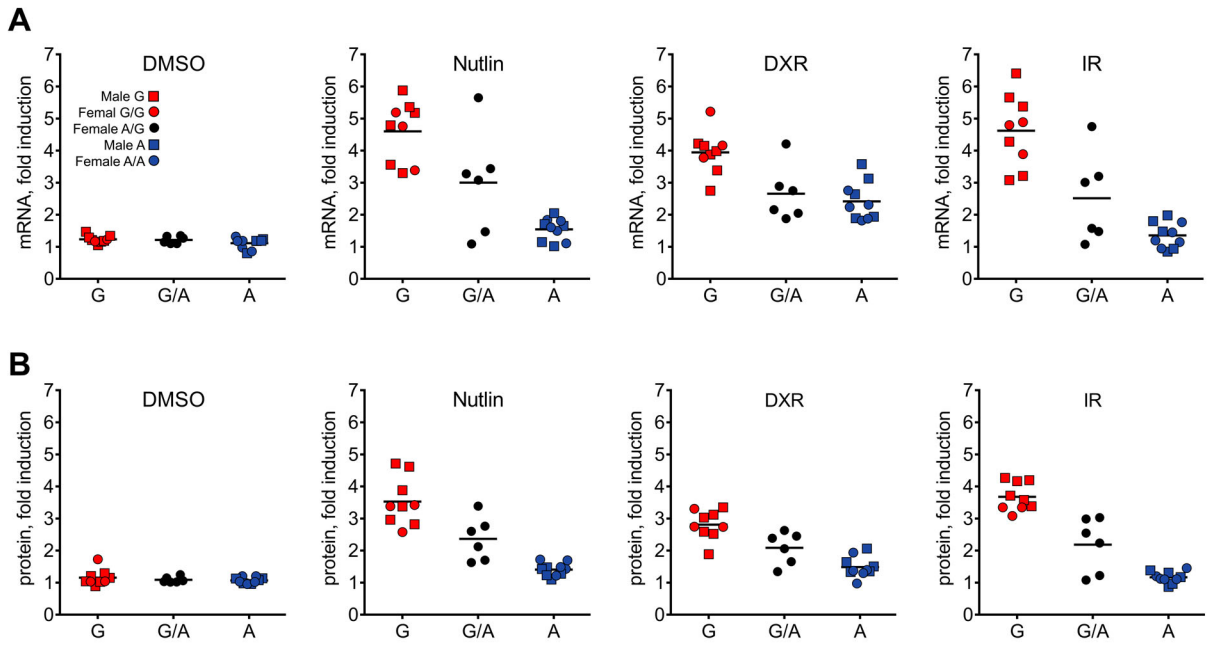
Number of observations = 239 LR χ^2 (1) = 4.33 Prob > χ^2 = 0.0375
Log likelihood = -142.36 Pseudo R2 = 0.0150

Supplemental Table 4.

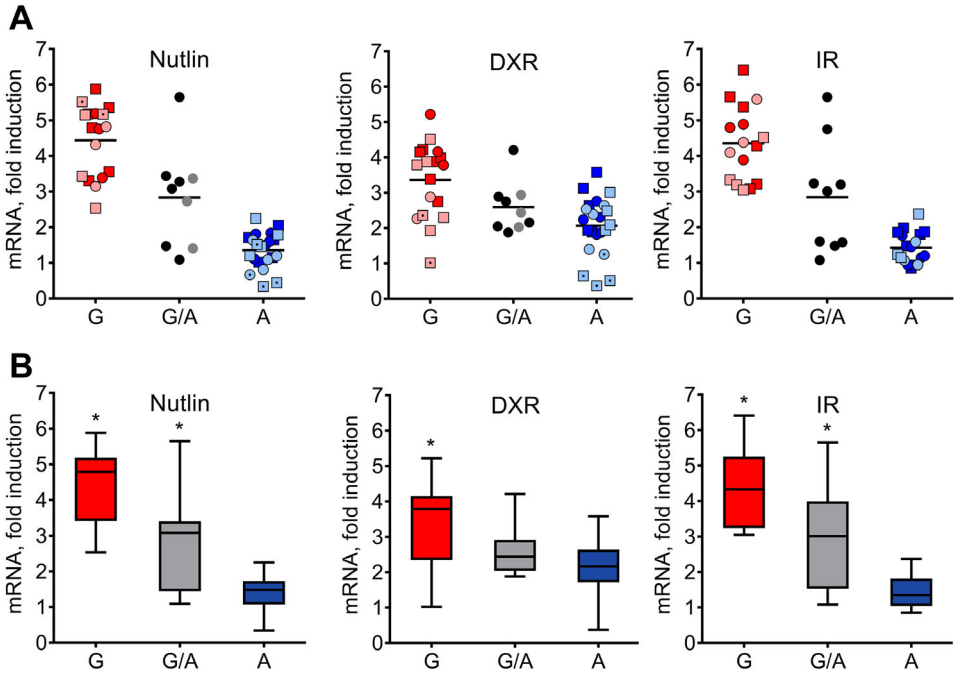
Severity	Odds ratio	Std. Error	z	P value	95% Cofidence Interval
Male G/- Female G/G	1.5	0.26	2.3	0.021	1.06-2.12
Age (months)	0.86	0.03	-4.47	<0.001	0.81-0.92

Number of observations = 585 LR χ^2 (2) = 28.56 Prob > χ^2 = 0.0000
Log likelihood = -386.89 Pseudo R2 = 0.0356

Supplemental Figure 1



Supplemental Figure 2



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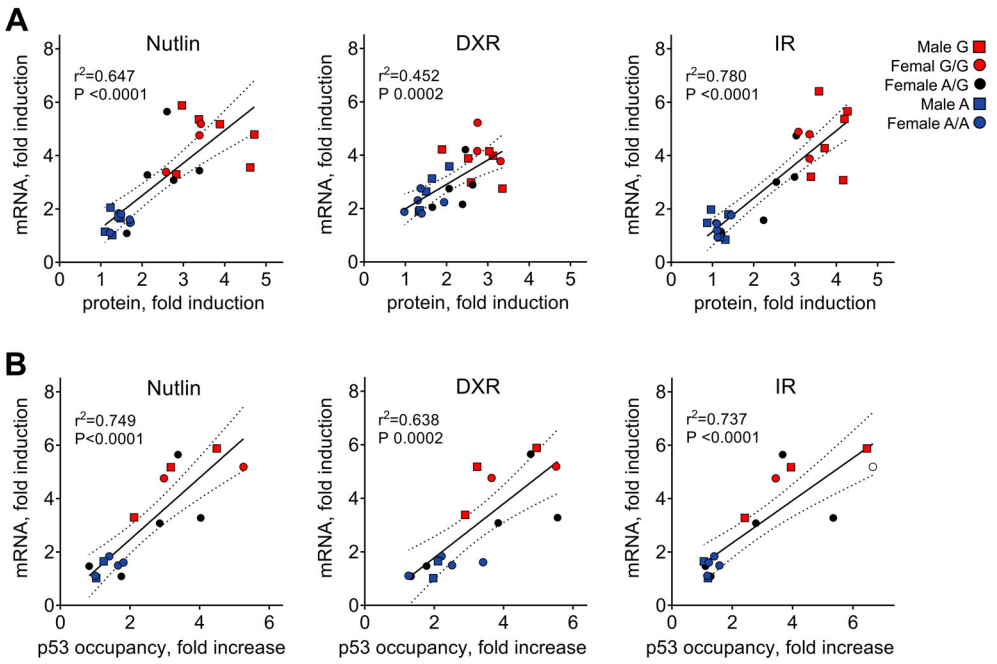

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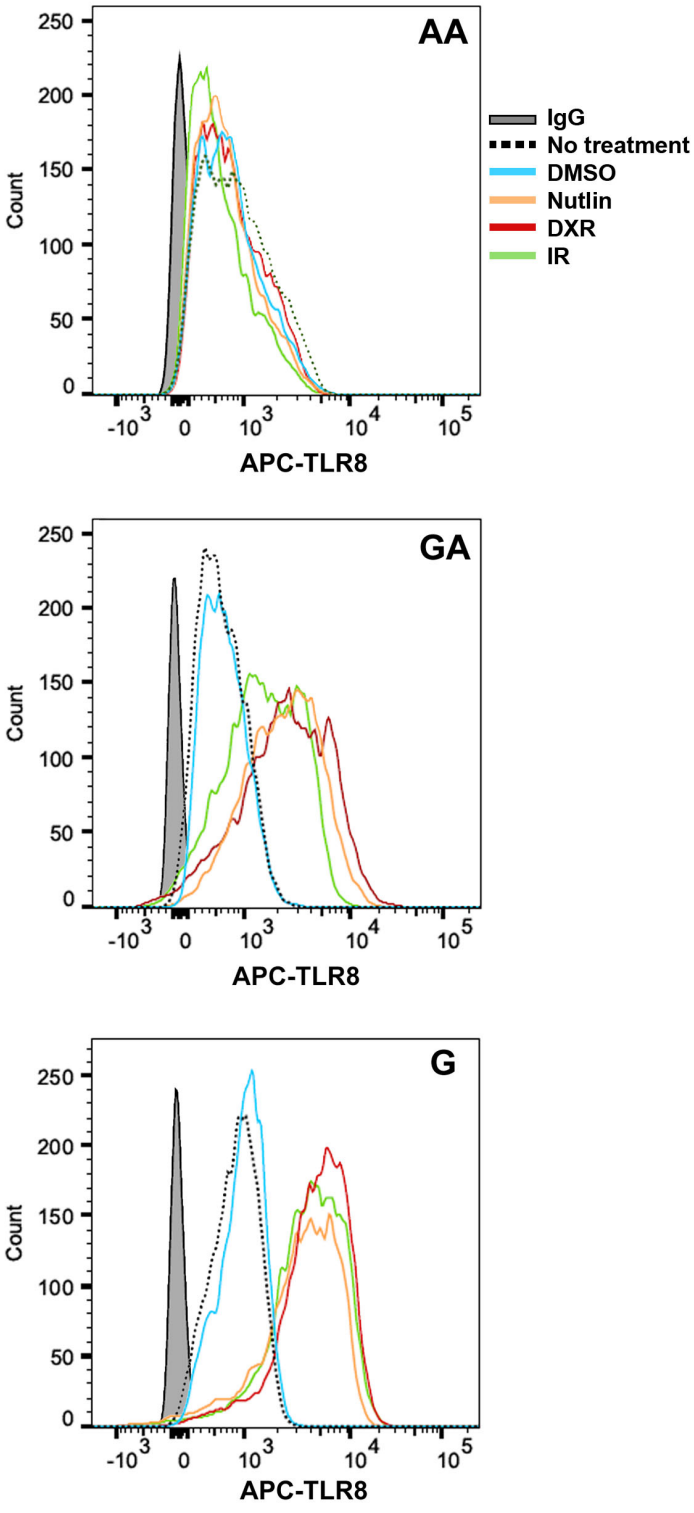

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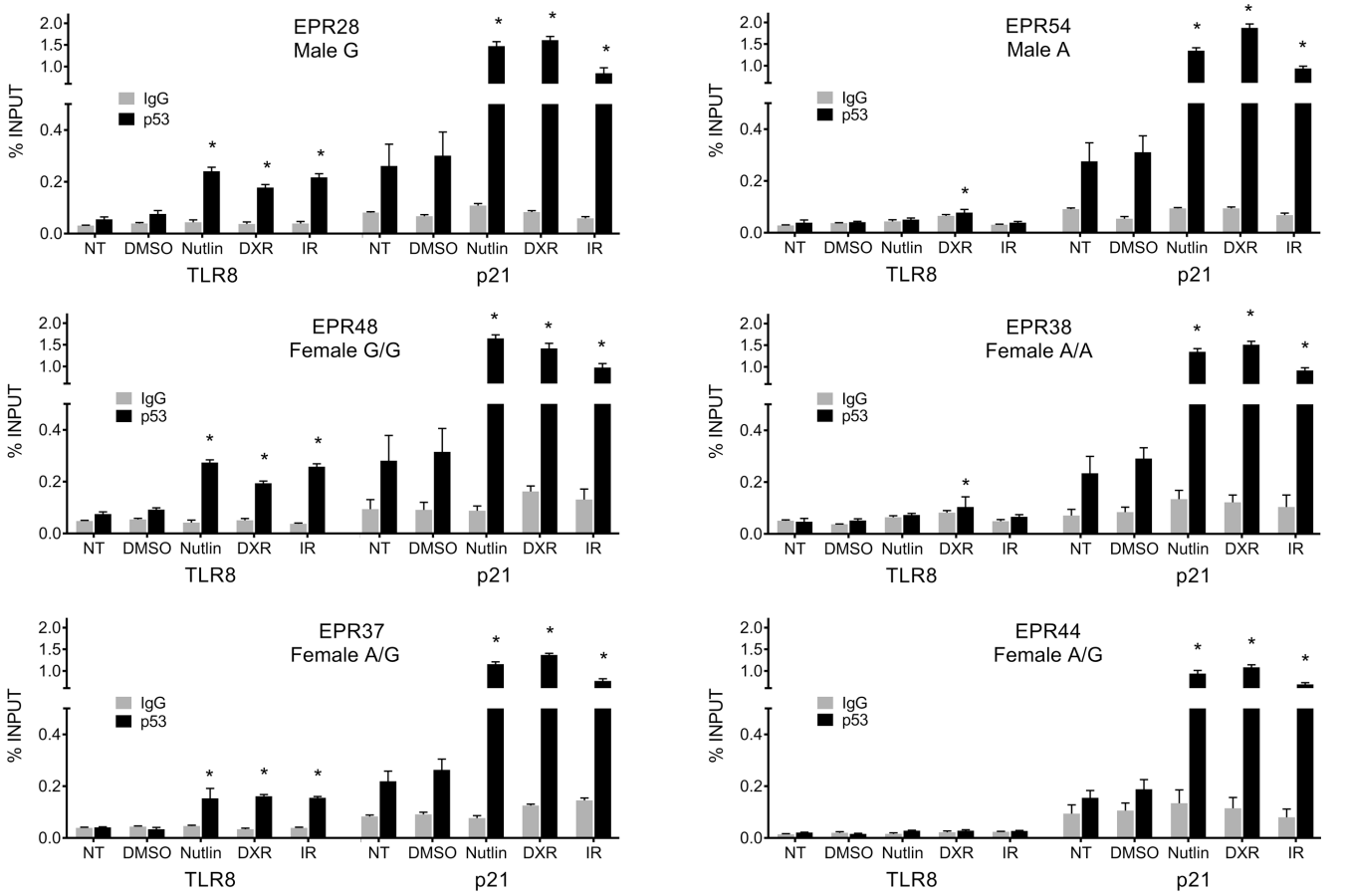

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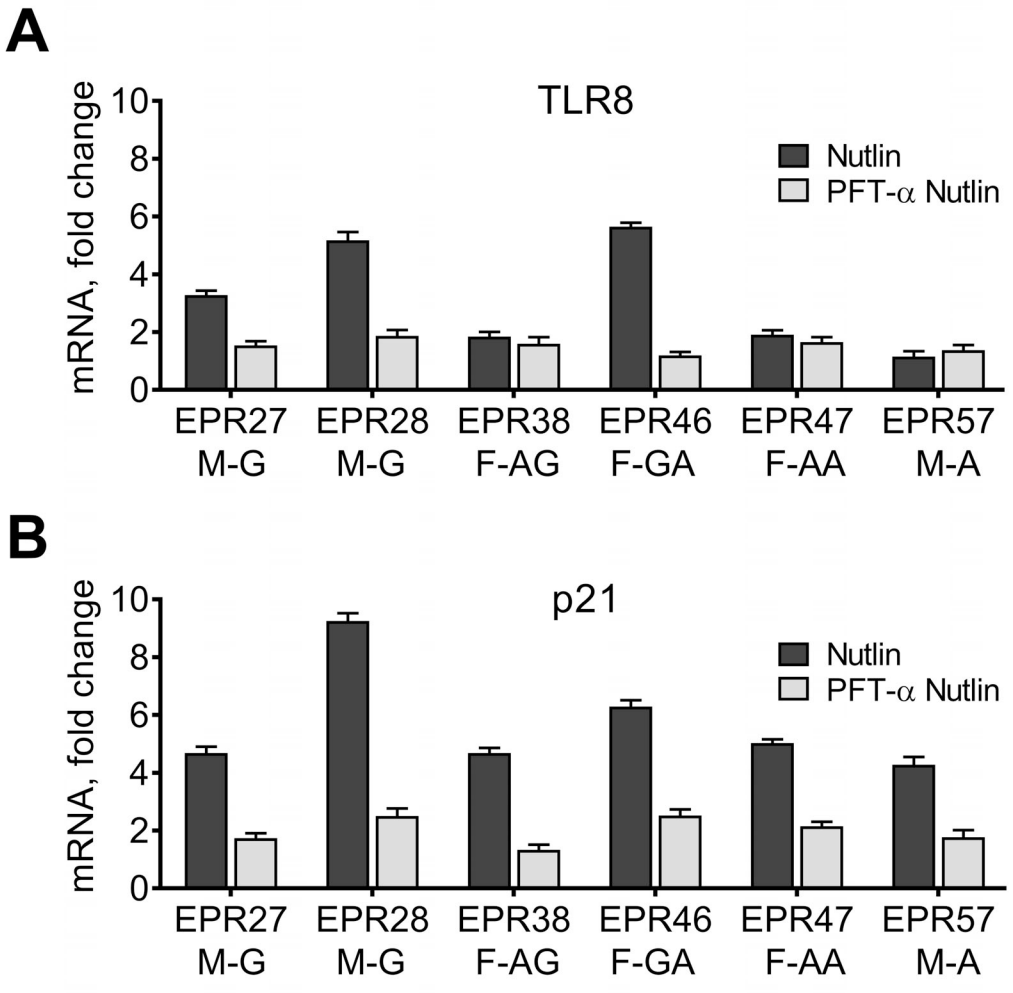

Supplemental Figure 7