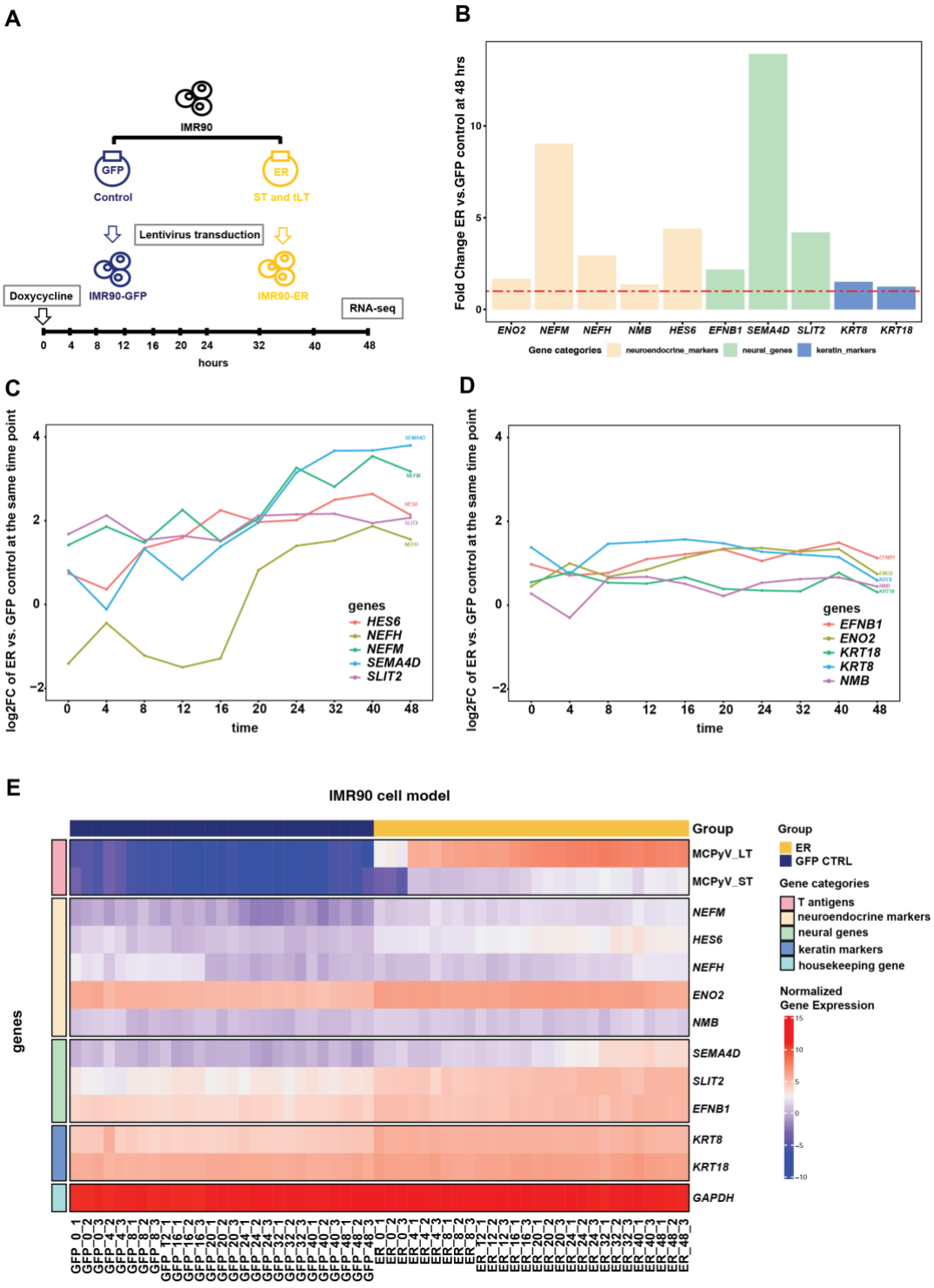
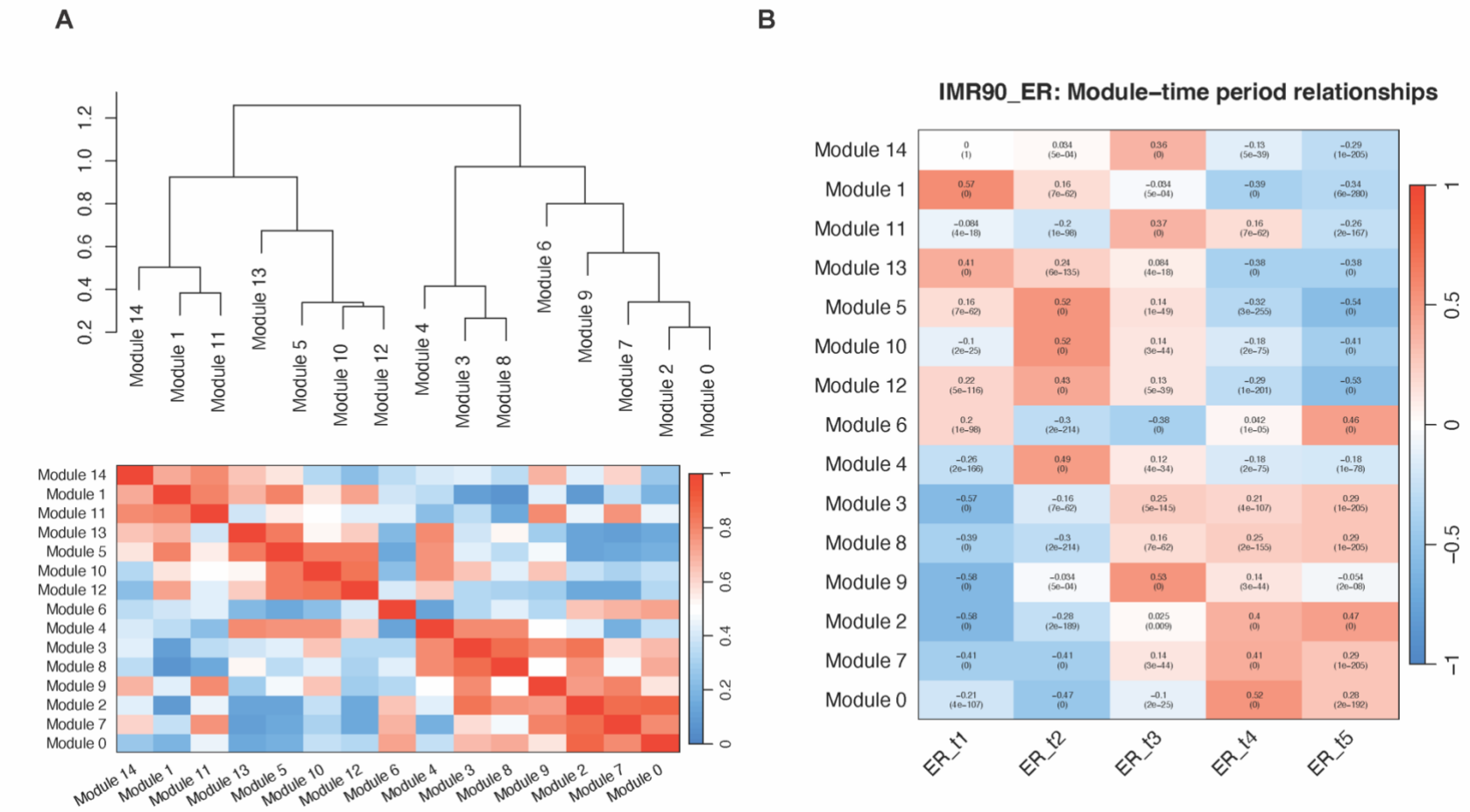
Supplemental data for

Integrative analysis reveals therapeutic potential of pyrvinium pamoate in Merkel cell carcinoma

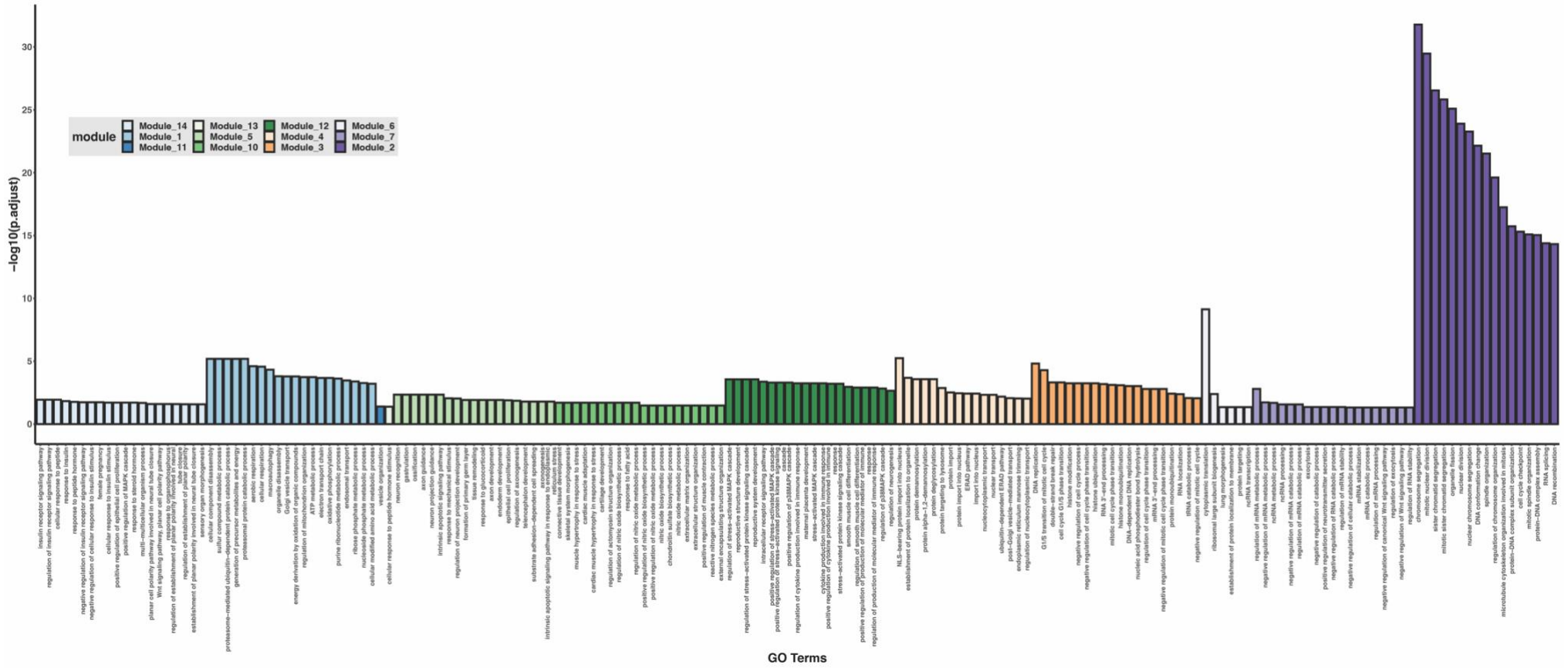
Supplemental Figures



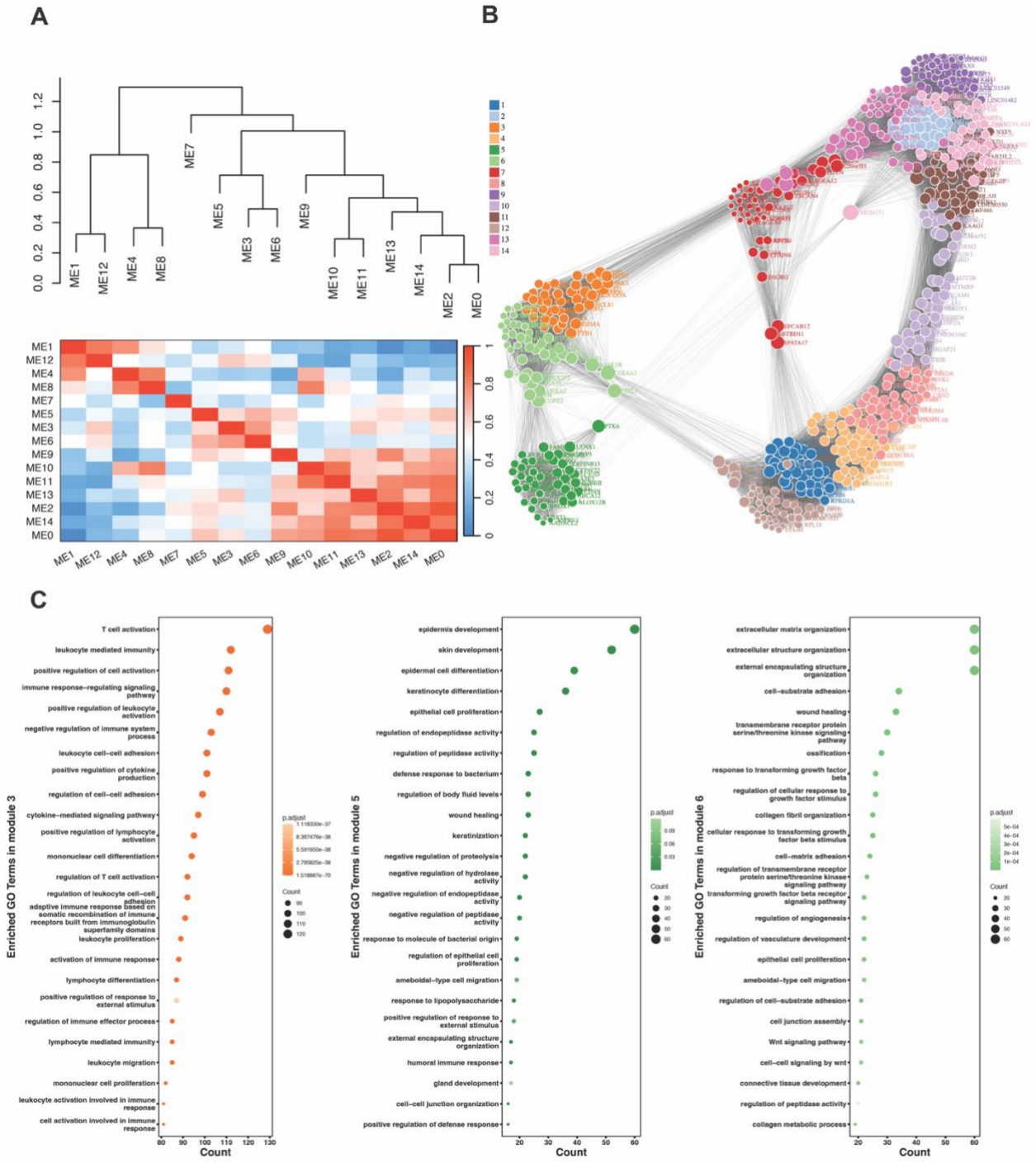
Supplemental Figure 1. MCPyV-ER upregulates a subset of Merkel cell carcinoma markers. (**A**) Schematic diagram of the IMR90-ER and IMR90-GFP induction model. (**B**) Fold change in the expression of some MCC marker genes in IMR90-ER compared to IMR90-GFP at 48 hours. (**C-D**) Temporal fold change in a subset of MCC marker genes in IMR90-ER versus IMR90-GFP inducible samples at each time point. (**E**) Heatmap showing normalized gene expression levels for T antigen genes, subset of MCC marker genes, and the housekeeping gene *GAPDH*.



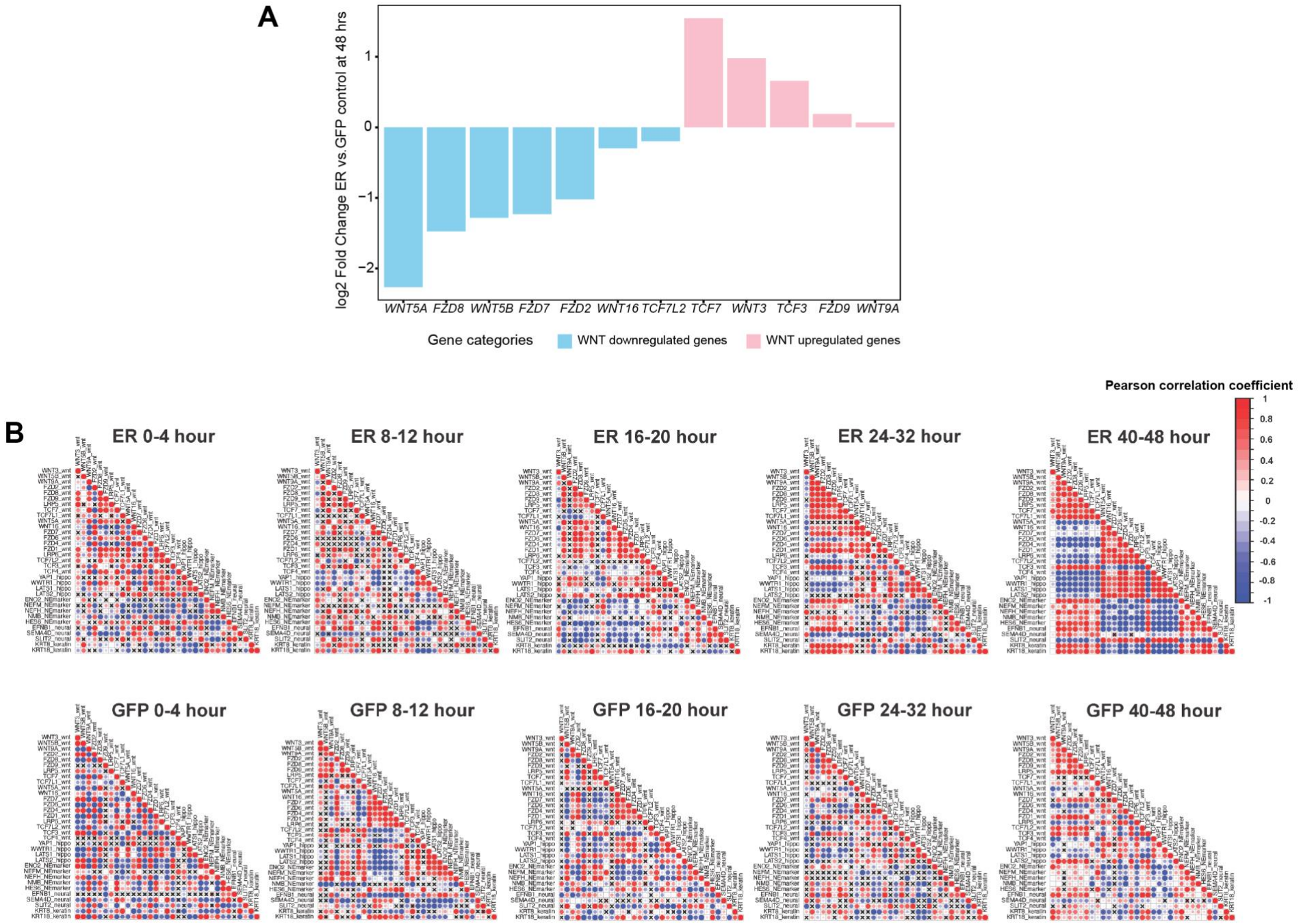
Supplemental Figure 2. WGCNA eigengenes analysis on IMR90-ER data. (**A**) Heatmap and dendrogram illustrating eigengenes correlations and the hierarchical clustering of eigengenes. (**B**) Correlations between module eigengenes and time period, calculated using the Kendall rank correlation coefficient.



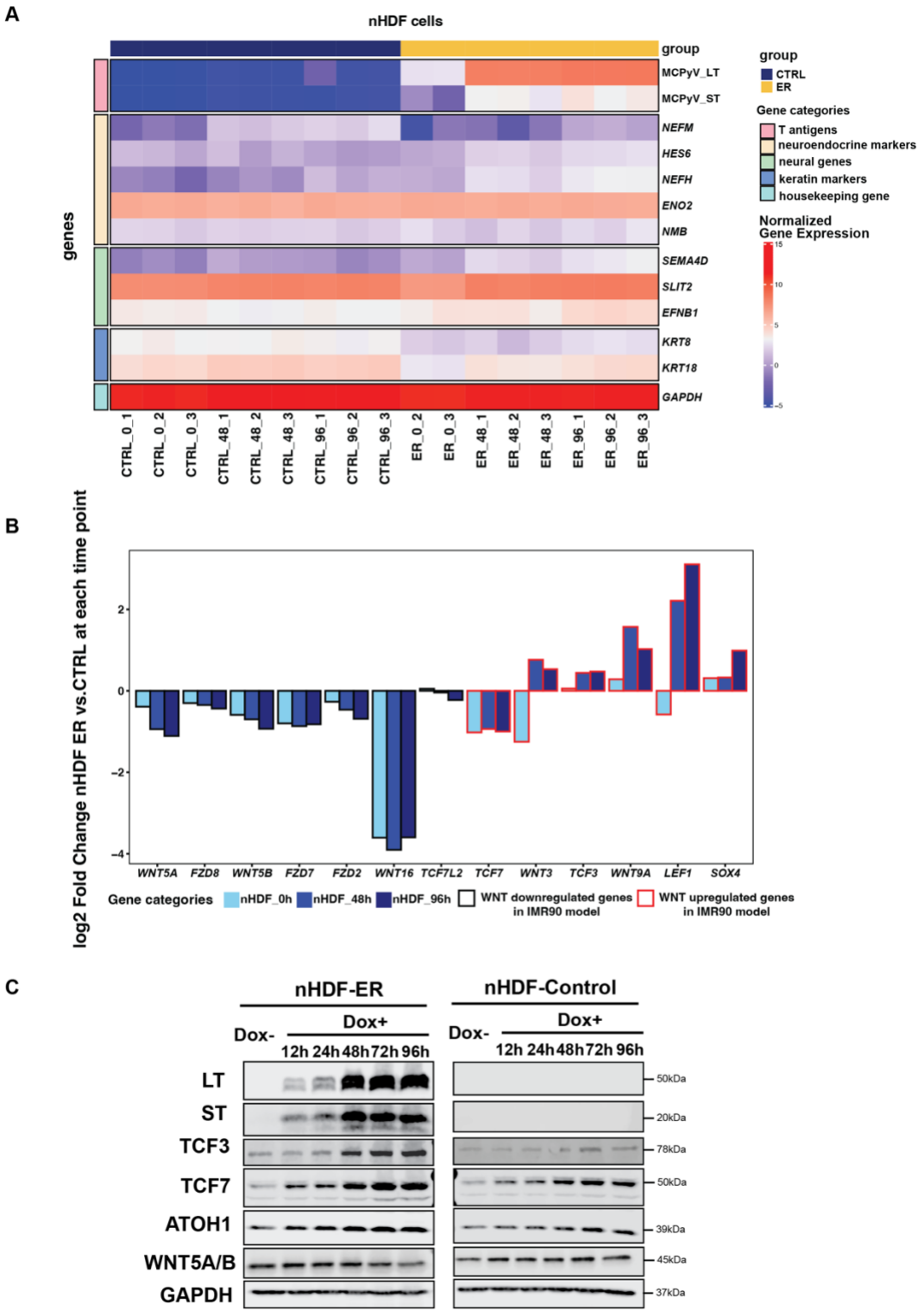
Supplemental Figure 3. Gene co-expression modules in MCPyV-ER and their enriched biological functions. Over-representation GO term enrichment results for 14 gene co-expression modules in MCPyV-ER samples. Only significant (P_{adj} < 0.05) terms from each module are presented.



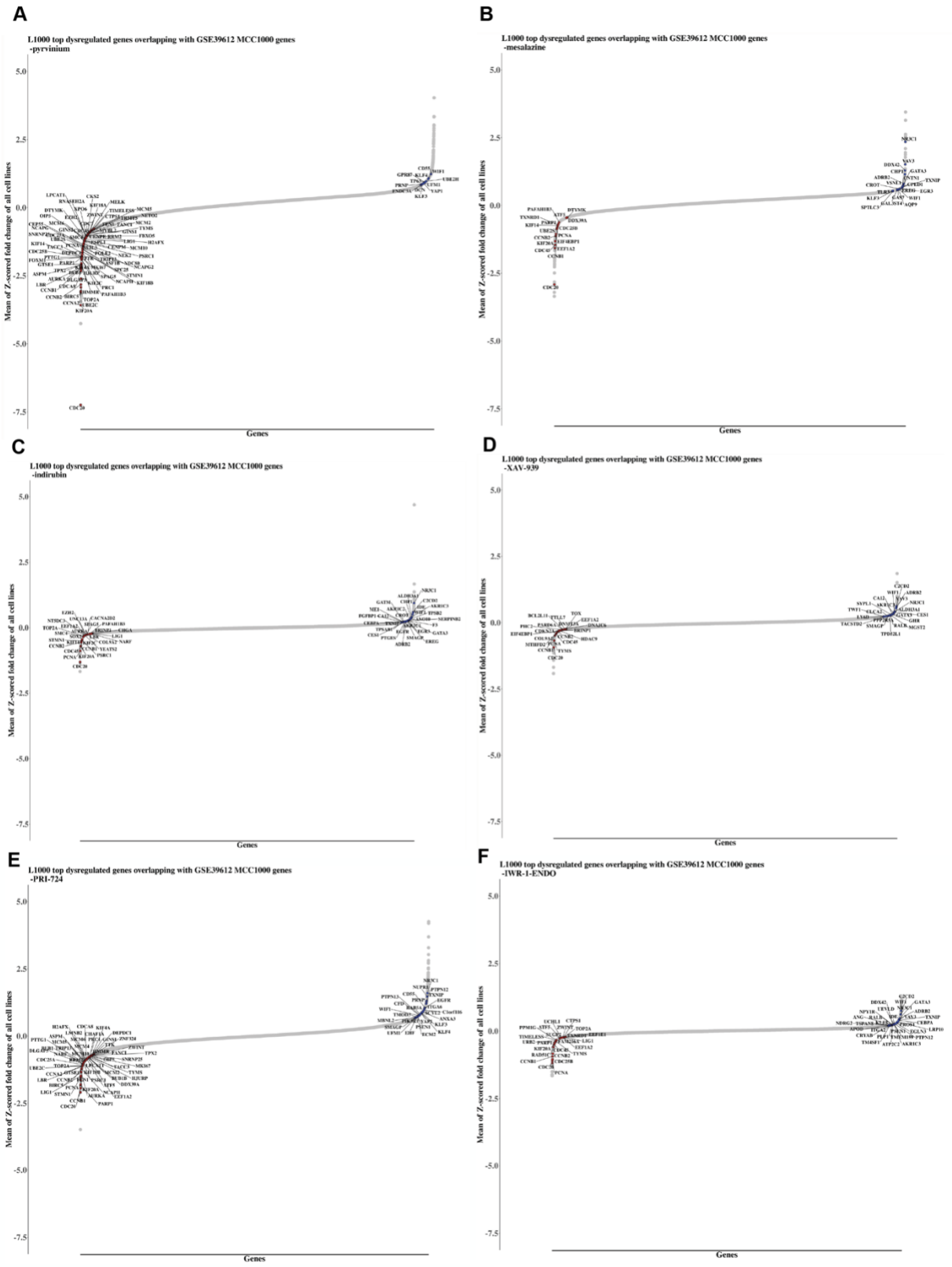
Supplemental Figure 4. WGCNA analysis on MCC patient samples reveals that Wnt signaling pathway is highly co-expressed with MCC-related pathways. (**A**) Heatmap and dendrogram illustrating eigengenes correlations and the hierarchical clustering of eigengenes, based on differentially expressed genes between MCC tumor samples and normal skin samples in a cohort of 30 MCC tumor samples. (**B**) Force-directed network of hub genes in 14 modules derived from MCC tumor samples. The attraction forces between genes were defined by their topological overlaps and were inversely proportional to the length of the edges in the graph. (**C**) GO term enrichment results for genes in module 3, 5 and 6 respectively


A
log2 Fold Change ER vs.GFP control at 48 hrs
1
0
−1
−2
WNT5A FZD8 WNT5B FZD7 FZD2 WNT16 TCF7L2 TCF7 WNT3 TCF3 FZD9 WNT9A
Gene categories
WNT downregulated genes
WNT upregulated genes
B
Pearson correlation coefficient
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
ER 0-4 hour
ER 8-12 hour
ER 16-20 hour
ER 24-32 hour
ER 40-48 hour
GFP 0-4 hour
GFP 8-12 hour
GFP 16-20 hour
GFP 24-32 hour
GFP 40-48 hour

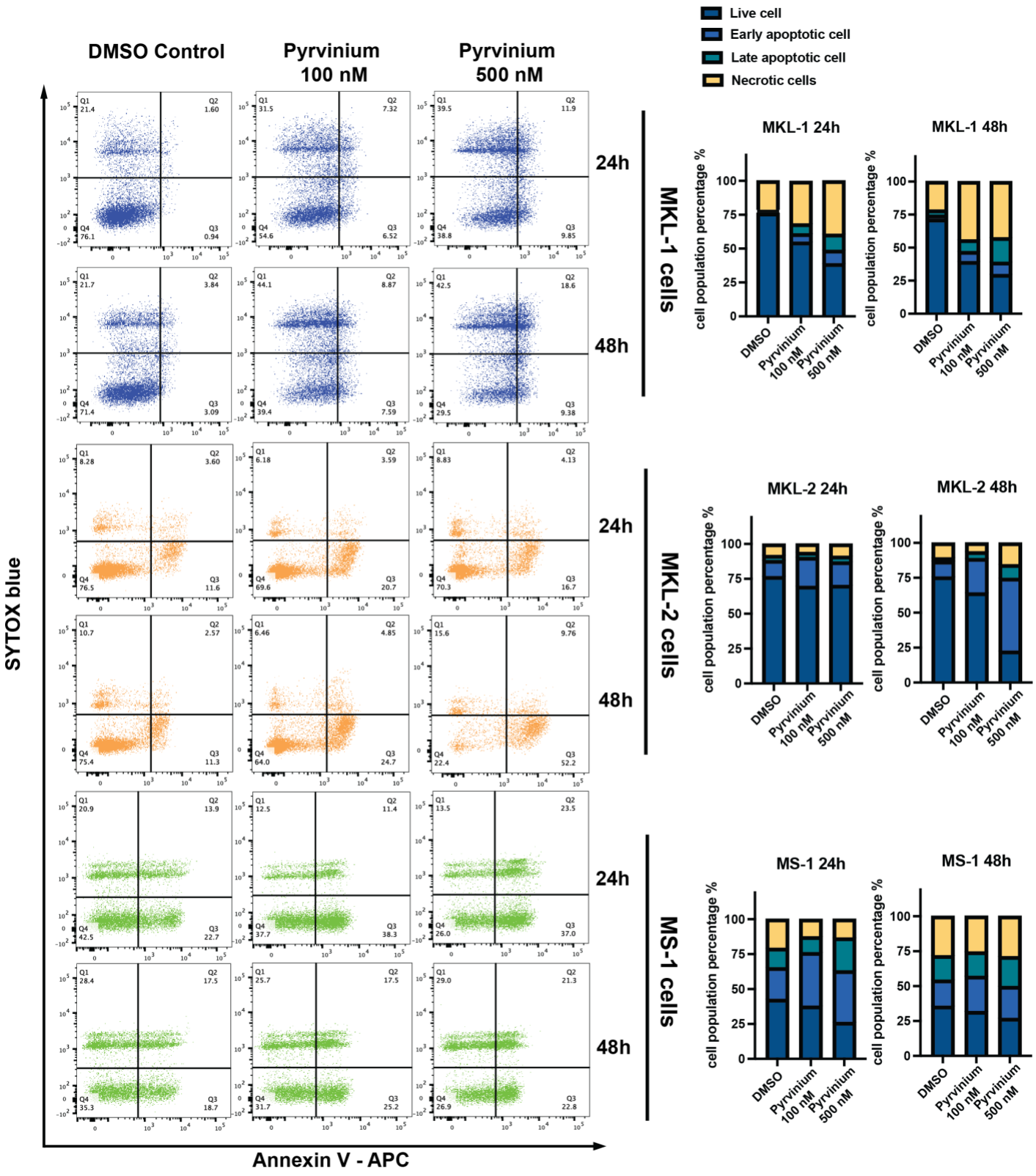
Supplemental Figure 5. MCPyV-ER induces characteristic changes in the expression and correlation of Wnt and neuroendocrine marker genes. (**A**) Bar plot showing selected Wnt gene expression levels in IMR90-ER samples relative to the IMR90-GFP samples at the 48-hour time point. The log2 fold change values were estimated from a linear model using the R package LIMMA. (**B**) The Pearson correlation coefficient was calculated to evaluate the relationship between Wnt genes and NE marker genes (*ENO2, NEFM, NEFH, NMB* and *HES6*), as well as MCC-related genes from previous studies, including hippo pathway genes (*YAP1* and *WWTR1*), neural markers (*EFNB1*, *SEMA4DM*), and keratin markers (*KRT8* and *KRT18*). The resulting correlation coefficients are color-coded according to the legend, with circle size reflecting the absolute value. Statistical significance was determined using a two-tailed t-test with a threshold of $P < 0.05$. Significant correlations are highlighted in color, while non-significant ones are crossed out.



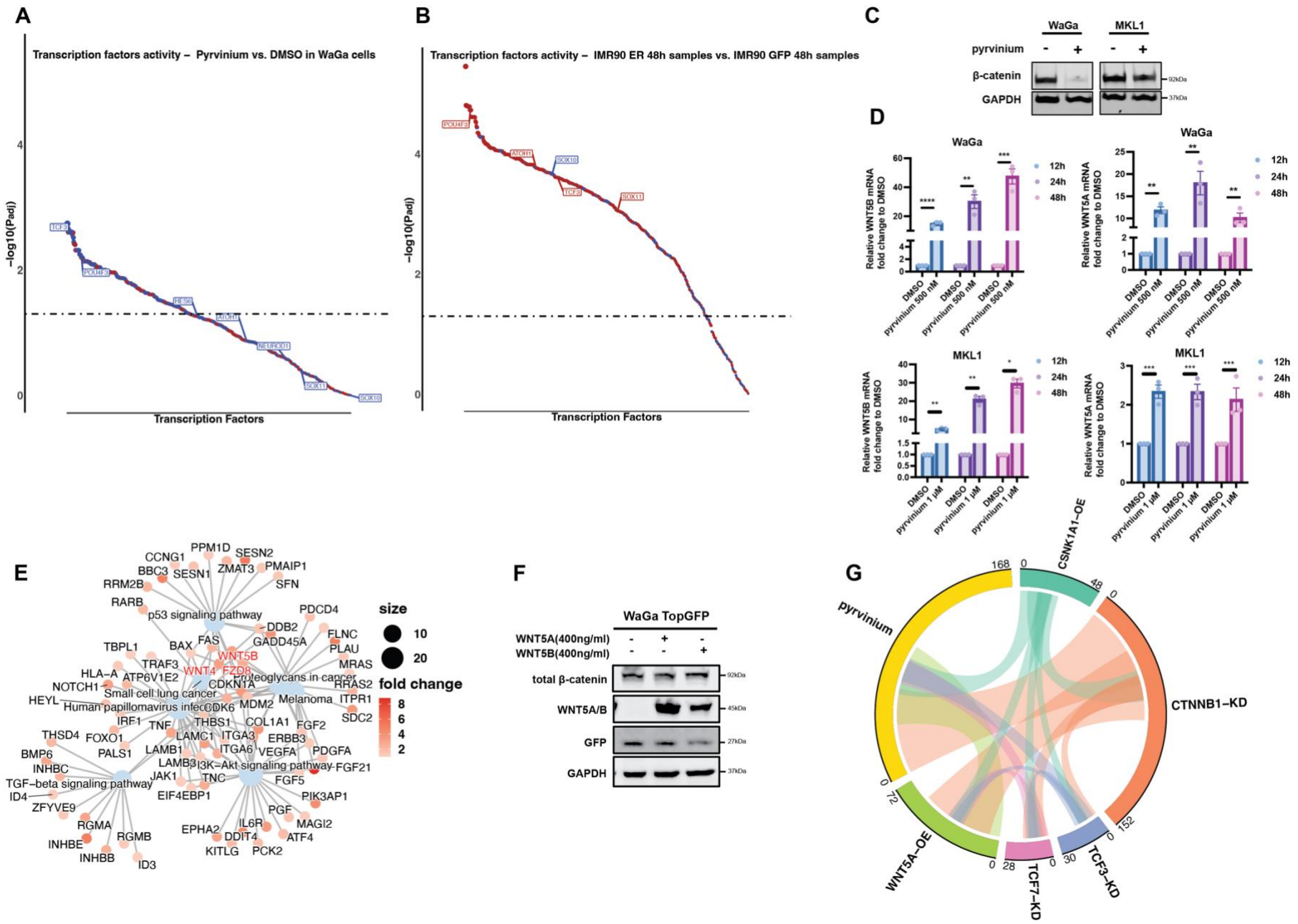
Supplemental Figure 6. The MCPyV-ER inducible model in nHDF cells supports findings from the IMR90 model. (**A**) Heatmap showing normalized gene expression levels for T antigen genes, a subset of MCC marker genes, and housekeeping gene *GAPDH* in nHDF-ER and nHDF-Control samples. (**B**) Bar plot illustrating the log2 fold changes of WNT gene expression levels in nHDF-ER samples across all time points, relative to the nHDF-Control samples at the corresponding time points. (**C**) Protein levels of MCPyV large T antigen, small T antigen, Wnt genes and NE marker ATOH1 in nHDF-ER and nHDF-Control cells at 0, 12, 24, 48, 72 and 96 hours following ER expression induction.



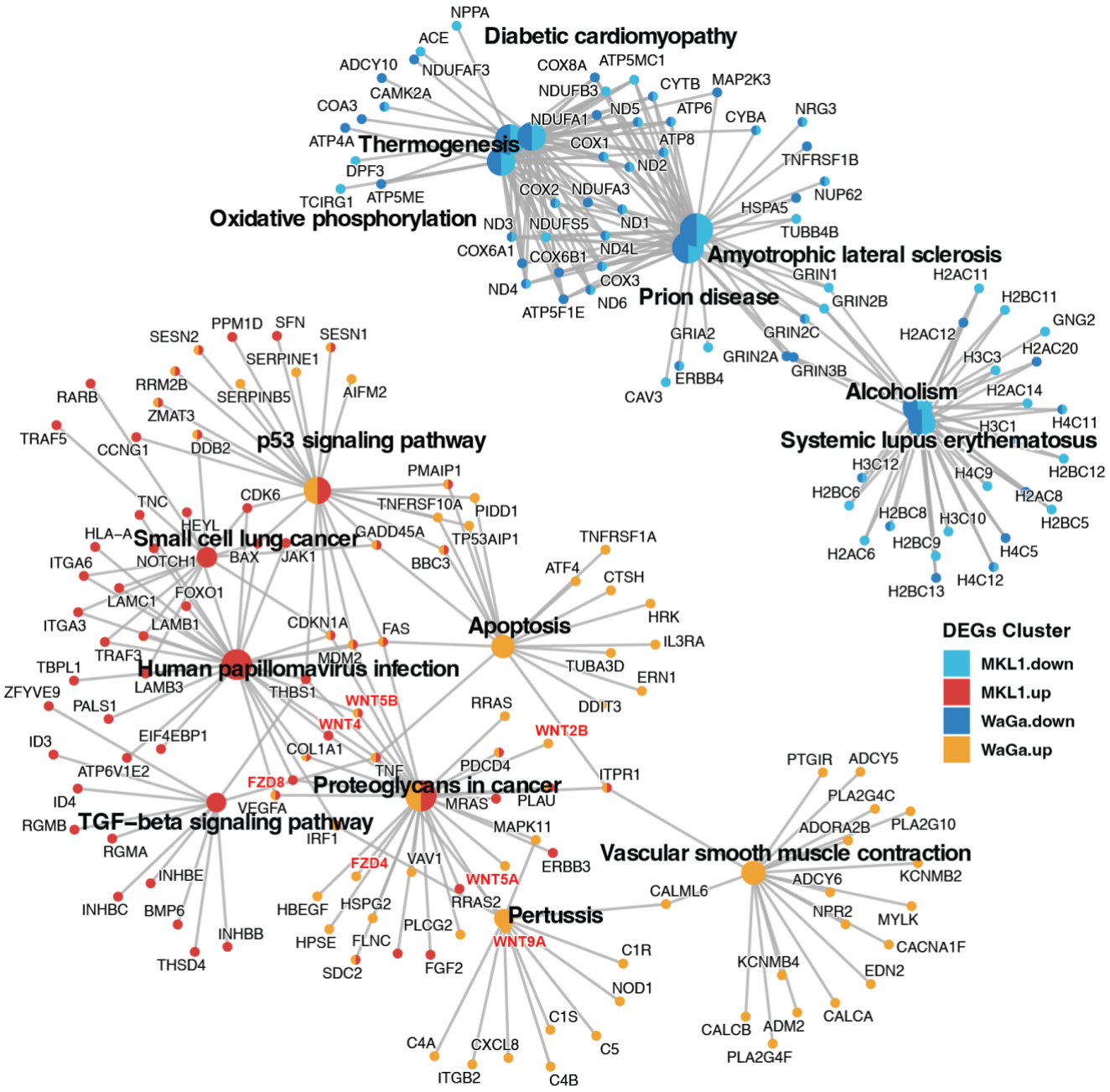
Supplemental Figure 7. Transcriptomic analysis of Wnt signaling perturbagens in LINCS L1000 and MCC tumor databases. (**A - F**) Six commercially available WNT signaling perturbagens reverse MCC1000 genes in the L1000 dataset. All cell lines from LINCS L1000 dataset were analyzed, and the top 500 reversed MCC1000 and L1000 genes from both sides were annotated.



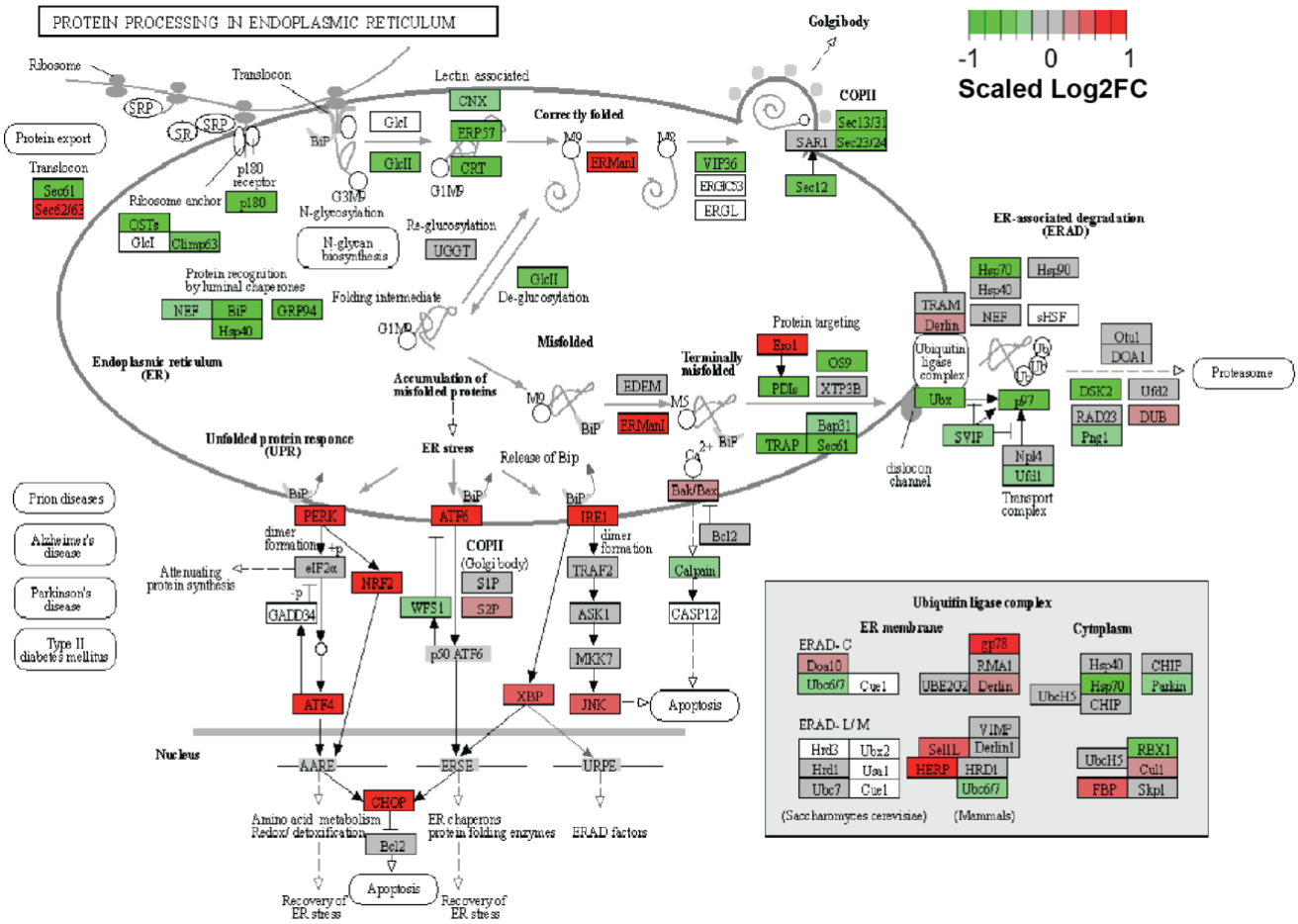
Supplemental Figure 8. Pyrvinium pamoate induces apoptosis in MCC cells. Flow cytometry analyses showing the levels of Annexin V-APC and SYTOX blue staining to assess the apoptotic population in MKL-1, MKL-2, and MS-1 cells treated with varying doses of pyrvinium pamoate over different time periods. Bar graphs on the right display the quantification of different populations in pyrvinium-treated MCC cells at 24h and 48h, respectively.


A
Transcription factors activity – Pyrvinium vs. DMSO in WaGa cells
−log10(Padj)
Transcription Factors
TCF3
POU4F3
HES6
ATOH1
NEUROD1
SOX11
SOX10
B
Transcription factors activity – IMR90 ER 48h samples vs. IMR90 GFP 48h samples
−log10(Padj)
Transcription Factors
POU4F3
ATOH1
SOX10
TCF3
SOX11
C
WaGa
MKL1
pyrvinium
β-catenin
GAPDH
92kDa
37kDa
D
WaGa
MKL1
Relative WNT5B mRNA fold change to DMSO
Relative WNT5A mRNA fold change to DMSO
12h
24h
48h
DMSO
pyrvinium 500 nM
pyrvinium 1 μM
E
size
10
20
fold change
8
6
4
2
PPM1D
CCNG1
SESN2
BBC3
SESN1
ZMAT3
PMAIP1
RRM2B
SFN
RARB
p53 signaling pathway
PDCD4
DDB2
GADD45A
FLNC
PLAU
TBPL1
BAX
FAS
WNT5B
MRAS
TRAF3
WNT4
FZD8
HLA−A
ATP6V1E2
Proteoglycans in cancer
RRAS2
NOTCH1
CDKN1A
Small cell lung cancer
HEYL
Human papillomavirus infec
CDK6
MDM2
Melanoma
ITPR1
SDC2
IRF1
TNF
THBS1
COL1A1
FGF2
THSD4
FOXO1
LAMC1
ITGA3
ERBB3
BMP6
PALS1
LAMB1
ITGA6
VEGFA
PDGFA
INHBC
LAMB3
PI3K−Akt signaling pathway
FGF21
TGF−beta signaling pathway
JAK1
TNC
FGF5
ID4
EIF4EBP1
PIK3AP1
ZFYVE9
PGF
RGMA
EPHA2
IL6R
MAGI2
RGMB
DDIT4
ATF4
INHBE
KITLG
PCK2
INHBB
ID3
F
WaGa TopGFP
WNT5A(400ng/ml)
WNT5B(400ng/ml)
total β-catenin
WNT5A/B
GFP
GAPDH
92kDa
45kDa
27kDa
37kDa
G
CSNK1A1−OE
CTNNB1−KD
TCF3−KD
TCF7−KD
WNT5A−OE
pyrvinium
168
48
152
30
28
72

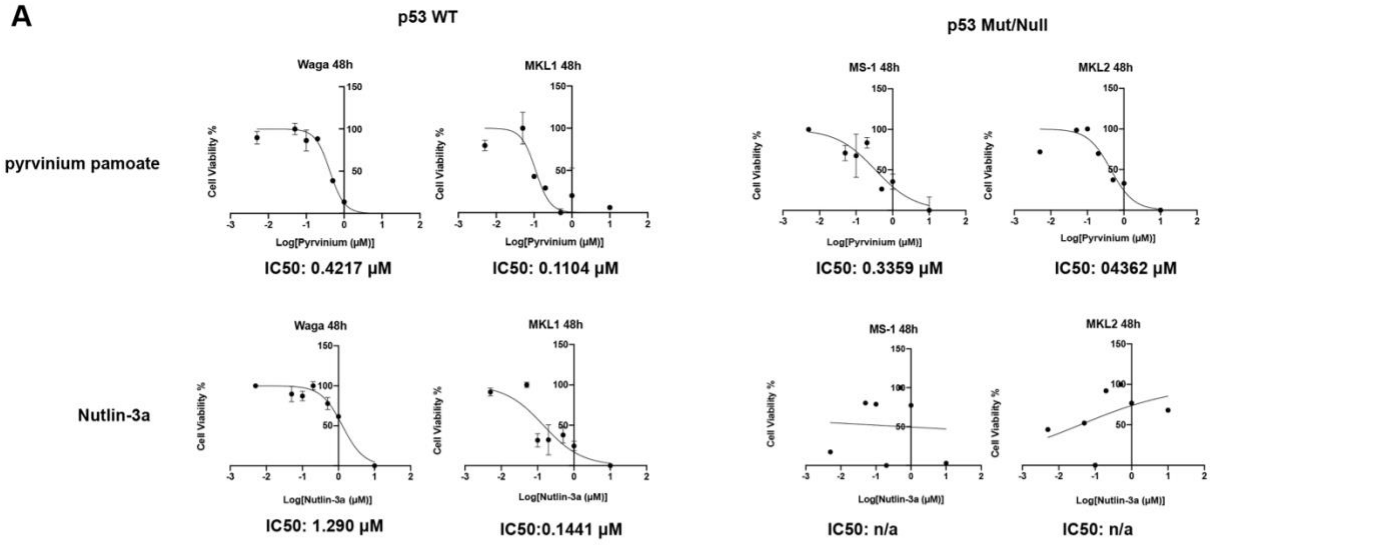
Supplemental Figure 9. Pyrvinium reverses Wnt signaling in MCC by targeting multiple proteins. (**A-B**) Scatter plots showing the predicted activity levels of master transcription factors in pyrvinium-treated vs. DMSO-treated MCC cells and in IMR90-ER vs. IMR90-GFP cells at 48 hours, respectively. The MCCP specific regulon used in VIPER was constructed using ARACNe on 13 virus-positive MCC patient samples. (**C**) Total β-catenin protein levels in WaGa and MKL-1 cells under DMSO and 1μM pyrvinium treatment, as measured by WB. (**D**) RT-qPCR validation of relative *WNT5B* and *WNT5A* mRNA levels in WaGa and MKL-1 cells treated with pyrvinium at 500 nM and 1 μM respectively for 12, 24, and 48 hours, using *GAPDH* as the internal control. The mean relative mRNA levels in vehicle control samples were set to 1. Statistical significance was determined by an unpaired two sample t-test (n = 3). (****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). (**E**) Gene-biological concepts network showing KEGG pathway enrichment analysis results of significantly upregulated DEGs ($P_{adj} \leqslant 0.05$, log2 fold change $\geqslant 1$) in WaGa cells. (**F**) Total β-catenin and GFP protein levels in WaGa TopGFP cells treated with 400 ng/ml human WNT5A and WNT5B recombinant proteins, as measured by WB. (**G**) Circos plot depicting the pairwise comparison of overlapping MCC1000 signature genes and the top dysregulated genes across different perturbations of the Wnt signaling pathway by pyrvinium.

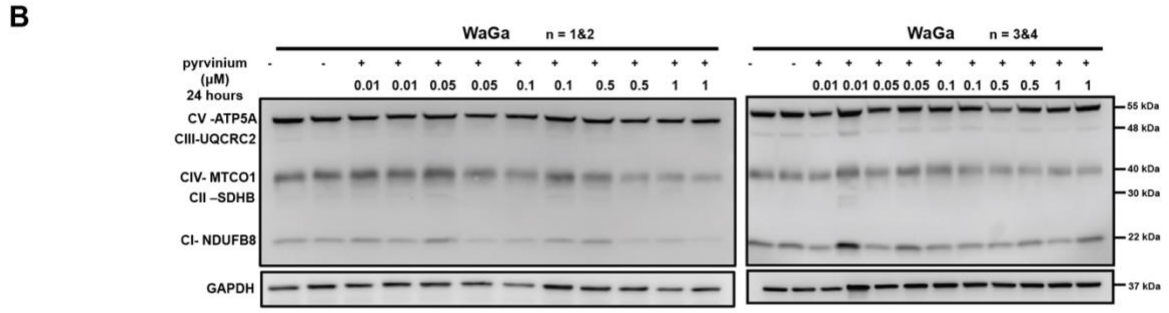


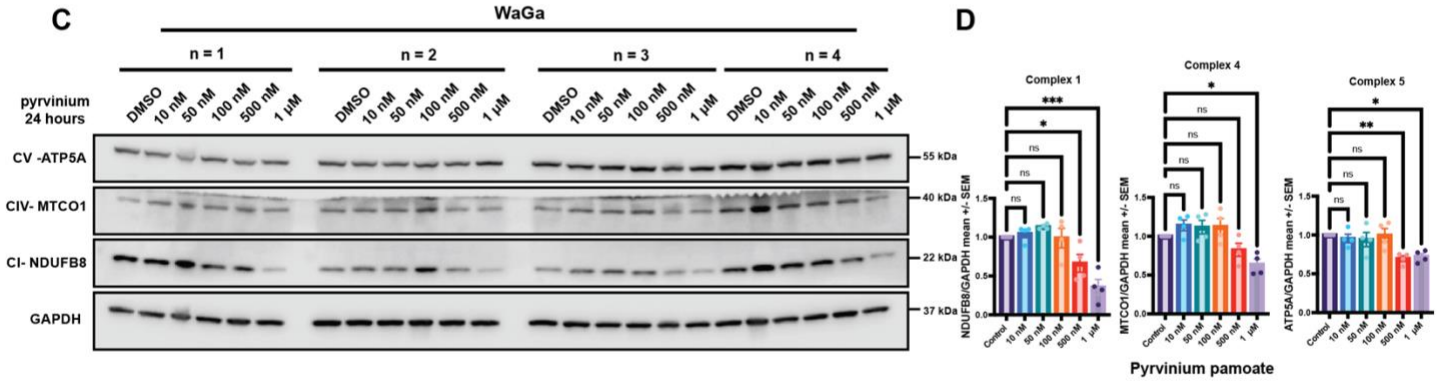
Supplemental Figure 10. KEGG pathway enrichment visualization of DEGs in WaGa and MKL-1 cells treated with pyrvinium. Gene-biological concepts network showing KEGG pathway enrichment for significantly upregulated ($P_{adj} \leq 0.05$, log2 fold change ≥ 1) and downregulated ($P_{adj} \leq 0.05$ and log2 fold change ≤ -1) DEGs in WaGa and MKL-1 cell lines.

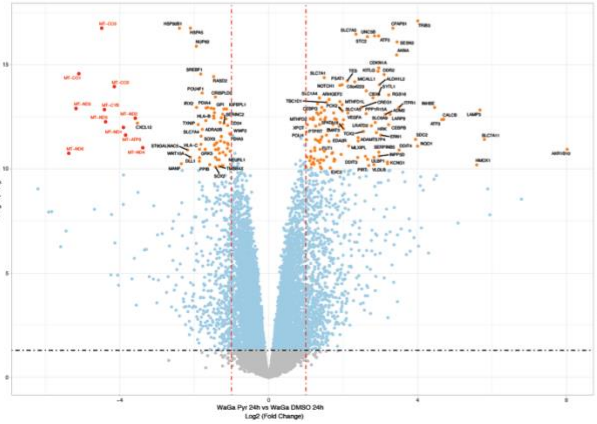


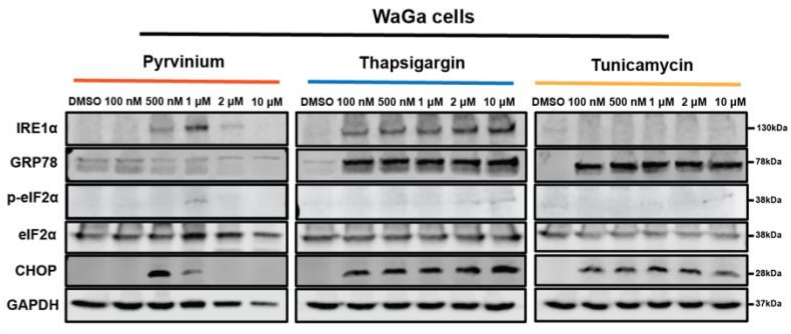
Supplemental Figure 11. Pyrvinium pamoate activates endoplasmic reticulum (ER) stress. KEGG pathway visualization of UPR and ER stress related genes. Gene coloring reflects the log2 fold change from the comparison between pyrvinium- and DMSO-treated WaGa cells after 24 hours. The log2 fold change values are scaled between -1 and 1.


A
p53 WT
p53 Mut/Null
pyrvinium pamoate
Nutlin-3a
Waga 48h
MKL1 48h
MS-1 48h
MKL2 48h
Cell Viability %
Log[Pyrvinium (μM)]
Log[Nutlin-3a (μM)]
IC50: 0.4217 μM
IC50: 0.1104 μM
IC50: 0.3359 μM
IC50: 04362 μM
IC50: 1.290 μM
IC50:0.1441 μM
IC50: n/a
IC50: n/a

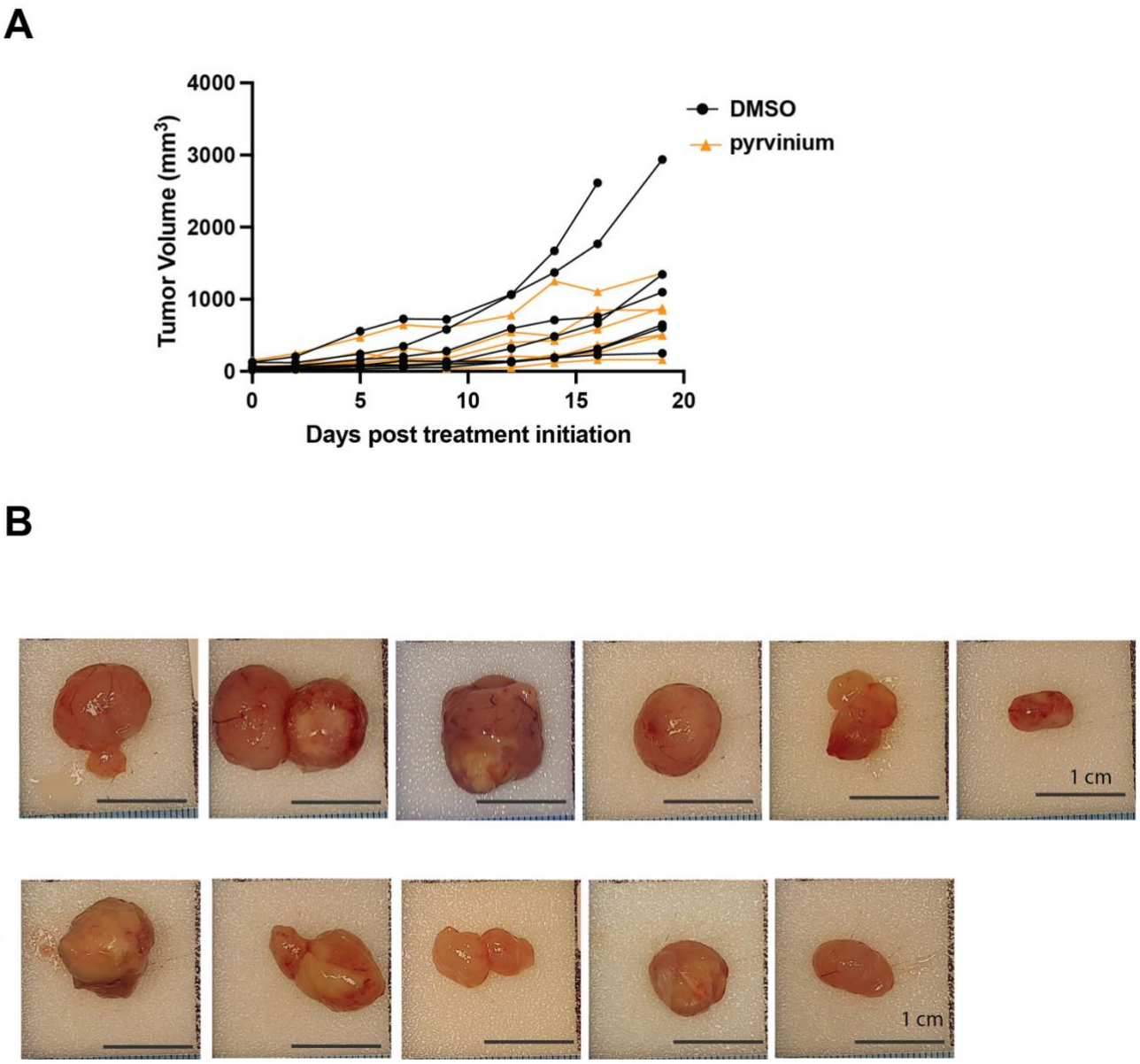

B
WaGa n = 1&2
WaGa n = 3&4
pyrvinium (μM) 24 hours
0.01 0.01 0.05 0.05 0.1 0.1 0.5 0.5 1 1
CV -ATP5A
CIII-UQCRC2
CIV- MTCO1
CII –SDHB
CI- NDUFB8
GAPDH
55 kDa
48 kDa
40 kDa
30 kDa
22 kDa
37 kDa


C
WaGa
n = 1
n = 2
n = 3
n = 4
pyrvinium 24 hours
DMSO 10 nM 50 nM 100 nM 500 nM 1 μM
CV -ATP5A
CIV- MTCO1
CI- NDUFB8
GAPDH
55 kDa
40 kDa
22 kDa
37 kDa
D
Complex 1
Complex 4
Complex 5
NDUFB8/GAPDH mean +/- SEM
MTCO1/GAPDH mean +/- SEM
ATP5A/GAPDH mean +/- SEM
Pyrvinium pamoate

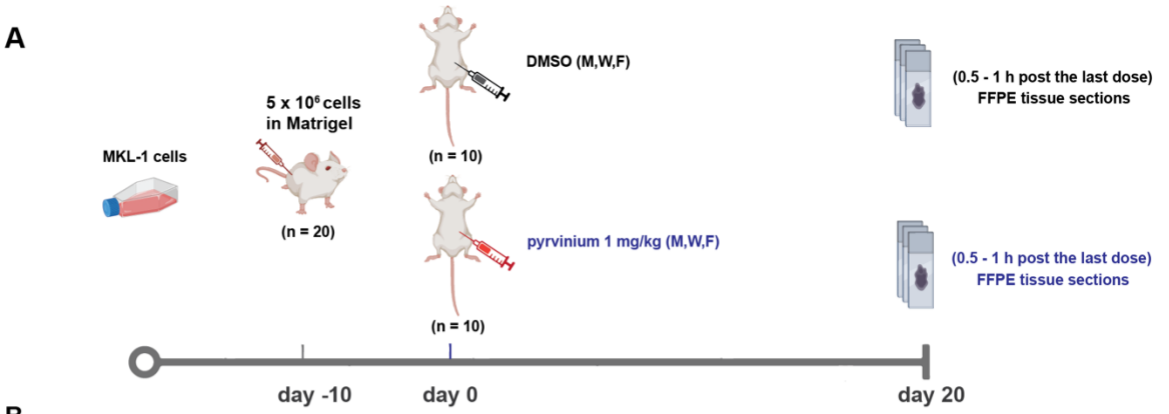

E
WaGa Pyr 24h vs WaGa DMSO 24h
Log2 (Fold Change)

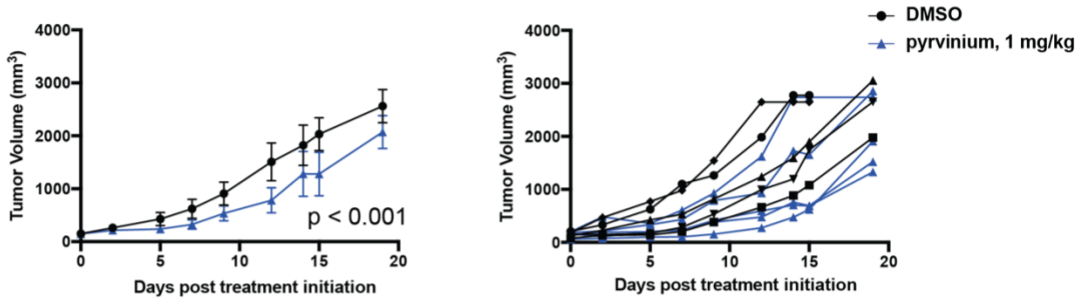

F
WaGa cells
Pyrvinium
Thapsigargin
Tunicamycin
DMSO 100 nM 500 nM 1 μM 2 μM 10 μM
IRE1α
GRP78
p-eIF2α
eIF2α
CHOP
GAPDH
130kDa
78kDa
38kDa
38kDa
28kDa
37kDa

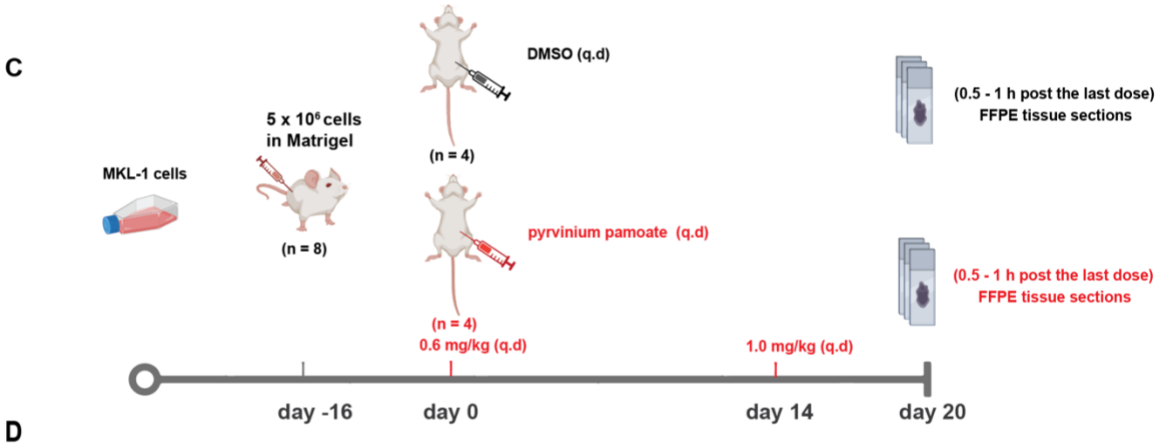
Supplemental Figure 12. Mechanisms of action of pyrvinium pamoate in MCC. (**A**) Half-maximal inhibitory concentration (IC50) values for pyrvinium and Nutlin-3a after 48 hours of treatment in p53 wild-type cell lines (WaGa, MKL-1) and *TP53*^{Mut}/*TP53*$^{-/-}$ cell lines (MS-1, MKL-2), measured using the MTT assay. (**B-C**) Two independent sets of OXPHOS Western blot analyses showing levels of mitochondria complex proteins in WaGa cells treated with pyrvinium at various concentration for 24 hours (n = 4). (**D**) Quantification of protein levels for NDUFB8 (Complex I subunit), MTCO1 (Complex IV subunit), ATP5A (Complex V subunit), and internal control GAPDH in panel (**C**). Data are presented relative to mean GAPDH expression in vehicle control samples (means ± SEM, n = 4 biological replicate blots). Statistical analysis was performed using ordinary ANOVA followed by Dunnett's multiple comparison test. (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). Quantitative data for western blot result in (**B**) are included in Figure 6G. (**E**) Volcano plot illustrating results from DEG analysis. The x-axis represents log2 fold change, and the y-axis indicates -log10(P_{adj}). (**F**) Protein levels of CHOP and ER-stress markers were detected by WB in WaGa cells treated with pyrvinium, thapsigargin and tunicamycin at various concentration for 24 hours.

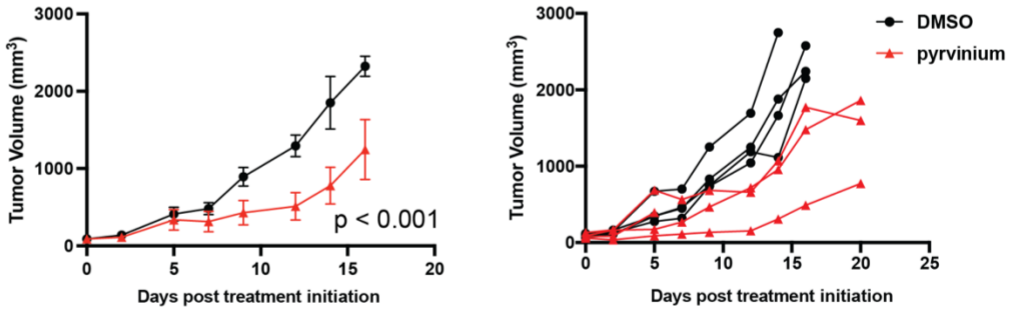


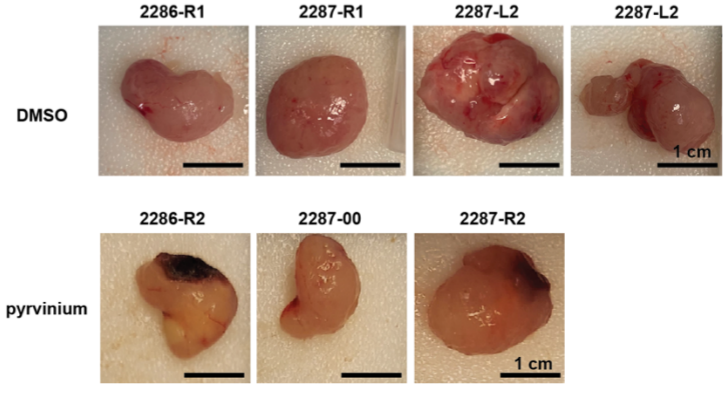
Supplemental Figure 13. Pyrvinium pamoate inhibits tumor growth in an MCC xenograft model. (**A**) MKL-1 xenograft tumor growth curve from in vivo Study #3 depicting individual tumor volumes in the vehicle control and pyrvinium-treated groups from day 0 to day 20. Treatment followed a gradually increasing dosage schedule of 0.1-1.0 mg/kg per day (q.d.). (**B**) Images of xenograft tumor collected from in vivo Study #3 with the 0.1 -1.0 mg/kg q.d. dosing schedule. Tumors were harvested 0.5 – 1 hour after the final treatment on day 20. (One mouse in the vehicle control group was sacrificed on day 16 because its tumor volume exceeded 2000 mm^3. In the treatment group, one mouse was euthanized on day 7 due to severe dermatitis on its neck, which was determined to be unrelated to pyrvinium toxicity. Another mouse in the treatment group was sacrificed on day 20, prior to the final injection, due to treatment-related side effects.) All images are scaled uniformly.


A
MKL-1 cells
5 x 10⁶ cells in Matrigel
(n = 20)
DMSO (M,W,F)
(n = 10)
pyrvinium 1 mg/kg (M,W,F)
(n = 10)
(0.5 - 1 h post the last dose) FFPE tissue sections
(0.5 - 1 h post the last dose) FFPE tissue sections
day -10
day 0
day 20


B
DMSO
pyrvinium, 1 mg/kg
Tumor Volume (mm³)
Days post treatment initiation
p < 0.001


C
MKL-1 cells
5 x 10⁶ cells in Matrigel
(n = 8)
DMSO (q.d)
(n = 4)
pyrvinium pamoate (q.d)
(n = 4)
0.6 mg/kg (q.d)
1.0 mg/kg (q.d)
(0.5 - 1 h post the last dose) FFPE tissue sections
(0.5 - 1 h post the last dose) FFPE tissue sections
day -16
day 0
day 14
day 20


D
DMSO
pyrvinium
Tumor Volume (mm³)
Days post treatment initiation
p < 0.001


E
2286-R1
2287-R1
2287-L2
2287-L2
DMSO
1 cm
2286-R2
2287-00
2287-R2
pyrvinium
1 cm

Supplemental Figure 14. Pyrvinium pamoate inhibits tumor growth in MCC xenograft models – pilot studies.

(**A**) Schematic representation of the experimental design for in vivo Study #1, featuring a treatment schedule of 1.0 mg/kg administered three days per week. (**B**) Tumor growth curve illustrating both the mean and individual tumor sizes in vehicle control and pyrvinium-treated mice from day 0 to day 20 in in vivo Study #1. (**C**) Schematic representation of the experimental design for in vivo Study #2, which employed a daily treatment schedule with doses ranging from 0.6 -1.0 mg/kg per day. (**D**) Tumor growth curve showing the mean and individual tumor volumes in vehicle control and pyrvinium-treated groups from day 0 to day 19 in in vivo Study #2. (**E**) Images of xenograft tumor tissues collected from in vivo Study #2. Tumors were harvested following the final 1.0 mg/kg dose of pyrvinium or DMSO control. (One mouse in the treatment group was sacrificed prior to the dose escalation to 1.0 mg/kg due to treatment-related side effects.) All images are scaled uniformly.

Supplemental Tables

Supplemental Table 1. MCPyV T antigens transcripts read count matrix from IMR90 ER samples

	MCPyV_gp1 (capsid protein)	MCPyV_gp2 (VP1, major capsid protein)	MCPyV_gp3 (large T-antigen)	MCPyV_gp4 (small T-antigen)
ER_0_1	0	0	261	3
ER_0_2	0	0	268	3
ER_0_3	0	0	271	2
ER_4_1	0	0	2297	68
ER_4_2	0	0	1769	50
ER_4_3	0	0	1285	35
ER_8_1	0	0	2640	51
ER_8_2	0	0	2851	52
ER_8_3	0	0	2507	55
ER_12_1	0	0	4720	96
ER_12_2	0	0	3659	82
ER_12_3	0	0	4336	86
ER_16_1	0	0	5370	96
ER_16_3	0	0	5299	90
ER_20_1	0	0	7276	167
ER_20_2	0	0	6043	151
ER_20_3	0	0	7510	180
ER_24_1	0	0	9560	169
ER_24_2	0	0	7018	146
ER_24_3	0	0	9645	193
ER_32_1	0	0	7492	175
ER_32_2	0	0	12703	252
ER_32_3	0	0	12368	273
ER_40_1	0	0	6080	80
ER_40_3	0	0	11485	255
ER_48_1	0	0	19499	475
ER_48_2	0	0	2820	65
ER_48_3	0	0	7869	203

Supplemental Table 2. In vivo Study #1 – 1 mg/kg IP injection tumor volume statistics with mixed-effects model

sqrvolume	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
1.Treatment	3.795161	4.610891	0.82	0.410	-5.242019	12.83234
day	2.241025	.1057545	21.19	0.000	2.03375	2.4483
Treatment#c.day						
1	-.5139983	.1395729	-3.68	0.000	-.7875561	-.2404404
_cons	-16.13178	3.324688	-4.85	0.000	-22.64805	-9.615512

[sqrvolume]day + [sqrvolume]1.Treatment#c.day = 0

sqrvolume	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	1.727027	.0910855	18.96	0.000	1.548503	1.905551

Supplemental Table 3. In vivo Study #2 – 0.6 – 1.0 mg/kg IP injection tumor volume statistics with mixed-effects model

sqrvolume	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
1.Treatment	20.4434	4.940943	4.14	0.000	10.75933	30.12747
day	2.613092	.1218058	21.45	0.000	2.374357	2.851827
Treatment#c.day						
1	-1.181657	.1646966	-7.17	0.000	-1.504456	-.8588573
_cons	-35.12977	3.600441	-9.76	0.000	-42.18651	-28.07304

[sqrvolume]day + [sqrvolume]1.Treatment#c.day = 0

sqrvolume	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	1.431436	.1108527	12.91	0.000	1.214168	1.648703

Supplemental Table 4. In vivo Study #3 – 0.1 – 1.0 mg/kg IP injection tumor volume statistics with mixed-effects model

sqrvolume	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
1.Treatment	6.616166	4.256251	1.55	0.120	-1.725932	14.95826
day	1.339024	.0823468	16.26	0.000	1.177627	1.500421
Treatment#c.day						
1	-.4020586	.1191159	-3.38	0.001	-.6355214	-.1685958
_cons	-11.69644	2.997815	-3.90	0.000	-17.57205	-5.820831

[sqrvolume]day + [sqrvolume]1.Treatment#c.day = 0

sqrvolume	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	.9369654	.0860674	10.89	0.000	.7682763	1.105654

Supplemental Table 5. Chemicals used in the study

Reagent	Supplier	Catalog number
Pyrvinium pamoate	MedChemExpress	MFCD00010090
Nutlin-3a	Selleck Chemicals	S8059
WNT5A	R&D systems Inc	645WN010
WNT5B	R&D systems Inc	7347WN025
Tunicamycin	Sigma-Aldrich	T7765
Thapsigargin	Sigma-Aldrich	T9033

Supplemental Table 6. Antibodies used in the study

Antibodies	Supplier	Catalog number	RRID number
Anti-GAPDH	Thermo Fisher Scientific	MA5-15738	AB_10977387
Anti-β-catenin	BD Bioscience	610154	AB_397555
Anti-MCPyV T antigens (1)	The DeCaprio Lab	Custom generation	
Anti-eIF2α	Cell Signaling Technology	3192	AB_2095847
Anti-phosphorylated eIF2α		3179	AB_2095853
Anti-PARP		9542	AB_2160739
Anti-PUMA		4976	AB_2064551
Anti-WNT5A/B		2530	AB_2215595
Anti-TCF3		2883	AB_2199136
Anti-TCF7		2203	AB_2199302
Anti-IRE1α		3294	AB_823545
Anti-GRP78		3177	AB_2119845
Anti-p53	Santa Cruz Biotechnology	sc-126	AB_628082
Anti-GFP		sc-9996	AB_627695
Anti-OXPHOS Cocktail	Abcam	ab110411	AB_2756818
Anti-Ki67		ab16667	AB_302459
Anti-ATOH1	Proteintech	21215-1-AP	AB_10733126

Supplemental Table 7. Optimized settings for FCCP-OCR test for WaGa cells

Settings	Cycles	Command	Time (min)
Calibrate			
Basal	3	Loop Start	3
		Mix	2
		Measure	2.50
Loop End			
Inject – Port A			
FCCP - 0.5 µM	3	Loop Start	3
		Mix	2
		Measure	2.50
Loop End			

Supplemental Table 8. Primers used for RT-qPCR assay

Primer	Supplier	Catalog number
AXIN2	Bio-Rad	qHsaCIP0031547
SOX2		qHsaCED0036871
WNT5A		qHsaCIP0028356
WNT5B		qHsaCID0038673
ATOH1		qHsaCED0019647
GAPDH		qHsaCED0038674
TCF3 (NM_003200)	IDT (Customized)	Forward: CGAGAAGCCCCAGACCAAAC Reverse: ACCTTTTCCTCTTCTCGCCG
TCF7 (NM_201632)	IDT (Customized)	Forward: ACCAGCGGCATGTACAAAGA Reverse: GTGGGATGTGGGCTGTTGAA

Supplemental Methods

Cell Viability and Cell Proliferation

MCC cells were seeded in 96 well plates at a density of 5×10^4 cells in 100 µl. Water soluble MTT (Thiazolyl blue tetrazolium bromide) (Sigma-Aldrich, cat: #M5655) compound was dissolved in DPBS to 5 mg/ml. Upon each measurement time point,10 µl of 5 mg/ml MTT was added to each 100 µl of cell suspension in each well of a 96 well plate and the plate was incubated at 37℃ for 3 hours. After incubation, 100 µl of acidified isopropanol was added into each well. Then, the plate was wrapped in foil and shaken on an orbital shaker for 10 mins at 37℃. The BioTek Synergy LX plate reader was used to read the 96 well plate at 562 nm.

Flow Cytometry

For apoptosis assay, MCC cells were seeded in 25 cm^2 cell culture flasks and were treated with pyrvinium for 24 hours before collection. Cells were centrifuged at 400 rcf for 5 min and washed in cold PBS once. Then, cells were washed with binding buffer. Next, cells were resuspended in 100 µl binding buffer at 5×10^6 cells/mL. 5 µl of Annexin V-APC (Thermo Fisher Scientific, cat: #A35110) was added to 100 µl of the cell suspension. After 15 minutes incubation at room temperature in the dark, the cells were spun down and resuspended in 500 µl binding buffer. 0.5 µl of SYTOX blue (Thermo Fisher Scientific, cat: #S34857) was added to each reaction and the cell suspension was transferred into 1ml Falcon tubes. The cells were incubated on ice for 15 min before measuring by a flow cytometer (BD FACS Canto II) in the allophycocyanin (APC) and Pacific Blue channels.

Immunofluorescence

WaGa cells were seeded in 25 cm^2 cell culture flasks and were treated with pyrvinium for 24 hours. Cells under different conditions were collected and washed with DPBS once. Then, DPBS was used to resuspend the cells in 2×10^5 cells/ml. After funnels were assembled for the Cytospin, 200 ul of cell suspension of each condition was added to the funnel, and the cells were centrifuged at 800 rpm for 5 min with high acceleration. Slides were air-dried for 5 min. Next, the cells were fixed on the slides with 4% paraformaldehyde in DPBS for 15 min at room temperature. After rinsing the cells two times in DPBS, the DPBS was aspirated and the cells were permeabilized with 0.1% Triton X-100 in DPBS for 10 min. The cells were again washed with DPBS twice, and samples were blocked in 3% BSA (in DPBS) for 30 min at room temperature. Then, incubation of primary and secondary

antibodies was performed under manufacturer recommended conditions. The Antifade (Thermo Fisher Scientific, cat: #P36935) mounting media with DAPI and 1.5 mm slides were added to cover the cells. The samples were imaged under a fluorescent microscope (Nikon Ti2 inverted Microscope) 24 hours later.

Immunohistochemistry

Immunohistochemistry was performed on paraffin embedded xenograft tumor tissue sections (5 μm). The sections were first deparaffinized in xylene for 5 mins twice. Then, samples were hydrated using 100%, 95% and 75% ethanol for 3 mins each. After ethanol hydration, the samples were rinsed with distilled water for 5 minutes twice. Antigen retrieval was done with citrate buffer (pH 6.2), under near-boiling temperature for 20 min. Antibodies were diluted 1:200 in goat serum and applied to sections overnight at 4℃ after 30 mins of blocking. UltraVision LP Detection System (Fisher Scientific, cat: # TL015HD) was used for signal detection. Staining procedures were performed followed the recommended conditions by the manufacturer. IHC profiler (2), an automated method that uses color deconvolution and computerized pixel profiling to assign scores to images, was applied to quantify the IHC images based on the DAB color spectrum.

RNA-seq processing pipeline

The raw reads were aligned with the reference genome “GDC.h38.d1.vd1 STAR2 Index Files (v36)” downloaded from https://gdc.cancer.gov/about-data/gdc-data-processing/gdc-reference-files using STAR 2.7.10a and counts were quantified using the Rsubread R package. The raw data and counts matrix can be found in the Gene Expression Omnibus (GEO) database (accession numbers: GSE130639, GSE229701, and GSE278335). The edgeR pipeline using TMM normalization and voom with default parameters was used to normalize the counts matrix. To filter out low-expressing genes, we performed calculations of counts per million (cpm) using the raw read counts matrix. Genes with less than half of the samples exceeding 1 cpm were subsequently removed.

Differential expression analysis

Differentially expressed genes were identified for RNA-seq data and for the publicly available microarray dataset GSE39612 dataset using limma R package.

Gene Ontology (GO) term enrichment analysis

GO term over-representation analysis was performed using R packages ClusterProfiler and msigdbr. Only the Biological Pathway GO terms were included in the analysis. Hypergeometric test followed by Benjamini-Hochberg (BH) adjustment was used to calculate the adjusted p-value (P_{adj}).

TF activities analysis

VIPER (3), a method for inferring the activity of transcription factors (TFs) from gene expression data, was utilized to predict TF activities in our samples. The human DoRothEA (4) TF-target interaction database was used to generate the regulon, and only interactions with high confidence score ("A", "B" and "C") were included. ARACNe-AP (5), an algorithm for gene regulatory network reconstruction, was used to create tissue-specific regulons. The normalized gene expression matrices containing GFP control and ER samples were then transformed to regulatory protein activity matrices by using the viper function in the R viper package. For each TF within the regulon dataset, a Student's t-test was performed and BH adjustment was used to calculate an adjusted p-value for altered TF activity.

L1000 data analysis

The LINCS L1000 Level 4 data was obtained from https://clue.io/releases/data-dashboard. The file "level4_beta_all_n3026460x12328.gctx" was downloaded to extract the z-scored fold change of both drug-treated and genetic modified samples relative to the plate vehicle control. Samples treated with pyrvinium pamoate, XAV-939, indirubin, IWR-1-ENDO, mesalazine, and PRI-724 with dosage ≤ 10 μM were selected to perform the Wnt signaling small molecule compound perturbagen analysis. Samples with CSNK1A1 and WNT5A overexpression, as well as TCF3/7 and CTNNB1 knockdown, were selected for Wnt signaling genetic modification perturbation analysis. The files "GSE92742_Broad_LINCS_cell_info.txt" and "GSE92742_Broad_LINCS_gene_info.txt" were downloaded to annotate the cell lines and gene symbols. The CMapR package was used to parse the .gctx file. MCC signature gene reversal analysis was performed using Fisher's exact test in R with its function fisher.test().

Supplemental Materials

Plasmids sequences:

>pLIX_402_ER_MCCL21 (9217 bp)

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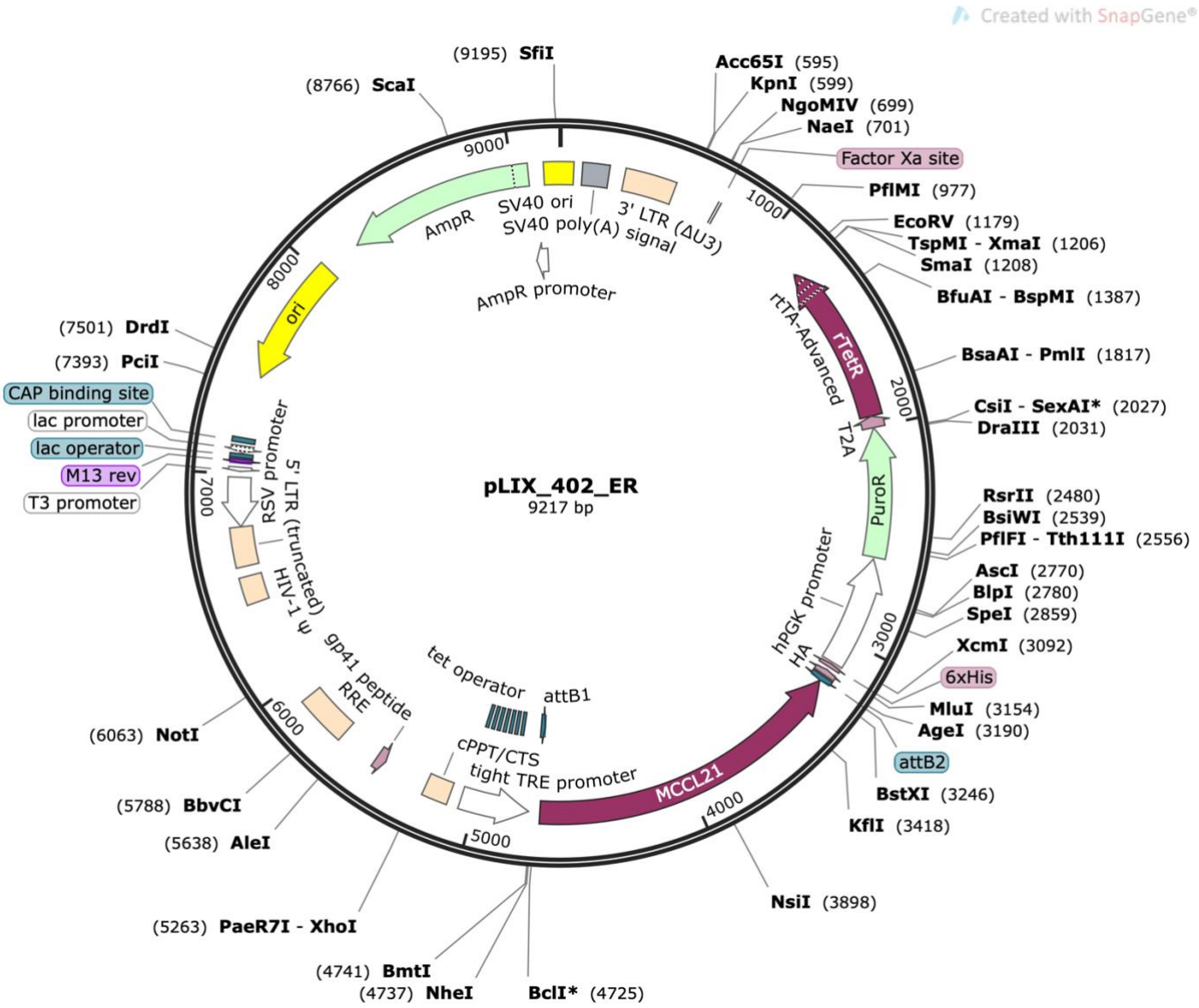

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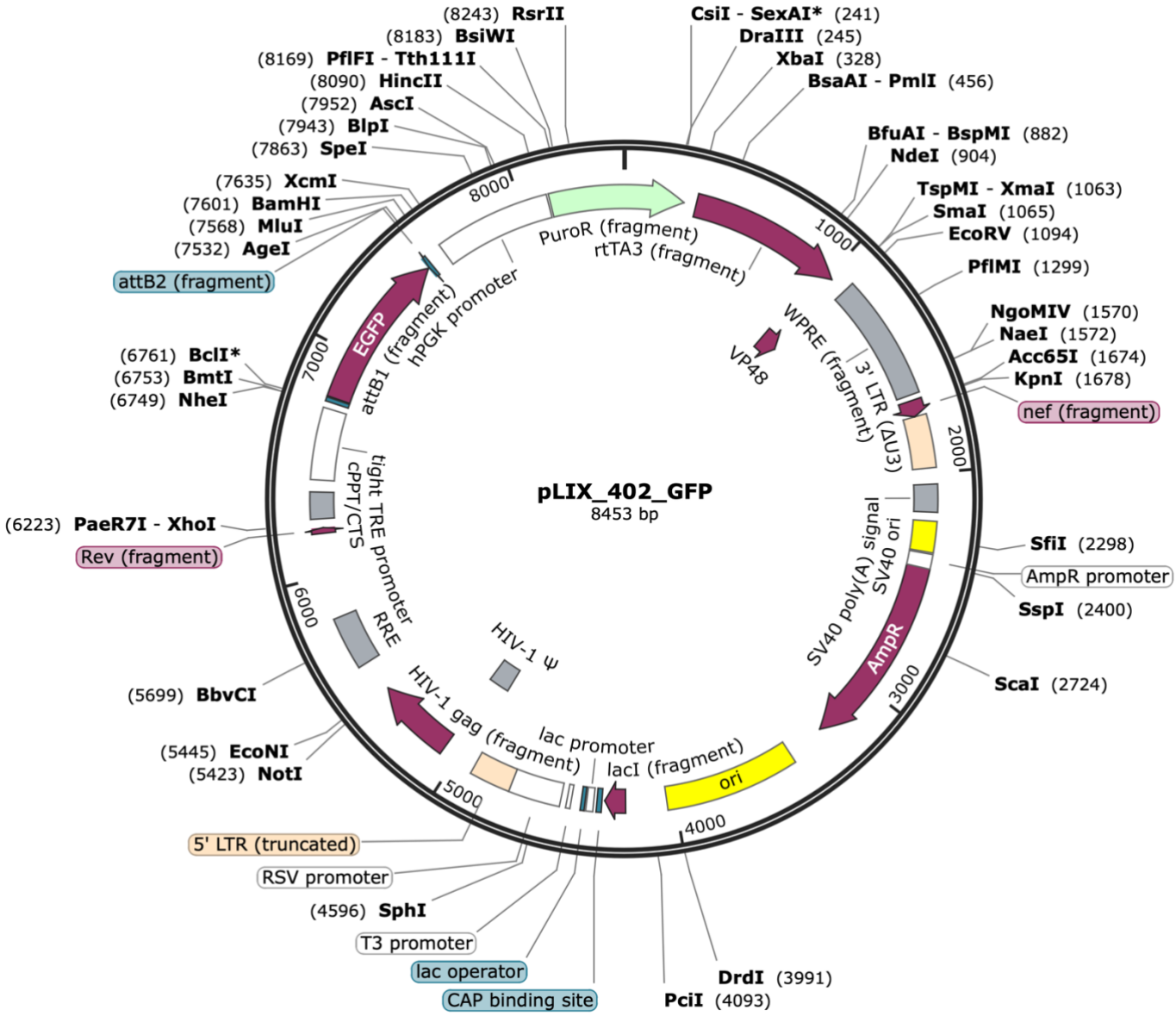

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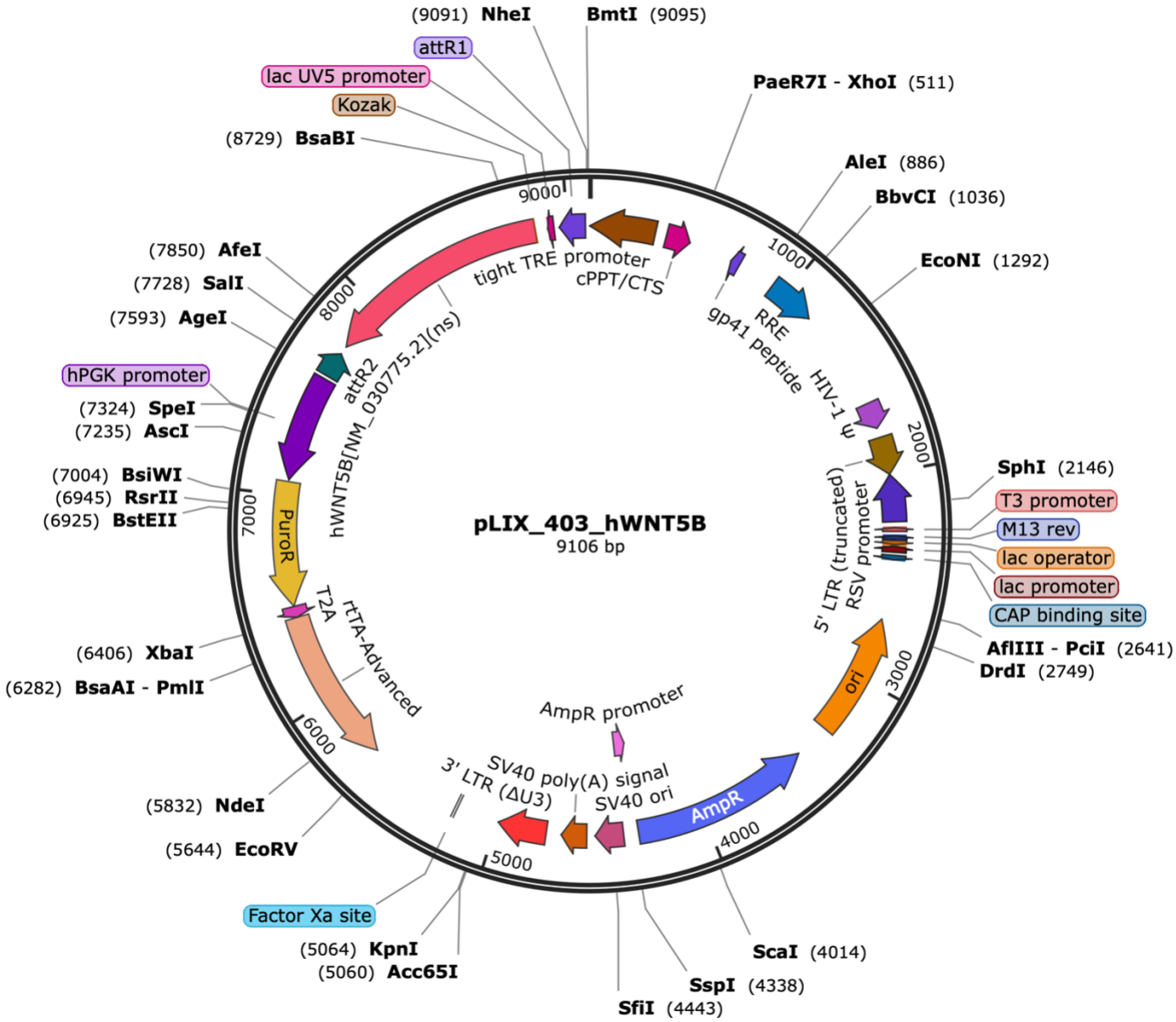
Created with SnapGene®
pLIX_403_hWNT5B
9106 bp
(9091) NheI
BmtI (9095)
attR1
lac UV5 promoter
Kozak
(8729) BsaBI
PaeR7I - XhoI (511)
AleI (886)
BbvCI (1036)
EcoNI (1292)
(7850) AfeI
(7728) SalI
(7593) AgeI
hPGK promoter
(7324) SpeI
(7235) AscI
(7004) BsiWI
(6945) RsrII
(6925) BstEII
(6406) XbaI
(6282) BsaAI - PmlI
(5832) NdeI
(5644) EcoRV
Factor Xa site
(5064) KpnI
(5060) Acc65I
SfiI (4443)
SspI (4338)
ScaI (4014)
SphI (2146)
T3 promoter
M13 rev
lac operator
lac promoter
CAP binding site
AflIII - PciI (2641)
DrdI (2749)
9000
1000
2000
3000
4000
5000
6000
7000
8000
tight TRE promoter
cPPT/CTS
RRE
gp41 peptide
HIV-1 Ψ
5' LTR (truncated)
RSV promoter
ori
AmpR
AmpR promoter
SV40 poly(A) signal
SV40 ori
3' LTR (ΔU3)
rtTA-Advanced
T2A
PuroR
attR2
hWNT5B[NM_030775.2](ns)

siRNA sequences

siRNA target	Sequences
siCTRL	commercially available from IDT CAT#: 51-01-14-03
siTCF3-1	Sense: 5'-CGUUCUGCAUAGAAUUCAAACGAGA-3' Anti-sense: 5'-UCUCGUUUGAAUUCUAUGCAGAACGCA-3'
siTCF3-2	Sense: 5'-GCAGAGUAAGAUAGAAGACCACCTG-3' Anti-sense: 5'-CAGGUGGUCUUCUAUCUUACUCUGCAG-3'
siTCF7-1	Sense: 5' -GGAGAAGCUCUGUUUAUAAAAACAA-3' Anti-sense: 5'-UUGUUUUUAUAAACAGAGCUUCUCCAU-3'
siTCF7-2	Sense: 5'-GAAAAAGAAAUGCAUUCGGUACUTA-3' Anti-sense: 5'-UAAGUACCGAAUGCAUUUCUUUUUCCU-3'

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