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14. ABSTRACT

Even though 80% of ovarian cancer patients will achieve a complete remission with a combination of surgery and chemotherapy, almost all will recur due to the development of chemoresistance. The WNT/beta-catenin pathway is involved in ovarian cancer growth and suppressing the ability of the immune system to fight off the cancer. Many ovarian cancers have a deficiency in the ability to repair its own DNA, called DNA repair deficiency. Recent clinical efforts have focused on using immune-directed therapies for the treatment of cancer, and specifically ovarian cancer. Although only a small subset of ovarian cancer patients respond to immunotherapy. Our goal to gain a better understanding of how DNA repair deficiency and upregulation of the WNT/beta-catenin pathway effect immune response and patient outcomes in ovarian cancer. Our preliminary data show that using a WNT inhibitor for the treatment of ovarian cancer in a mouse model improves the immune system's ability to fight off the cancer. Our central hypothesis is that inhibition of the WNT/beta-catenin signaling pathway will promote antitumor immune response and repress tumor growth, thereby improving clinical response. WNT/beta-catenin genes regulate cell proliferation, thereby mediating cancer initiation and progression and we have successfully targeted this pathway in cells isolated from patients with ovarian cancer and shown that a WNT inhibitor downregulates the WNT pathway and, in a subset of patient samples, caused cell kill. This project will examine the inhibition of the WNT/beta-catenin pathway using a mouse model of spontaneously developing ovarian cancer. In addition, we will also implant ovarian cancer cells with and without DNA repair deficiency into mice that have an intact immune system. The specific aims of this project are: 1) To determine the relationship between WNT/beta-catenin signaling, the DNA repair pathway, T cell responses and clinical outcomes in ovarian cancer. 2) To determine how inactivation of the WNT pathway by the tumor and dendritic immune cells impacts T cell responses and tumor growth using mouse models. 3) To determine whether mutations that affect DNA repair impact T cell responses following treatment with WNT inhibitors. The proposed research is significant because it will investigate the role of WNT-mediated T cell exclusion from the tumor and elucidate the therapeutic potential of novel WNT inhibitors against ovarian cancer cells with and without a mutation that causes deficiency in the DNA damage repair mechanism.

15. SUBJECT TERMS

None listed

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1. **INTRODUCTION:** Ovarian cancer is the fifth leading cause of death in women in the USA and the most lethal gynecologic malignancy. Given the poor overall survival, high recurrence rates, and rapid development of resistance to chemotherapy in HGSOC, we urgently require new methods to treat this disease. In addition to classic prognostic factors like stage and debulking status, mutations in *BRCA1* and *BRCA2*, components of the homologous recombination (HR) DNA repair pathway, are linked to long-term prognosis in HGSOC. *BRCA1/2* mutations are found in ~20% of HGSOC tumors and are associated with improved prognosis. Interestingly, most BRCA-deficient tumors have activated tumor-infiltrating lymphocytes (TILs), which are also linked to improved outcomes. In contrast, immunosuppressive T regulatory cells (Tregs) and tumor-associated macrophages (TAMs), correlate with a worse prognosis. Despite the link between TILs and positive clinical outcomes, the use of immune checkpoint inhibitors (ICIs) in HGSOC has been disappointing in part due to the "cold" immune landscape surrounding these tumors. A "cold" immune landscape is characterized by the lack of TILs, which sensitize tumors to ICI. Thus, in order to develop better treatments for HGSOC, we need to understand the mechanisms that regulate anti-tumor immune responses, including the role of BRCA1/2 and the HR DNA repair pathway. WNT/β-catenin signaling is also linked to the progression of HGSOC by directly triggering tumor growth (1, 23), and promoting resistance to platinum agents. Interestingly, melanoma-intrinsic activation of the WNT pathway leads to the exclusion of TILs and immune escape by repressing local chemokine expression. In addition, WNT/β-catenin signaling in DCs triggers the expression of immunosuppressive molecules like IL- 10, TGFβ and RALDH, which in turn promote the differentiation of Tregs. Given that infiltrating Tregs and TAMs correlate with a worse prognosis in HGSOC, and that these cells are associated with WNT/β-catenin signaling in other tumors, it is likely that WNT signaling in ovarian cancer also leads to immune suppression. In fact, expression data from 8890 tumor samples (including HGSOC) in The Cancer Genome Atlas (TCGA), show that activating mutations in the WNT/β-catenin pathway, such as *CTNNB1*, encoding β-catenin, and inactivating mutations in negative regulators, such as Axin1, Axin2, APC1, and APC2, are inversely related to inflammatory T cell gene expression signatures. Thus, the purpose of our research is to investigate the inhibition of WNT/β-catenin signaling and its effects on tumor growth and anti-tumor immune responses in ovarian cancer patients with an intact or altered HR DNA repair pathway.

2. **KEYWORDS:** Ovarian cancer, murine ovarian cancer, WNT, β-catenin, WNT inhibition, tumor microenvironment, ID8 cells, ID8p53$^{-/-}$ cells, PORCN, WNT-974, CGX-132, DKK1, DKN-01

3. **ACCOMPLISHMENTS:**

   - **What were the major goals of the project?**

	Timeline (Months)	% of Completion
Specific Aim 1		
Major Task 1: Obtain RNA sequencing results from 917 patients from GOG 218	2	
Major Task 2: Categorize the patients into T cell inflammation subtype, WNT pathway score, HRD status, correlate with survival stratified by treatment	5	
Milestone(s) Proposed: (1) RNA sequencing data downloaded for 917 patients with HGSOC (2) The relationship between WNT signaling, DNA repair pathway, T cell inflammation subtype and clinical outcomes in HGSC		
Specific Aim 2		
Major Task 1: Determine whether inhibition or activation of the WNT pathway impacts T cell response	9	

Major Task 2: Create a CRISPR/Cas9-mediated β-catenin knockout model and access T cell response	12	
Milestone(s) Proposed: (1) Identify the T cell response and tumor response in mice treated with a T cell inhibitor and T cell activator (2) Validate the role of β-catenin in T cell and dendritic cell response		
Specific Aim 3		
Major Task 1: Evaluate the number and types of T cells that are present after treating BRCA2 mutated and wild-type cells	32	
Milestone(s) Proposed: Determine the difference between immune response to WNT inhibition and activation in cells with and without HR DNA repair deficiency		

- **What was accomplished under these goals?**

Specific Aim 1

Major Task 1: Obtain RNA sequencing results from 917 patients from GOG 218

Subtask 1: Identify location of data

We were not able to contain RNA sequencing results from the 917 patients from GOG 218 trial. However, we were able to obtain data from an IRB-approved, research project conducted at the University of Alabama at Birmingham (UAB) titled, "Identifying the Effect of Neoadjuvant Chemotherapy on the Enrichment of Chemoresistant Ovarian Cancer Stem Cells in Patients with High Grade Serous Cancer (HGSC): A Pilot Study".

Subtask 2: Download data in a format that can be transferred to Hudson Alpha Institute for analysis

Dr. Sara Cooper at Hudson Alpha helped us analyze RNA sequencing data from the pilot study mentioned above.

Goals not met: We were not able to secure data from GOG 218 trial.

Major Task 2: Categorize the patient data into T cell inflammation subtype, WNT pathway score, HRD status, correlate with survival stratified by treatment

Subtask 1: Create categories based on the ovarian TCGA analysis of "hot" and "cold" tumors

/////When analyzed ovarian cancer samples from the TCGA dataset, we found an inverse correlation between a 13-gene T cell signature and Wnt pathway activating mutations (LRP5 LPR6, FRZ (a family of receptors), DVL, GSK3B, APC, APC2, AXIN (1 and 2), CTNNB1, LEF1, TCF7) (Pearson r= −0.358, p<0.001) (**Figure A**). In addition, we found a negative correlation between Wnt pathway activity and T cell signatures in 57 treatment naïve human high grade serous ovarian cancer (HGSOC) tissue from UAB. Using RNA-seq data to calculate relative Wnt activity and T cell scores (**Figure B**). ***Conclusion:*** **Wnt activating mutations correlated with a decreased "hot" T cell signature.** /

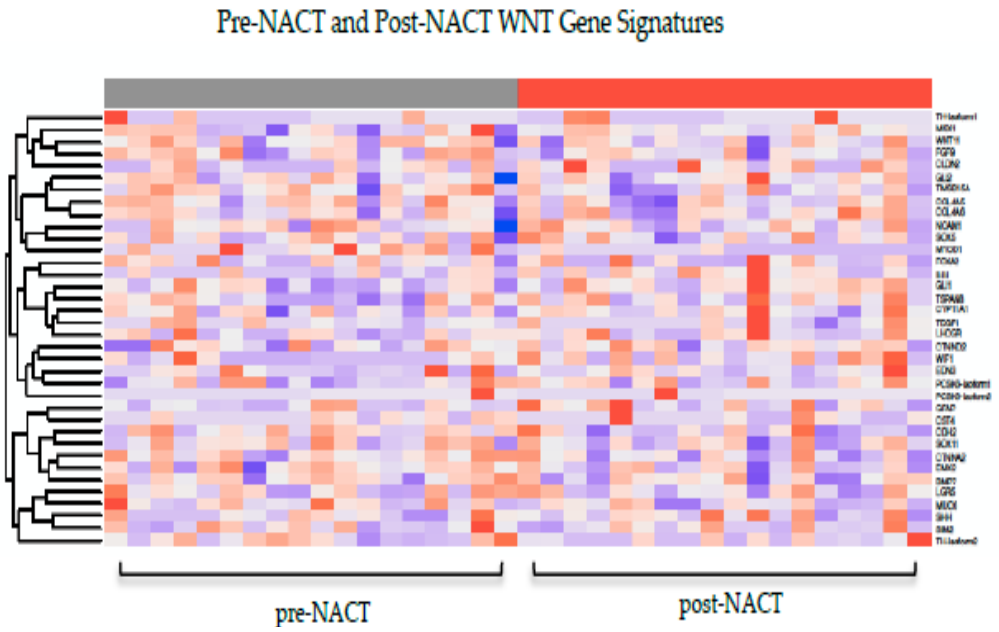
Subtask 2: Analyze the previously collected data (BROCA-HR assay) as it relates to the "hot" and "cold" signature

Subtask 3: Analyze the RNA sequencing data pertinent to HRD status

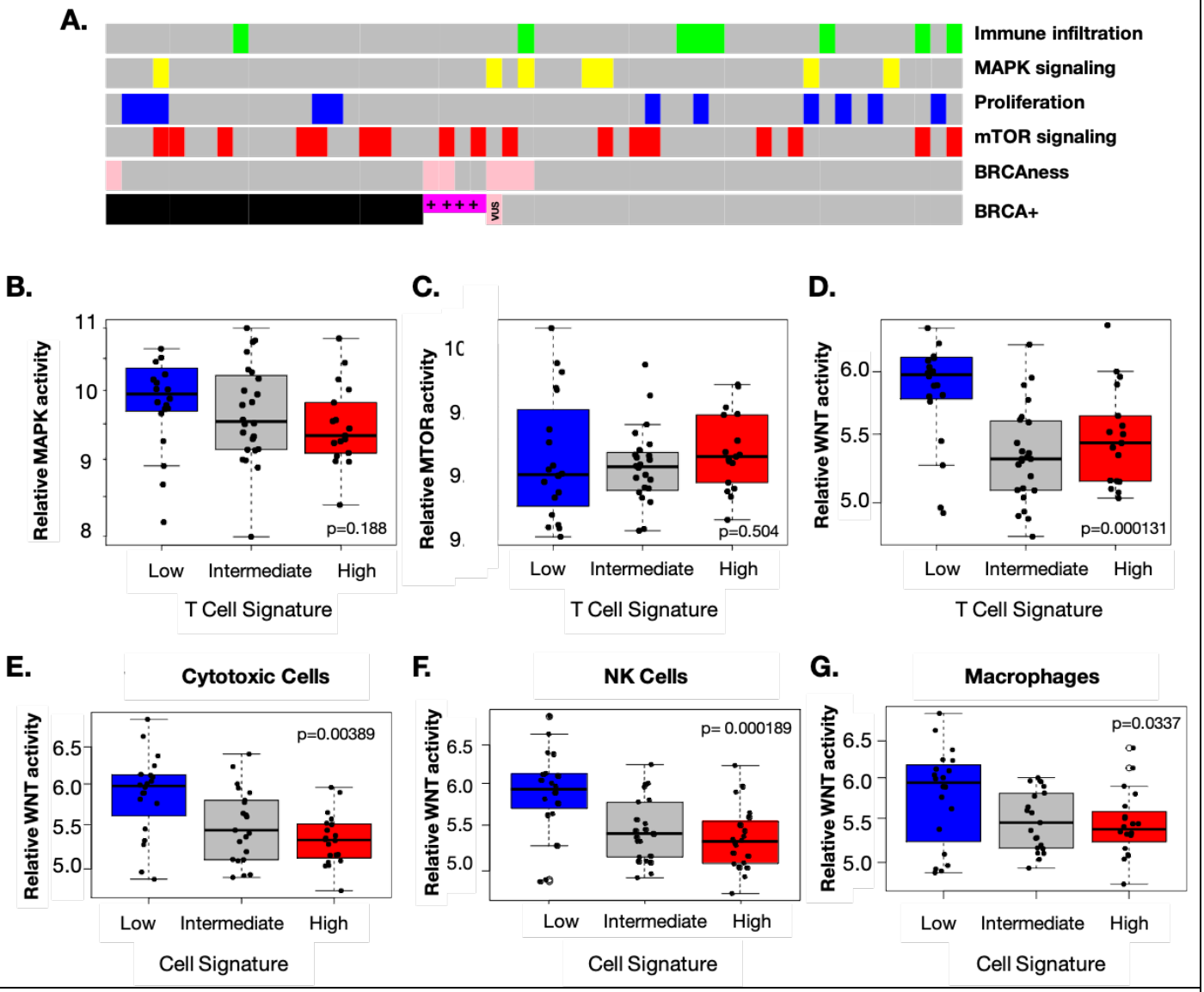
The HGSOC patient samples we were able to analyze from the pre- and post-NACT timepoint were also screened for HR status. Unfortunately, many of the patients had unknown HR status, potentially due to the lack of HR testing at the time of tissue collection. However, of the patients who had known status (n=11), only 25% of patients whose WNT signaling increased in response to NACT were BRCA(+) = HRd, and 34% of patients with high WNT signaling post-NACT were BRCA+.

Subtask 4: Categorize patient data based on WNT pathway gene expression

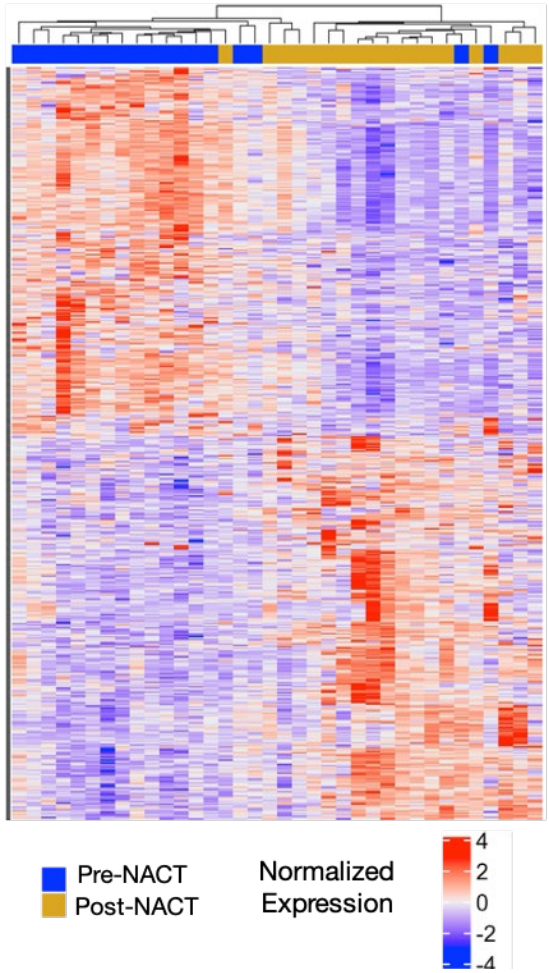
A subset (n = 17) of 57 treatment naïve high grade ovarian cancer samples had matched samples after receiving neo-adjuvant chemotherapy (NACT). Changes in Wnt activity were calculated for these samples and displayed in heatmap below. ***Conclusion: Differential Wnt-related gene expression occurred in responses to NACT.***



Transcriptional signatures were used to identify tumor tissues with evidence of alterations in **(A)** immune infiltration, MAPK signaling, increased proliferation, mTOR signaling, BRCAness, and/or BRCA+. Activity of **(B)** MAPK, **(C)** mTOR, and **(D)** WNT pathways was compared to T cell Infiltration Signature. A more detailed analysis of specific immune cell types estimated from transcriptional profiles using CIBERSORT was performed to calculate association of WNT signaling activity is negatively associated with infiltration of **(E)** cytotoxic cells, **(F)** natural killer **(NK)** cells, and **(G)** macrophages. ***Conclusion: WNT signaling was the only pathway that inversely correlated with T cell infiltration signature, and it also inversely correlated with cytotoxic, NK, and macrophage infiltration.***

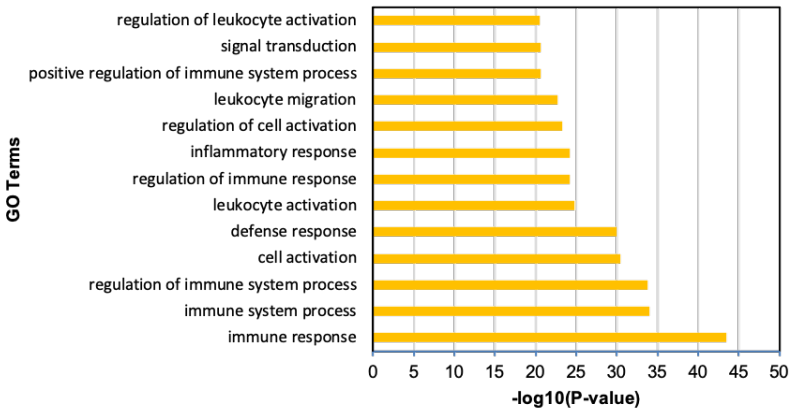


For 18 patients from which we collected treatment naïve tissue (pre-NACT) and transcriptomic data, we also analyzed tissue collected at the time of resection after 3-6 cycles of NACT (post-NACT). Heatmap of transcriptomic profiles from top differentially expressed genes (DEGs) (FDR<0.05) associated with NACT. ***Conclusion: NACT causes significant alterations in gene expression profiles in HGSOC.***

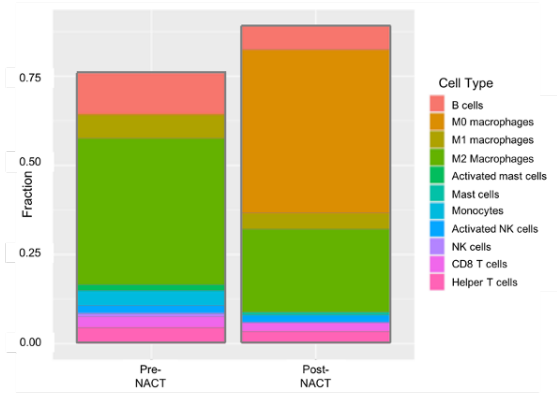


DEGs from pre- and post-NACT tumor samples were used for (**A**) pathway analysis. (**B**) Immune cell types/fractions were estimated from bulk RNA-seq data. ***Conclusions: NACT enriched genes associated with immune response and activation pathways. M0 (inactive) macrophage population was the only immune cell population that increased in response to NACT in our 18 matched pre- and post-NACT patient samples.***

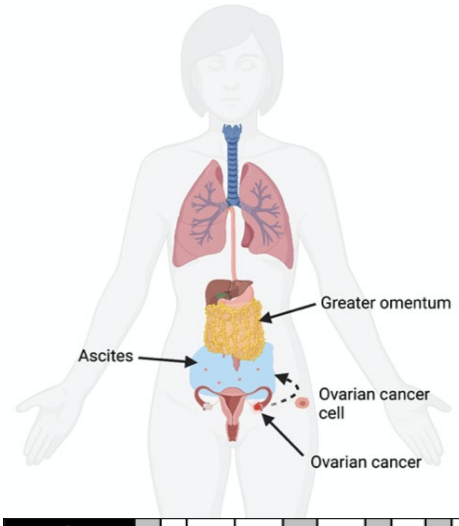
A.



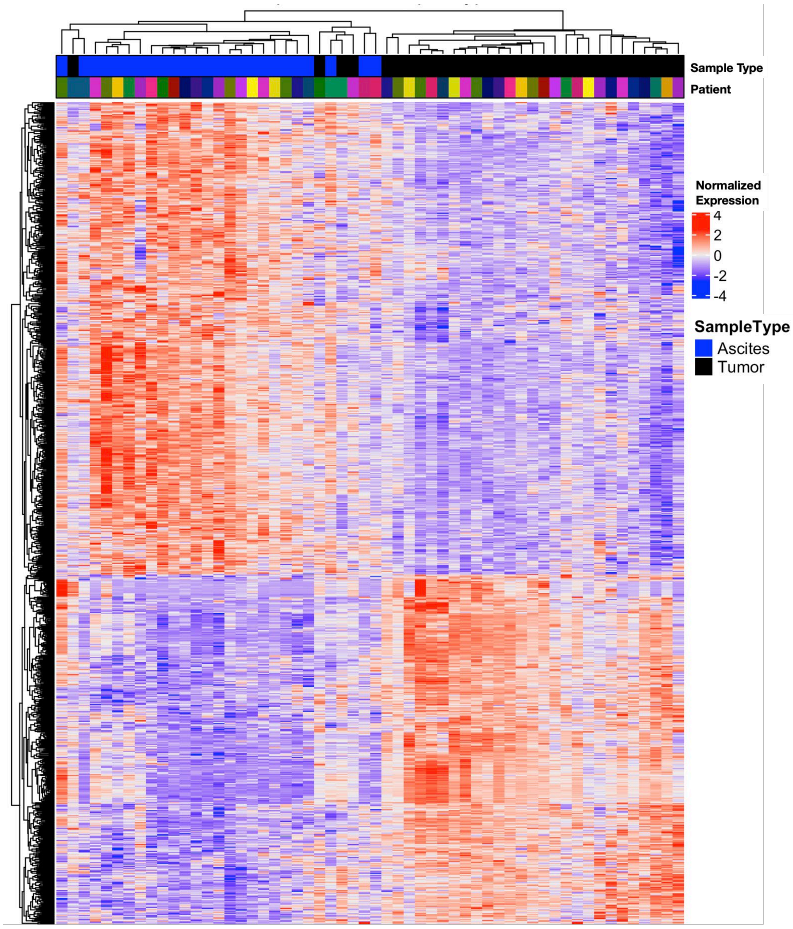
B.



Comparison of isolated ascites and primary tumors….



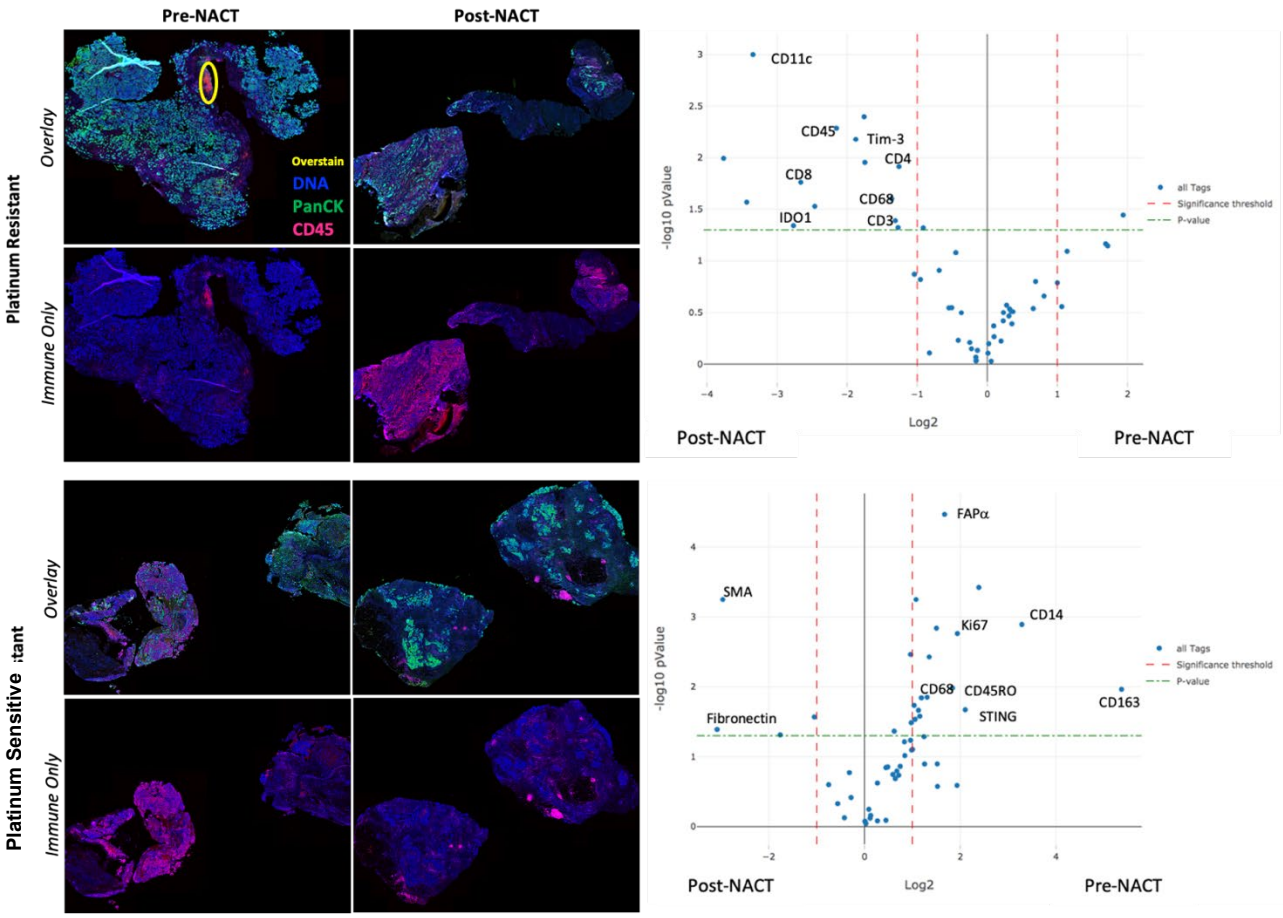
Sample ID	1	2	3	4	5	6	7	8	9	11	12	13	14	15	16	17	18	20	21	27	31	33	34	35	36	37	38	39	74	77	82	83
Tumor	P	I	AM	I	M	AM	I	M	I	AM	P	I	I	M	I	P	AM	I	I	P	M	I	AM	M	I	I	I	AM	M	M	AM	P
Ascites	P	P	M	AM	M	P	I	D	I	D	P	I	I	M	M	I	AM	D	D	M	M	P	AM	P	AM	M	M	D	I	P	AM	AM



Since we have yet to obtain data from the GOG 218 trial, we decided to move forward with an analysis of matched tumor samples from individual patients with high-grade serous ovarian cancer (HGSOC) pre- and post-neoadjuvant chemotherapy (pre- and post-NACT, respectively) (18-Study). We were able to obtain unstained slides from formalin-fixed paraffin-embedded (FFPE) blocks from each timepoint from 18 patients and sent them to NanoString for GeoMx Digital Spatial Profiling (DSP) Analysis using their Human Immuno-Oncology Protein Panel. This panel allowed for high-plex and high-throughput spatial profiling that enabled us to rapidly and quantitatively assess over 70 proteins, highlighted in table below, of interest simultaneously.

Immune Cell Profiling Panel		IO Drug Target Panel	Immune Activation Status Panel	Immune Cell Typing Panel	Pan-Tumor Panel	Cell Death Panel	PI3K/AKT Signaling Panel
CD3	CTLA-4	4-1BB CD137	CD25	CD14	Bcl-2	BAD	p-AKT (S473)
CD4	Fibronectin	ARG1	CD27	CD34	EpCAM	BCL6	p-GSK (S9)
CD8	GZMB	B7-H3	CD40	CD45RO	ER alpha	BCLXL	p-GSK3ab (S21/S9)
CD11c	HLA-DR	GITR	CD44	CD66b	HER2	BIM	INPP4B
CD20	Ki-67	IDO1	CD80	CD163	MART1	CD95/Fas	MET
CD45	Pan-cytokeratin	LAG3	CD127	FAPalpha	NY-ESO-1	Cleaved caspase 9	Pan-AKT
CD56	PD-1	OX40L	ICOS	FOXP3	PR	GZMA	PLCG1
CD68	PD-L1	STING	PD-L2		OTEN	NF1	p-PRAS40 (T246)
	SMA	Tim-3			S100B	p53	p-Tuberin (T1462)
		VISTA				PARP	

DSP allows for 6 regions of interest (ROIs) to be further segmented into tumor (PanCK+CD45-) and immune (PanCK-CD45+) areas resulting in 6 tumor and 6 immune areas with 70 protein readouts per area. The innovation behind DSP is the ability to quantitate tumor vs. immune markers of interest, capture heterogeneity across patient tissue, and visualize the spatial distribution of tumor and immune cells. The immunosuppressive proteins of interest included (CTLA-4, PD-1, PD-L1, ARG1, Lag3, Tim-3, CD25, CD163 and FOXP3). Furthermore, we were assisted by NanoString's TAP Team lead by trained scientists and research staff to perform and aid in DSP data analysis. Due to the expensive nature of this analysis we were only able to send in 2 patients matched pre- and post-NACT tissue; one patients was deemed platinum sensitive (PS) with low WNT gene expression signature pre-NACT, and 1 patient platinum resistant (PR) with high WNT gene expression signature pre-NACT. ***Conclusion: PS patient had low WNT activity with high expression of interferon (STING), immune memory (CD45RO,) and proliferation (Ki67) markers in pre-NACT. The PR patient had high WNT activity in pre-NACT tissue, and elevated immune exhaustion (Tim-3 and IDO1) in their post-NACT tissues. The PS patient did not have an increase in these markers in response to NACT. However, the PS patient did have increased.***


Pre-NACT
Post-NACT
Platinum Resistant
Overlay
Immune Only
Overstain
DNA
PanCK
CD45
CD11c
CD45
Tim-3
CD4
CD8
CD68
IDO1
CD3
-log10 pValue
Log2
all Tags
Significance threshold
P-value
Post-NACT
Pre-NACT
Platinum Sensitive
Overlay
Immune Only
FAPα
SMA
CD14
Ki67
CD68
CD45RO
CD163
Fibronectin
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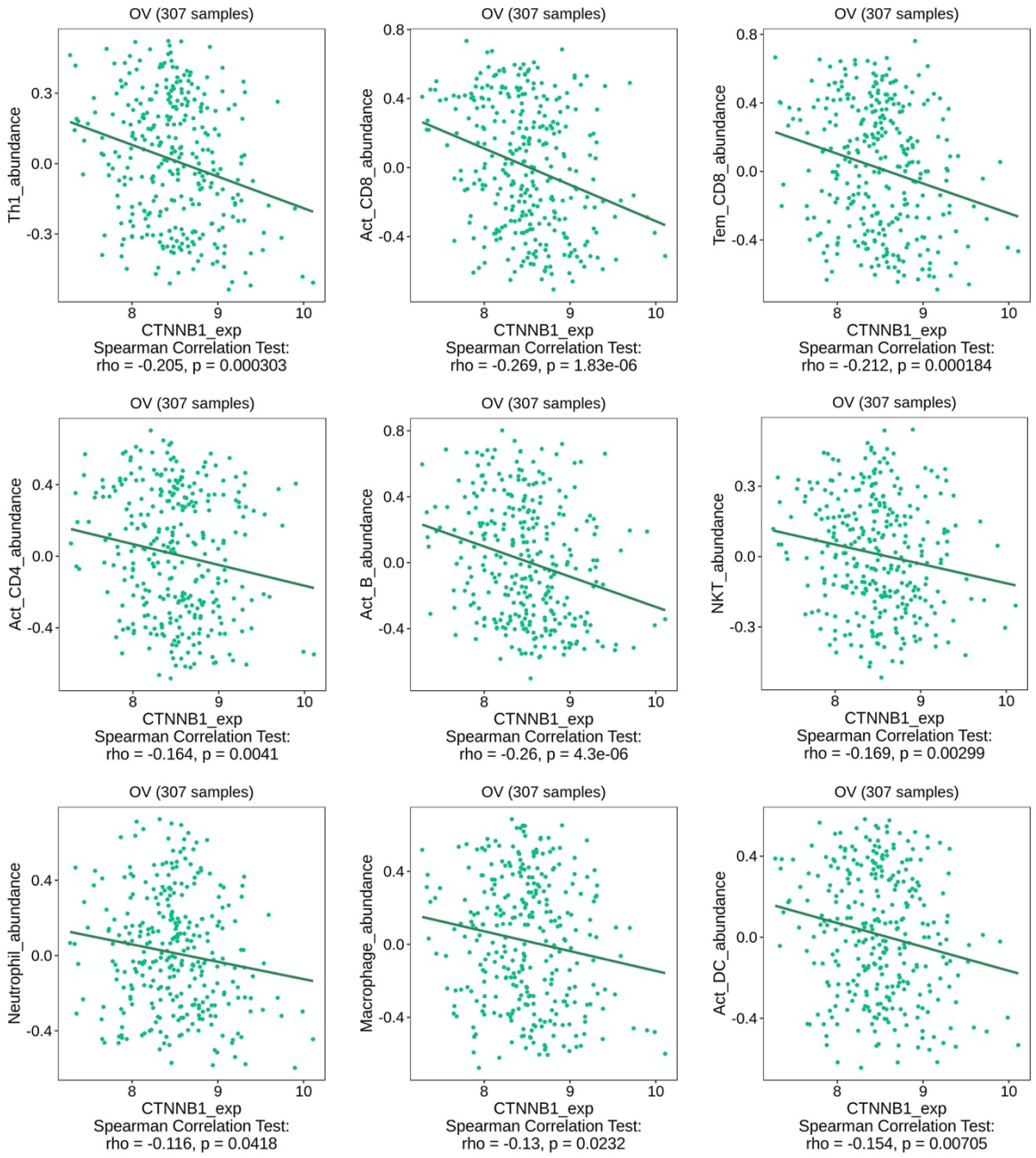
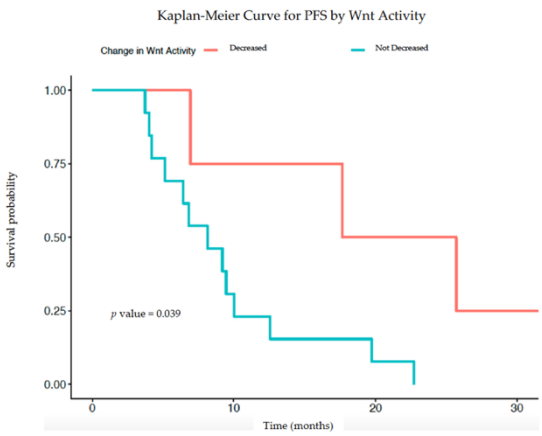
Log2
all Tags
Significance threshold
P-value
Post-NACT
Pre-NACT

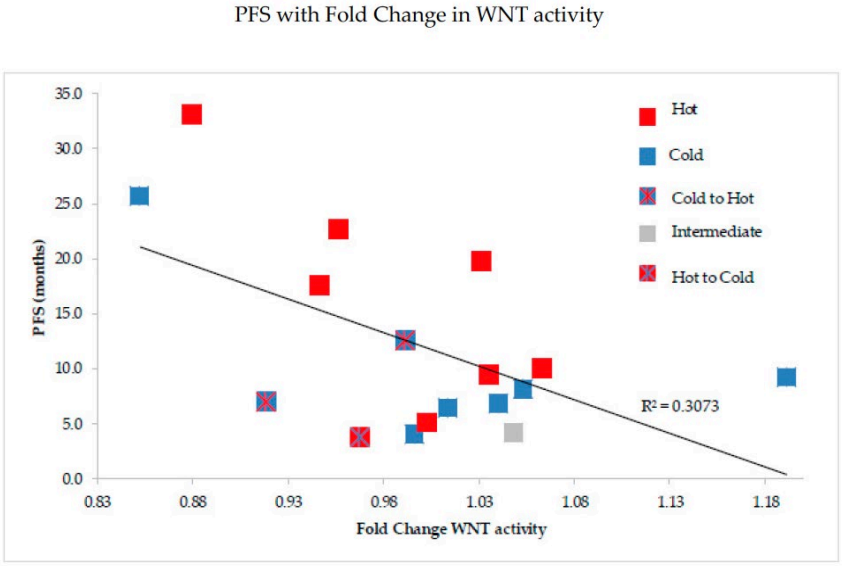


Subtask 5: Combine the data collected in subtask 1-4 and correlate to patient outcomes and treatment

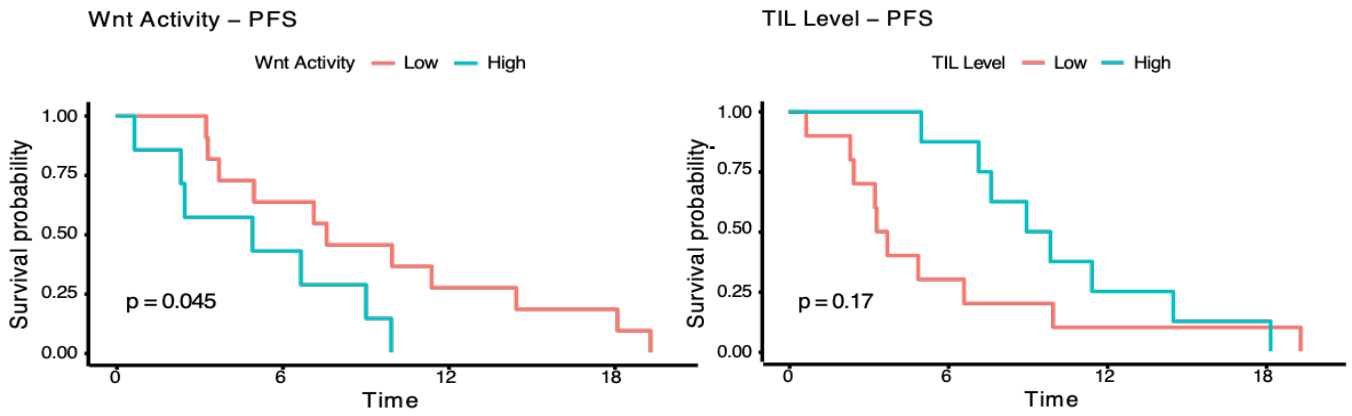
Kaplan-Meier curve for progression-free survival (PFS) (n=17) based on decreases in Wnt activity after neoadjuvant chemotherapy (NACT). ***Conclusion: High Wnt signaling activity corresponds to decreased ovarian cancer patient PFS.***



Patients are labeled based on T cell signature change, with solid color indicating no change, blue square with red lines indicating cold-to-hot signature change, and red square with bluelines indicating a hot-to-cold signature change. ***Conclusion: PFS shows a negative correlation with fold-change in Wnt activity measured by signature genes in matched post- versus pre-NACT in 17 HGSOC patients.***

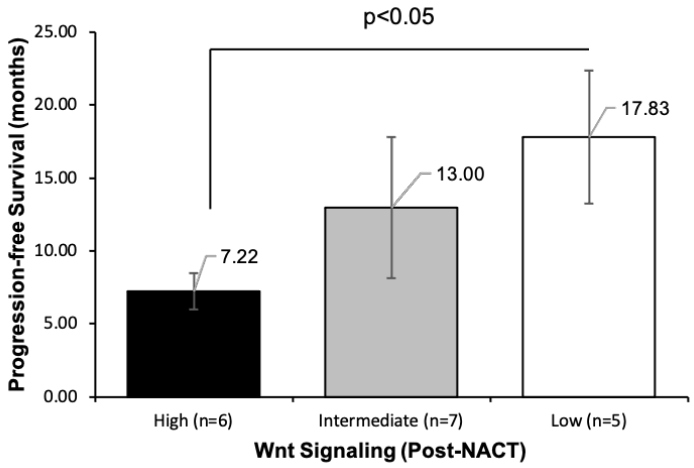


In our pilot study we evaluated WNT activity and tumor-infiltrating lymphocyte (TIL) level via gene expression signatures and correlated to patient survival. ***Conclusion: High WNT activity and low TIL levels corresponded to reduced progression-free survival (PRF) in HGSOC patients.***

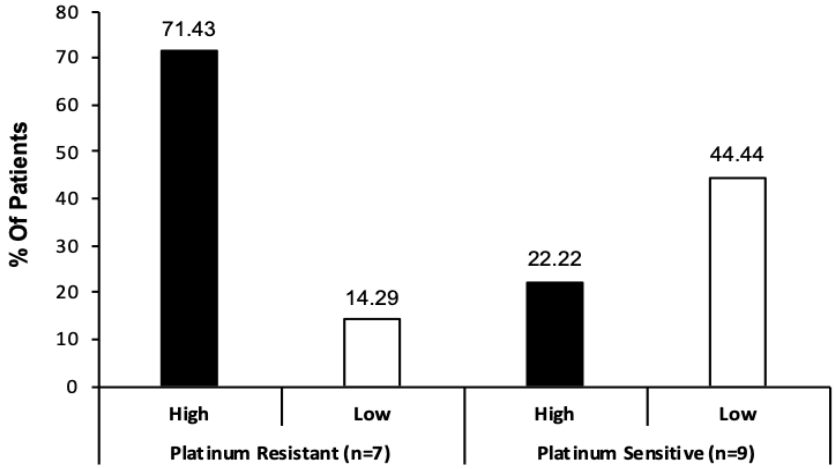


In our samples from 18-Study, we calculate WNT activity score (high, intermediate, or low) from gene expression profiles. We calculated a score that represents WNT and immune signatures from gene expression data. First, we used the variant StabilizingTransformation function in the DESeq2

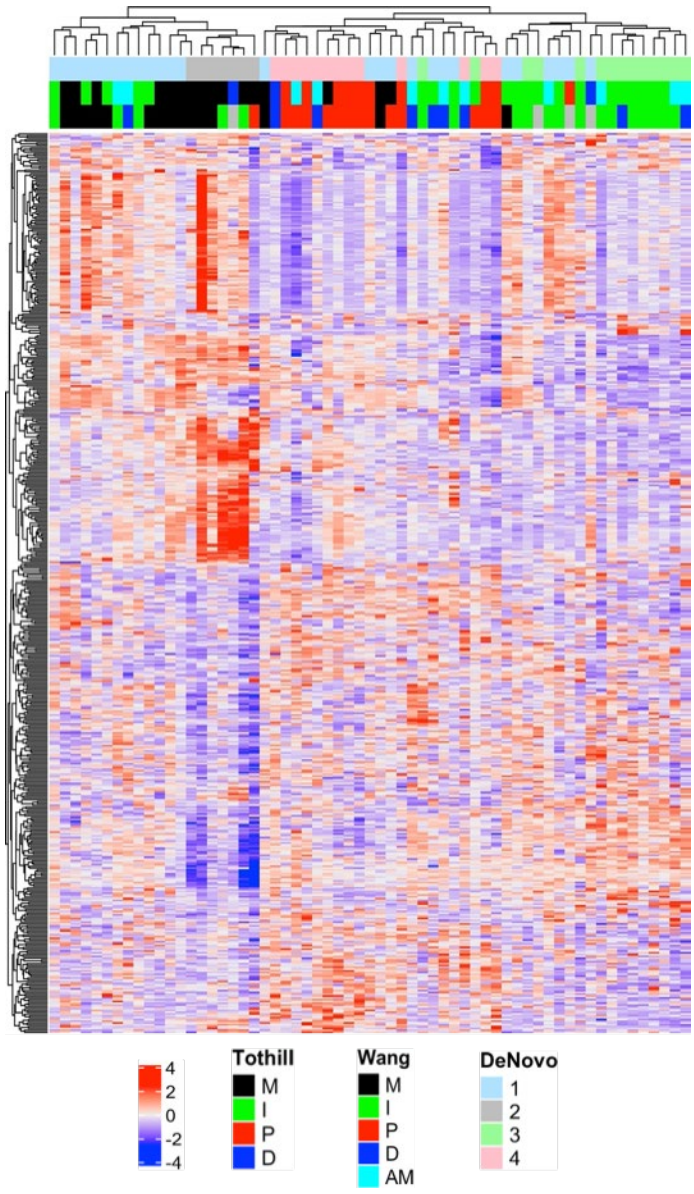
package to normalize all data. Next, we calculated a score for WNT signaling by determining the median normalized gene expression levels of all genes associated with WNT signaling function. ***Conclusion: HGSOC patients who had increased WNT signaling in response to NACT had decreased PFS compared to patients with decreased WNT signaling.***

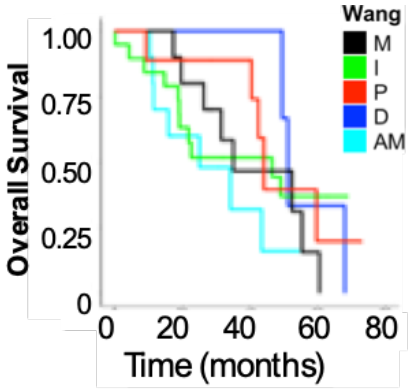


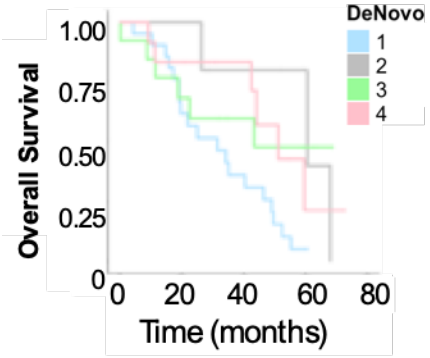
In our samples from the pilot study with classified as WNT high or low based on gene expression signatures in pre-NACT tissues. ***Conclusion: More PR HGSOC patients had high WNT signaling at baseline compared to PS patients (71 vs. 22 %).***



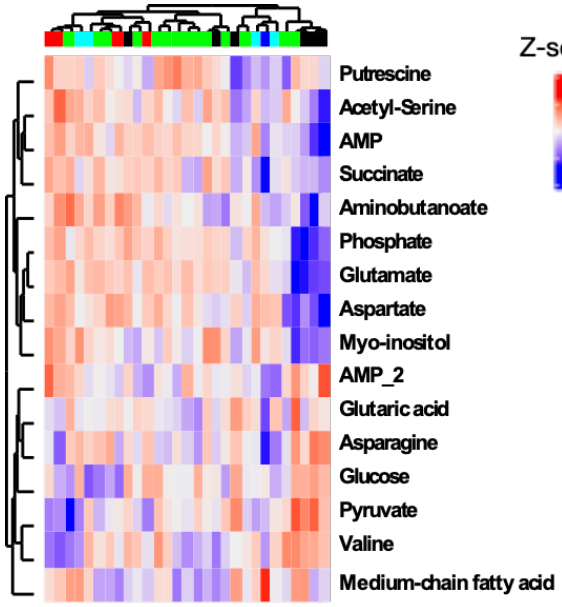
Metabolomics…

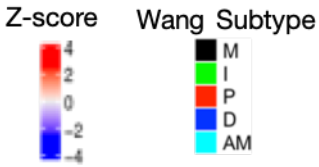


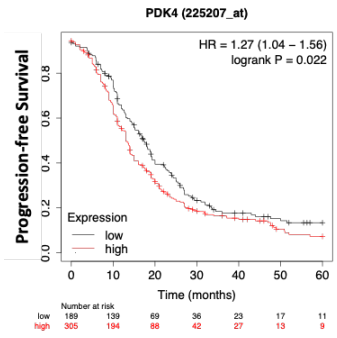


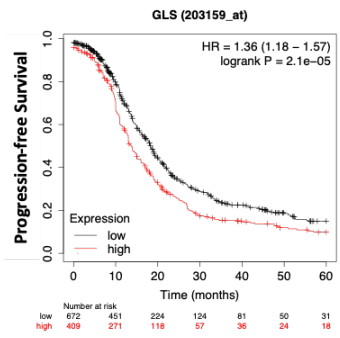


Metabolomics and survival…









Goals not met: We were not able to secure data from GOG 218 trial.

Specific Aim 2

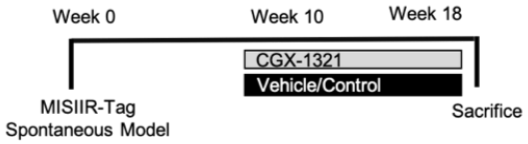
Major Task 1: Determine whether inhibition or activation of the WNT pathway impacts T cell response

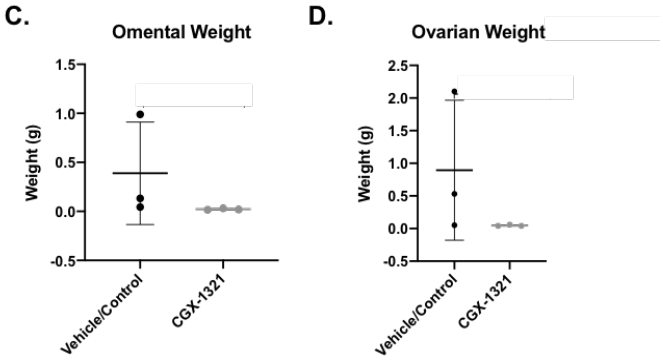
Subtask 1: Breed enough female *TgMISIIR-Tag-Low* mice (n=40)

Subtask 2: Inject mice with MOVCAR-luc cells

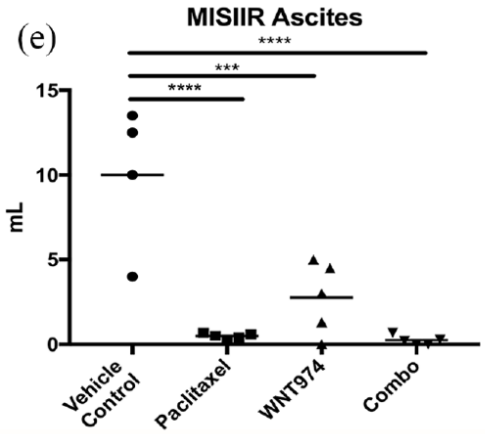
Subtask 3: Treat mice with vehicle control, WNT974, WNT7A (n=10 in each group) with one untreated group. (DKN-01 used as surrogate for WNT7a)

Previously, our group investigated the effects of PORCN inhibitor, CGX-1321, on tumor growth in ID8 parental and ID8p53$^{-/-}$ models. To make our current study more translatable, we used the MISIIR-Tag model. Treatment with CGX-1321 ablated the progression of tumors, as no increases were seen in omentum or ovarian weights compared to vehicle/control-treated mice. ***Conclusion: Inhibition of Wnt signaling via CGX-1321 significantly decreased MISIIR-Tag tumor burden via omentum weights and ascites volumes.***





MISIIR-Tag-Low mice injected IP with MISIIR ovarian cancer (MOVCAR) cells and treated with 14 days of paclitaxel, WNT974, or combination all have fewer ascites than vehicle control (n = 5 mice/group). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ***Conclusion: The addition of paclitaxel to WNT-974 further reduced ascites formation, but no better than paclitaxel treatment alone.***



Subtask 4: Sac 5 mice at day 14 and the remainder on day 28 and quantify tumor-specific CD8+ T cells, test whether the T cells are activated, and send whole tumor for RNA seq

Subtask 5: Dissociate fresh tumor tissue and sort-purify cells into CD3/4+ or CD3/8+ T cells

Subtask 6: Extract RNA from sorted cells and use TCR repertoire sequencing

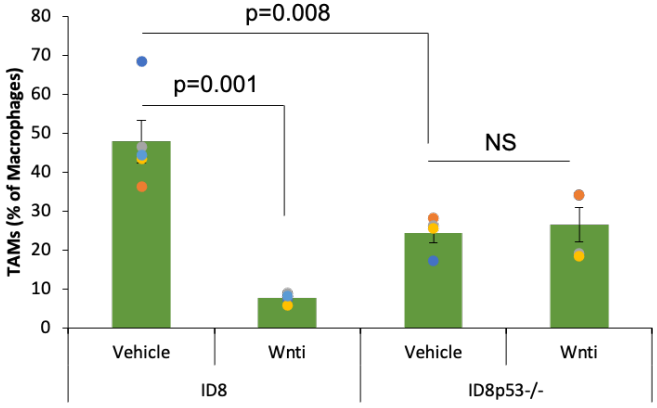
Goals not met: We have not been able to complete TCR repertoire sequencing for MISIIR model.

Major Task 2: Create a CRISPR/Cas9-mediated β-catenin knockout model and access T cell response

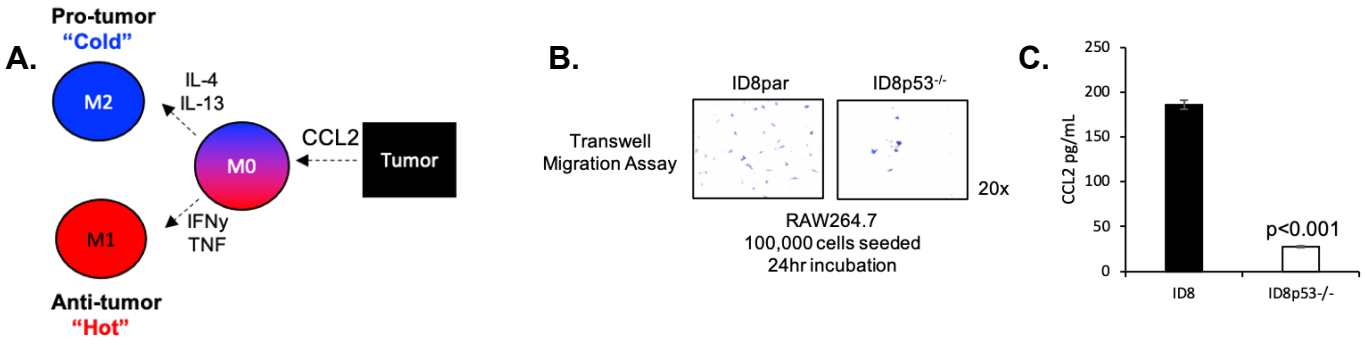
Subtask 1: Create MOVCAR knockout cell line

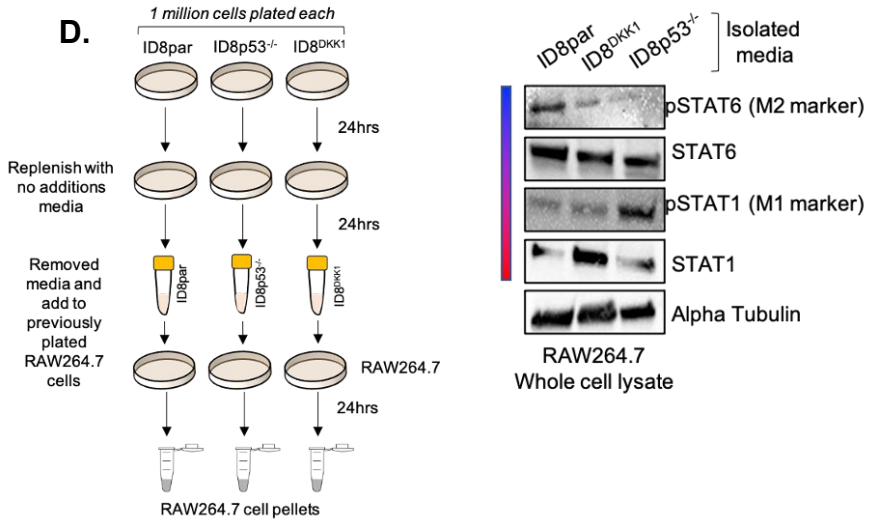
Subtask 2: Compare T cell infiltration, proportion of TAMs, proportion of Tregs, presence of CD103+ dendritic cells in the β-catenin knockout model compared to wild-type. (ID8p53-/- model was used as a surrogate for β-catenin knock-out due to its decreased β-catenin expression compared to ID8 model)

Flow cytometry (FACS) analysis of total tumor-associated macrophages (CD45+F4/80+CD206+) presence in the omenta of female, C57BL/6 mice injected with either ID8 or ID8p53$^{-/-}$ cells treated with vehicle or WNT inhibitor (WNTi). ***Conclusion: ID8 model had elevated TAM infiltration in omenta compared to ID8p53$^{-/-}$ model. In addition, WNT inhibition only resulted in decreased TAM infiltration in ID8 model compared to ID8p53$^{-/-}$ model.***



Macrophage (**A**) recruitment and polarization in the tumor microenvironment (TME). We performed (**B**) transwell migration assays to measure macrophage migration in response to isolated ID8 model media, as well as (**C**) ELISA assays to determine the level of CCL2, macrophage chemokine, in the isolated media from ID8 models. In addition, we performed (**D**) western blot analyses on macrophages that had been exposed to isolated media from ID8 models to determine protein expression of pSTAT6 (M2 macrophage) and pSTAT1 (M1 marker). ***Conclusion: ID8 cells with high WNT activity had enhanced CCL2 secretion and enhanced macrophage migration compared to ID8p53$^{-/-}$ cells with low WNT activity. In addition, isolated media from ID8 cells increased macrophage polarization towards an M2-like state compared to isolated media from ID8p53$^{-/-}$ cells shifting macrophages more towards an M1-like state.***





Goals not met: We recently got our *TgMISIIR-Tag-Low* colony back and will continue analyses proposed with this model. However, we have found that our ID8p53$^{-/-}$ mice have low beta-catenin compared to ID8 parental cells and will use this model to substitute for beta-catenin knockout model. Furthermore, we need to further profile macrophage subtypes in response to Wnt signaling alteration in our models.

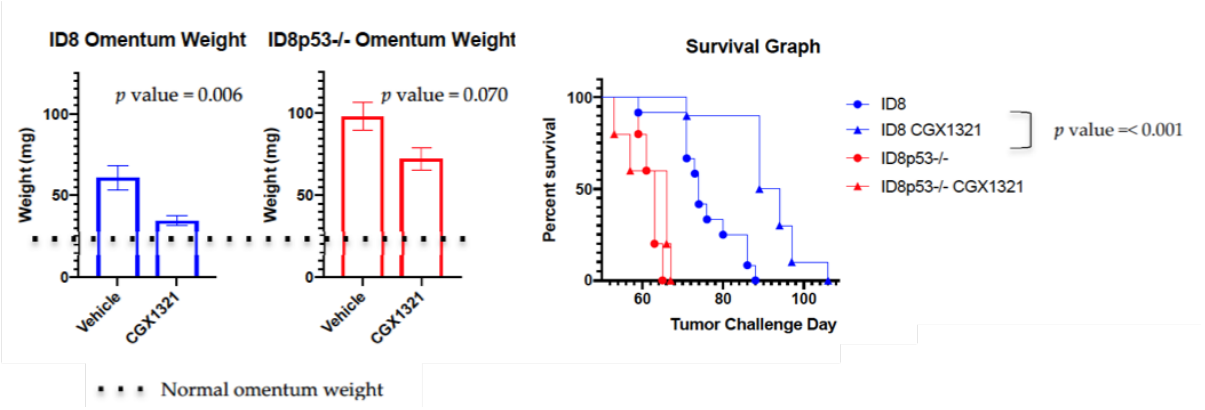
Specific Aim 3

Major Task 1: Evaluate the number and types of T cells that are present after treating BRCA2 mutated and wild-type cells

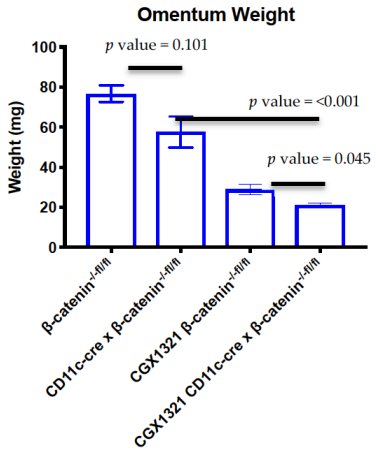
Subtask 1: Implant C57BL/6 mice with ID8 cells that have been modified using CRISPR/Cas9 to lack TRP53 or both TRP53 and BRCA2.

Survival was increased with ID8 intraperitoneal tumor challenge in C57Bl/6 mice compared with ID8p53-/- tumor challenge. On tumor challenge day 42, omentum weights between ID8 and ID8p53-/- tumor challenge was statistically different. ***Conclusion: ID8 and ID8p53-/- had differences of survival and tumor burden. //***

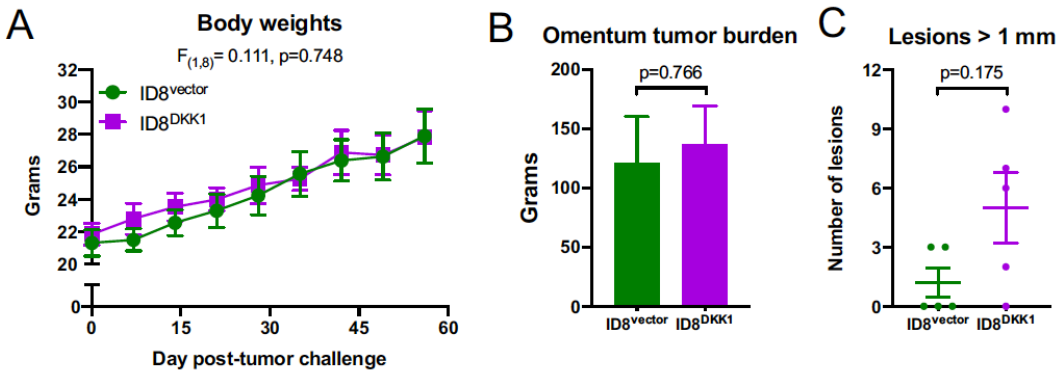
In vivo inhibition of Wnt signaling using an additional PORCN inhibitor, CGX-1321. ID8 or ID8p53$^{-/-}$ tumors were challenged in C57BL/6 mice for both cell lines, and treatment groups were divided into vehicle and CGX-1321. At day 42, omentum weight had significantly decreased with CGX-1321 treatment in ID8 model, but not with ID8p53$^{-/-}$ tumor challenge. *In vivo* inhibition of Wnt signaling using CGX-1321 increased survival in ID8 tumor challenge, but not tumor challenge with ID8p53$^{-/-}$. Western blot analysis revealed reduced beta-catenin levels in the ID8p53$^{-/-}$ cell line comparted to ID8 cell line. ***Conclusion: CGX-1321 exerts anti-tumor effects only on ID8 parental model.***



Omentum weight decreased with loss of beta-catenin in CD11c-cre x Beta-catenin$^{-/-fl/fl}$ mice, and treatment with CGX-1321 further increased this difference. ***Conclusion: Inhibition of beta-catenin signaling in dendritic cells decreases tumor burden, and CGX-1321 treatment enhances this effect.***

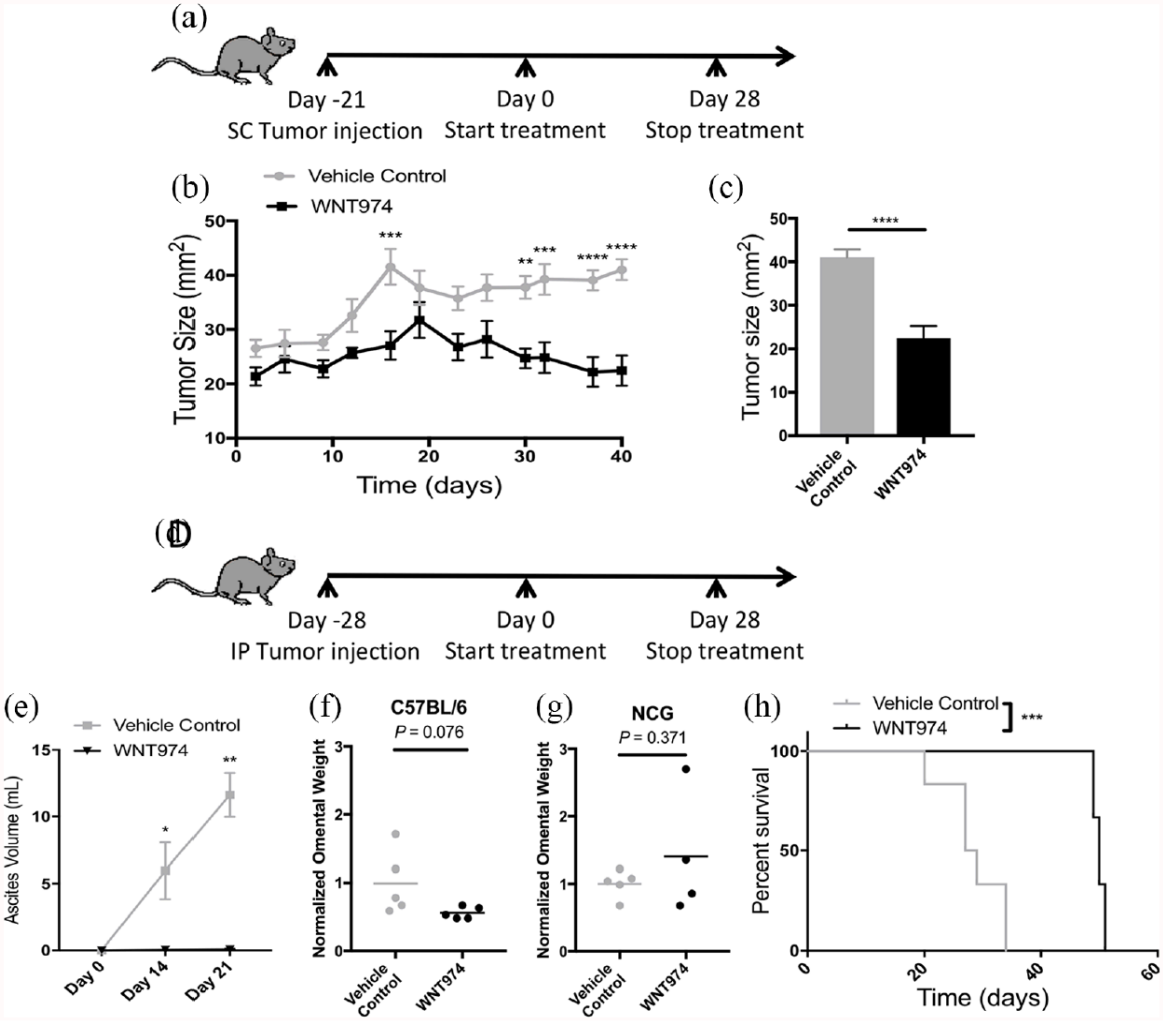


Ten C57BL/6 mice were randomized to be challenged with control ID8 or DKK1 overexpressing ID8 cells (n = 5/group) and monitored for 8 weeks. At day 56 animals were sacrificed. The overexpression of DKK1 does not alter **(A)** body weights, **(B)** omentum tumor burden, or **(C)** the number of lesions N1 mm compared to mice injected with parental ID8 cells. ***Conclusion: Over-expressing DKK1 in ID8 cell does not alter tumor burden but does enhance >1mm lesions.***



Subtask 2: Treat mice with vehicle control, WNT-974*, WNT7A** (n=10 in each group) with one untreated group (*CGX-1321 was used as a surrogate for WNT-97; **DKN-01 was used as a surrogate for WNT7A)

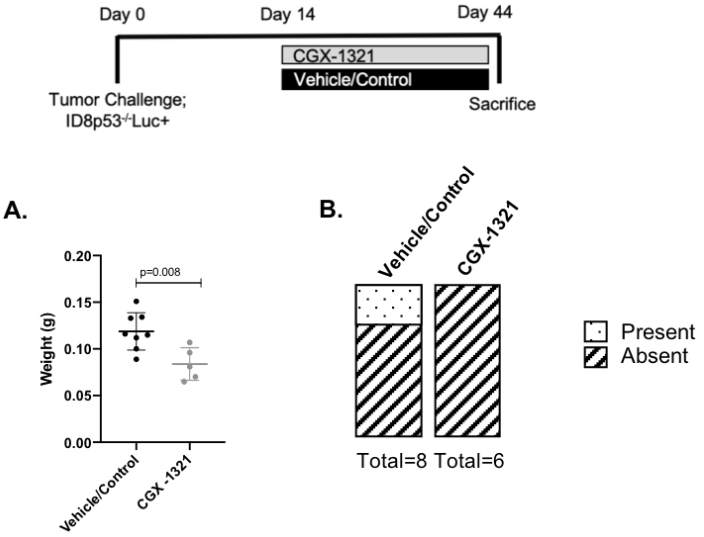
(A) Subcutaneous (SC) experimental design using ID8 parental cells. **(B)** Mice treated with WNT974 had decreased SC tumor growth compared to controls. **(C)** Average SC tumor size at day 40 was significantly smaller in WNT974-treated mice (n = 7 mice/group, data from one of two independent experiments). **(D)** Intraperitoneal (IP) experimental design. **(E)** WNT974-treated mice with IP tumors had fewer ascites than control mice (n = 5–7 mice/group, data from one of three independent experiments). **(F)** C57BL/6 mice treated with WNT974 had lower omental weights than control mice (n = 5 mice/group). **(G)** NCG mice treated with WNT974 had similar omental weights to control mice (n = 5 mice/group). **(H)** WNT974-treated mice with IP tumors had prolonged survival compared to control mice (n = 6 mice/group). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ***Conclusion: Wnt inhibition reduces tumor burden and ascites formation and prolongs survival in vivo.***



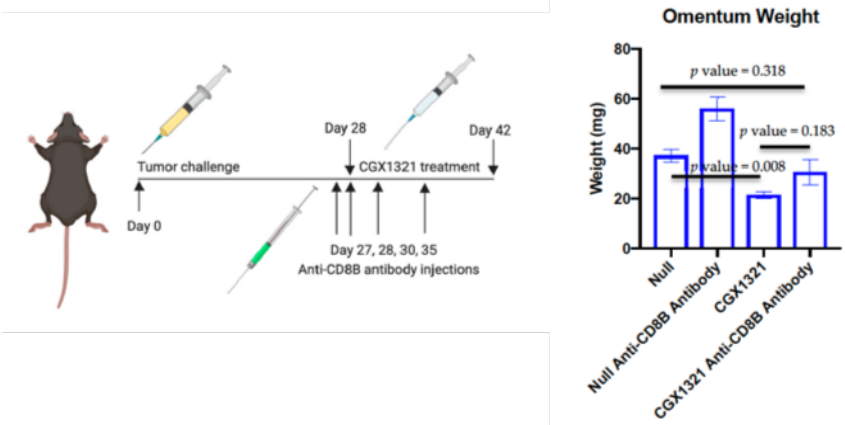
Subcutaneous (SC) tumor growth of ID8 parental cells (n = 7 mice/group, data from one of two independent experiments). *Indicates significance between WNT974 and Combo groups. φ indicates significance between vehicle control/paclitaxel and combo groups at endpoint. Treatment was initiated 21 days after SC tumor implantation. Treatment was initiated 28 days after intraperitoneal (IP) tumor injection. Survival curves of mice (n = 6 mice/group). ***Conclusion: Combination of paclitaxel with Wnt inhibition resulted in great reduction in tumor size and enhanced survival.***

//

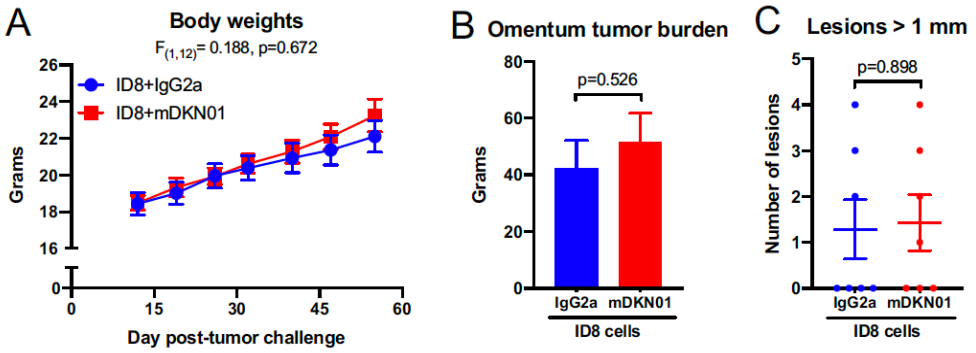
We challenged C57BL/6 mice with ID8p53$^{-/-}$ cells since the majority of ovarian cancers harbor *p53* mutations, and also lengthened CGX-1321 treatment. Here, treatment began 14-days post-tumor challenge and lasted for a total of 31 days. In doing so, we found that CGX-1321 significantly decreased omental weights (p=0.008) compared to vehicle/control-treated mice when treated for 31 days (**Figure A**). There were no ascites present in mice receiving treatment with CGX-1321 compared to vehicle/control-treated mice (**Figure B**). ***Conclusion: Inhibition of Wnt signaling via CGX-1321 significantly decreased ID8p53$^{-/-}$ tumor burden via omentum weights and ascites volumes.***



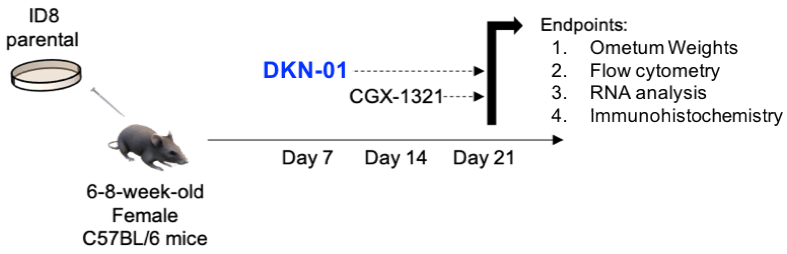
C57BL/6 mice were challenged with ID8 parental cells, and 28 days post-tumor injection the mice were depleted of CD8+ T cells followed by treatment with CGX-1321. ***Conclusion: CD8+ T cells were required for Wnt inhibition to significantly inhibit tumor progression in ID8 in vivo model.***

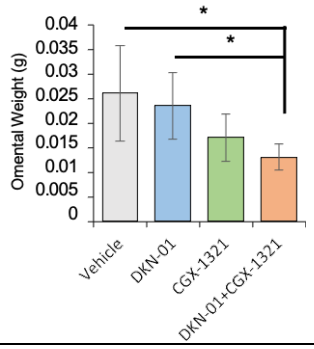


Fourteen C57BL/6 mice were challenged with parental ID8 cells and after 12 days were randomized to IgG2a control arm and DKN01 treatment (n=7/group) and monitored for 8weeks. At day 56 animals were sacrificed. mDKN01 treatment does not alter **(A)** body weights, **(B)** omentum tumor burden, or **(C)** the number of lesions (1mm) compared to isotype treated control mice. ***Conclusion: DKK1 monoclonal antibody, mDKN-01, does not alter ID8 tumor growth or lesions >1mm.***

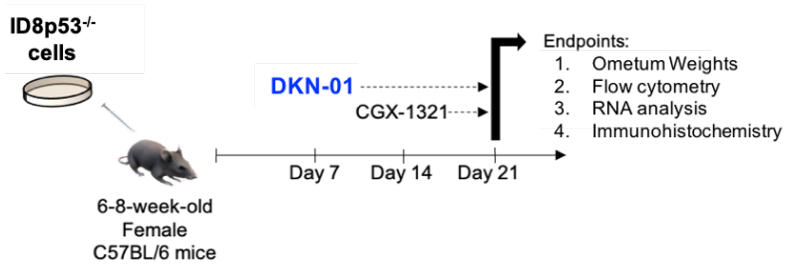


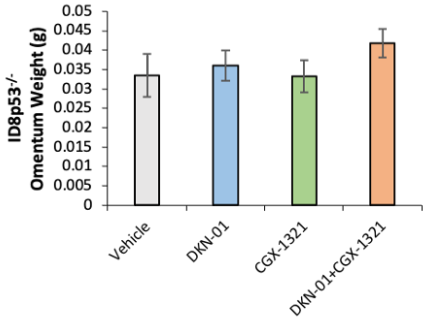
Schema of ID8 model sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week), n=8 per treatment arm and day 21 tumor burdens measured by omental weight (g). ***Conclusion: sequential therapy with DKN-01 and CGX-1321 in ID8 model resulted in decreased tumor burden in combo treatment group compared to DKN-01 and vehicle monotherapy groups.***



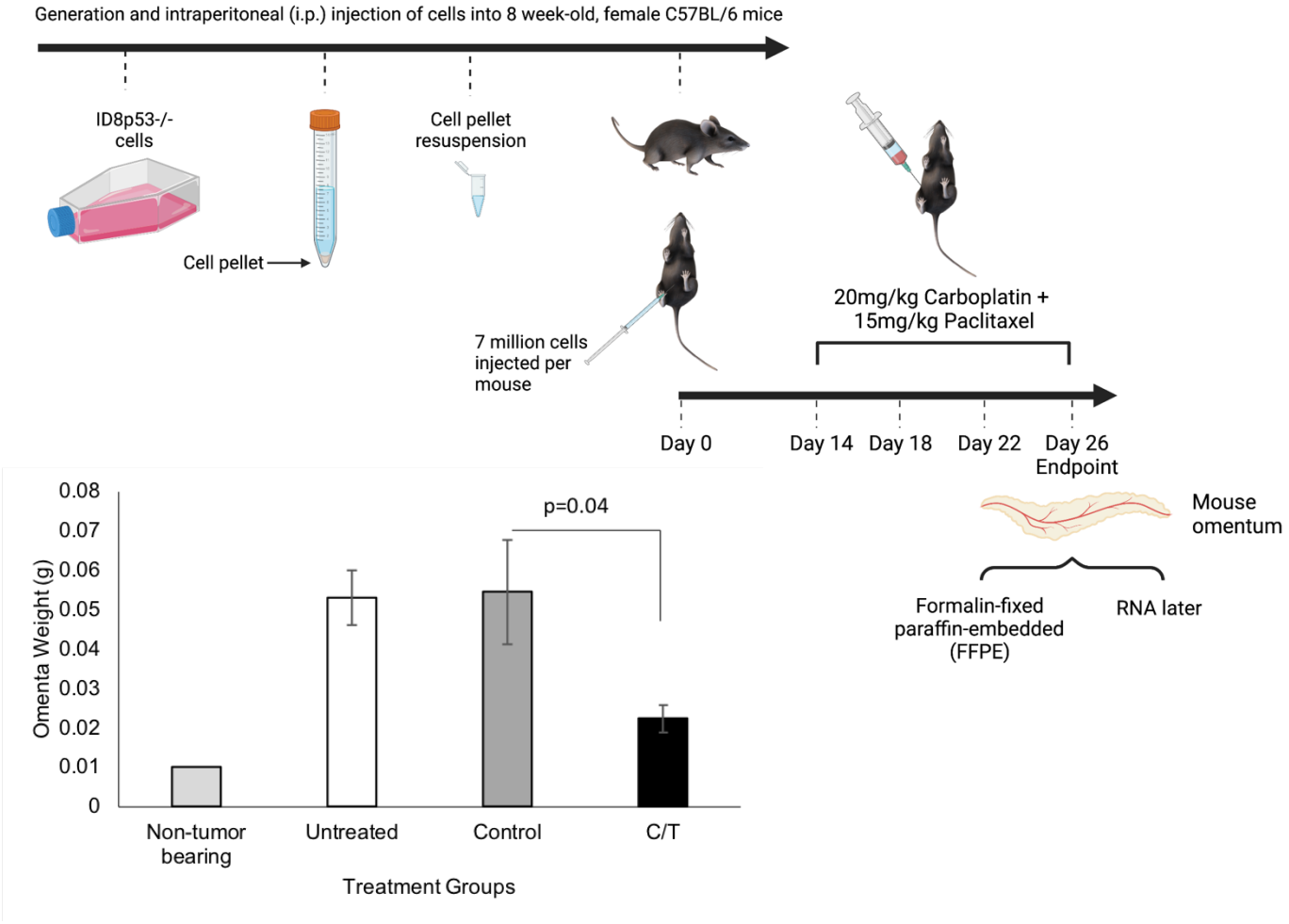


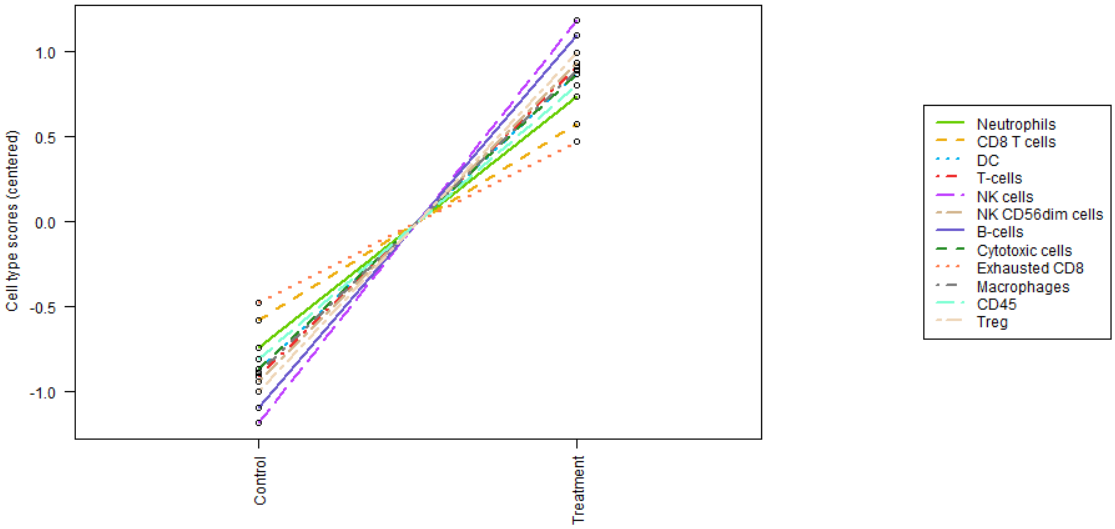
Schema of ID8p53$^{-/-}$ model sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week), n=8 per treatment arm and day 21 tumor burdens measured by omental weight (g). ***Conclusion: sequential therapy with DKN-01 and CGX-1321 in ID8p53$^{-/-}$ model did not result in decreased tumor burden in combo treatment group compared to DKN-01 and vehicle monotherapy groups.***

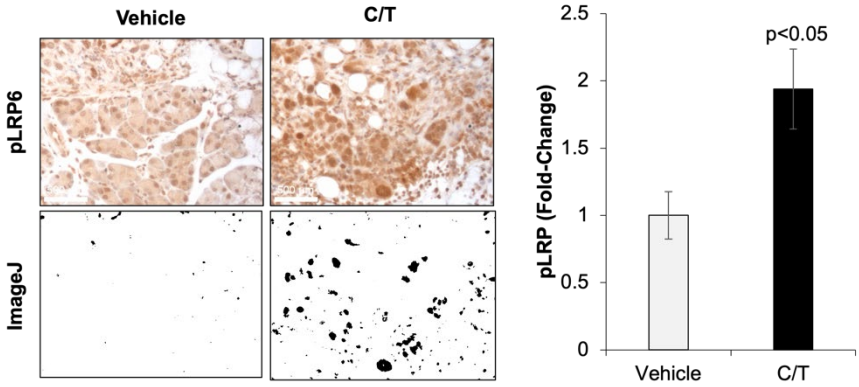




Schema of ID8p53-/- model….

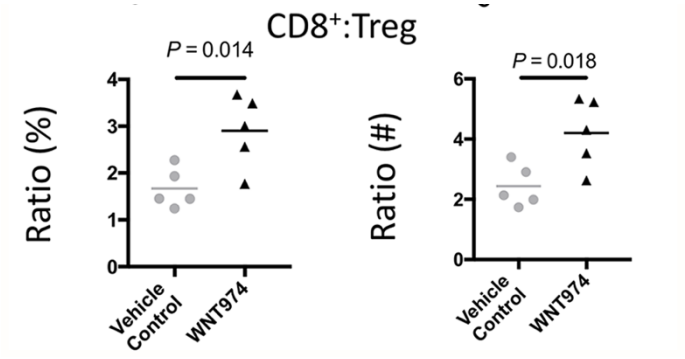

Generation and intraperitoneal (i.p.) injection of cells into 8 week-old, female C57BL/6 mice
ID8p53-/- cells
Cell pellet
Cell pellet resuspension
7 million cells injected per mouse
20mg/kg Carboplatin + 15mg/kg Paclitaxel
Day 0
Day 14
Day 18
Day 22
Day 26 Endpoint
Mouse omentum
Formalin-fixed paraffin-embedded (FFPE)
RNA later
Omenta Weight (g)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
p=0.04
Non-tumor bearing
Untreated
Control
C/T
Treatment Groups





Subtask 3: Quantify and sort CD8+ T cells, CD4+ T cells, and dendritic cells

Immune population CD8+ T cell and Treg populations were measured by flow cytometry in omental tumor with or without treatment with WNT-974 for 14 days in C57BL/6 mice with subcutaneous injection of ID8 parental cells. ***Conclusion: Wnt inhibition via WNT-974 significantly increased CD8+ T cell and regulatory T cell (Treg) ratio in the omental tumor of murine ovarian cancer model.***



MHC-II+CD11c^{hi} DC populations measured by flow cytometry in tumor-naive or tumor-bearing mice (ID8 parental cells injected into C57BL/6 mice) with treatment with vehicle control or WNT-974 for 14 days. Percentage frequency of parent shown on the left, total number of cells shown on the right (n = 5–7 mice/group, data from one of three independent experiments, dots represent individual mice). ***Conclusion: Wnt inhibition enhances anti-tumor DC function.***

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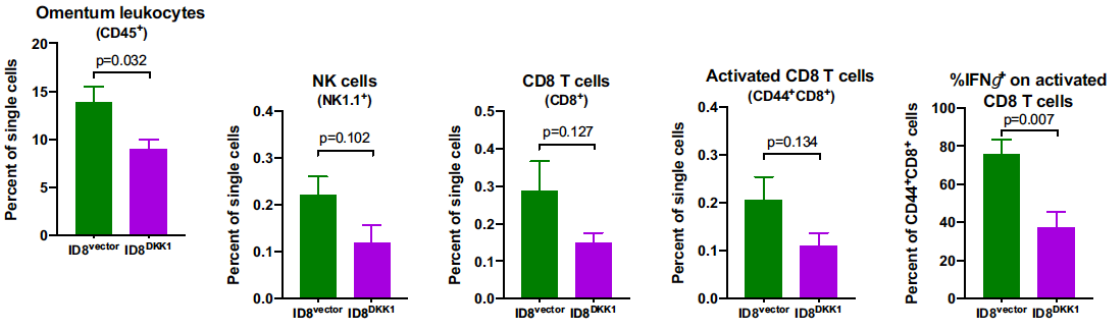
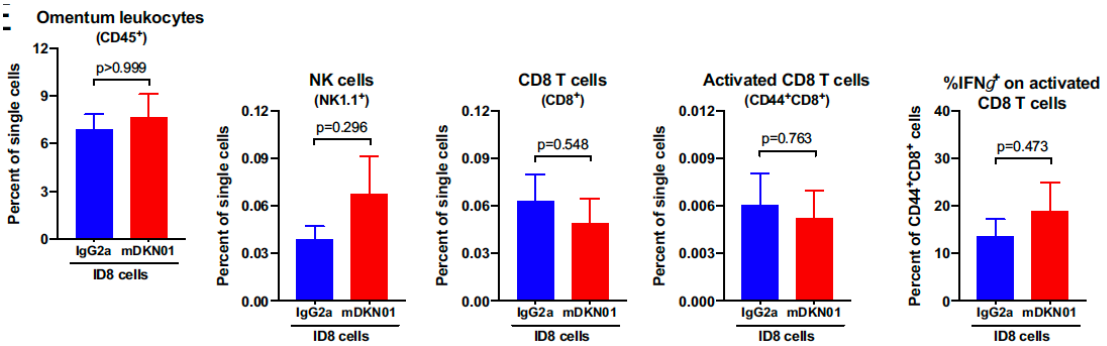
Subcutaneous (SC) tumor growth of ID8 parental cells (n = 7 mice/group, data from one of two independent experiments). Treatment was initiated 21 days after SC tumor implantation. CD8+ T cell:Treg ratio (measured by flow cytometry) and β TCR repertoire analysis of omental tumors after 14-26 days of treatment. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ***Conclusion: Combination of WNT-974 and paclitaxel results in the greatest increase in CD8+ T cell:Treg ratio and number of distinct CDR3 sequences (β TCR repertoire).***
/

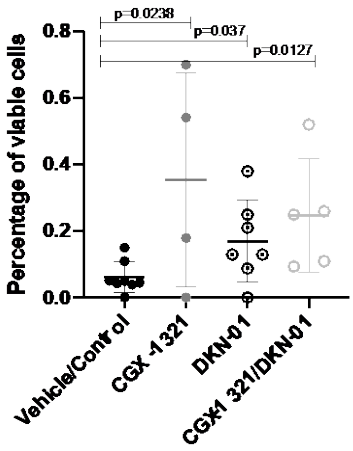
Ten C57BL/6 mice were randomized to be challenged with control ID8 or DKK1 overexpressing ID8 cells (n = 5/group) and monitored for 8 weeks. At day 56 animals were sacrificed. In the omentum, a significant reduction in total leukocyte infiltration (p = 0.032) was observed in mice injected with $ID8^{DKK1}$ cells. Investigating this leukocyte population, a non-significant reduction in NK cells, total CD8 T cells, and activated (CD44+) CD8 T cells was observed in $ID8^{DKK1}$ injected mice. The percent of activated CD8 T cells positive for IFNγ was significantly reduced (p= 0.007) in $ID8^{DKK1}$ injected mice compared to mice injected with parental ID8 cells. ***Conclusion: Over-expressing DKK1 reduces omentum immune cell infiltration in ID8 parental model.***



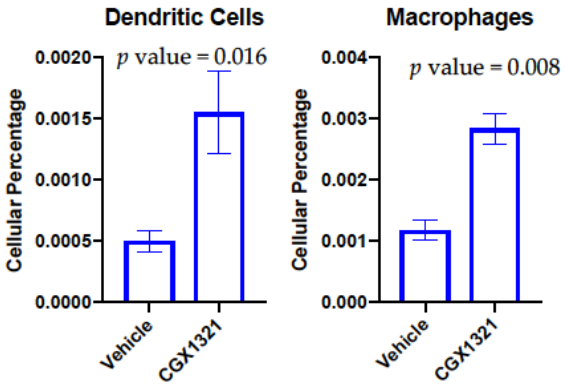
Fourteen C57BL/6 mice were challenged with parental ID8 cells and after 12 days were randomized to IgG2a control or mDKN01 treatment (n=7/group) and monitored for 8 weeks. At day 56 animals were sacrificed. In the omentum, no significant differences were observed between groups in total leukocyte infiltration, NK cells, total CD8 T cells, activated (CD44+) CD8 T cells, or the percent of activated CD8 T cells positive for IFNγ. ***Conclusion: DKK1 monoclonal antibody, mDKN-01, does not significantly alter omentum immune population infiltration into TME.***



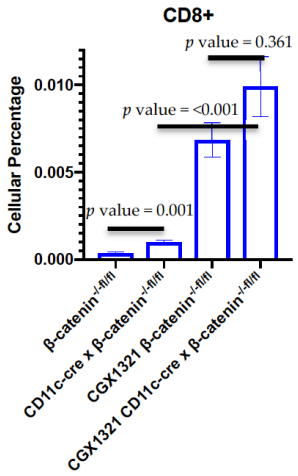
C57BL/6 mice were challenged with $ID8p53^{-/-}$ cells and treated with CGX-1321. Here, treatment began 14-days post-tumor challenge and lasted for a total of 31 days. Flow cytometry analysis was performed to measure infiltration of CD8+ T cells into the tumor microenvironment. ***Conclusion: treatment with CGX-1321 increased viable CD8+ T cells in TME.***



In vivo effect of Wnt signaling inhibition in omentum tumor and the immune cell population in the tumor microenvironment. ***Conclusion: After 42 days of ID8 tumor challenge, flow cytometry of omentum tumor showed an increase in macrophages and dendritic cells (DCs) with CGX-1321 treatment.***

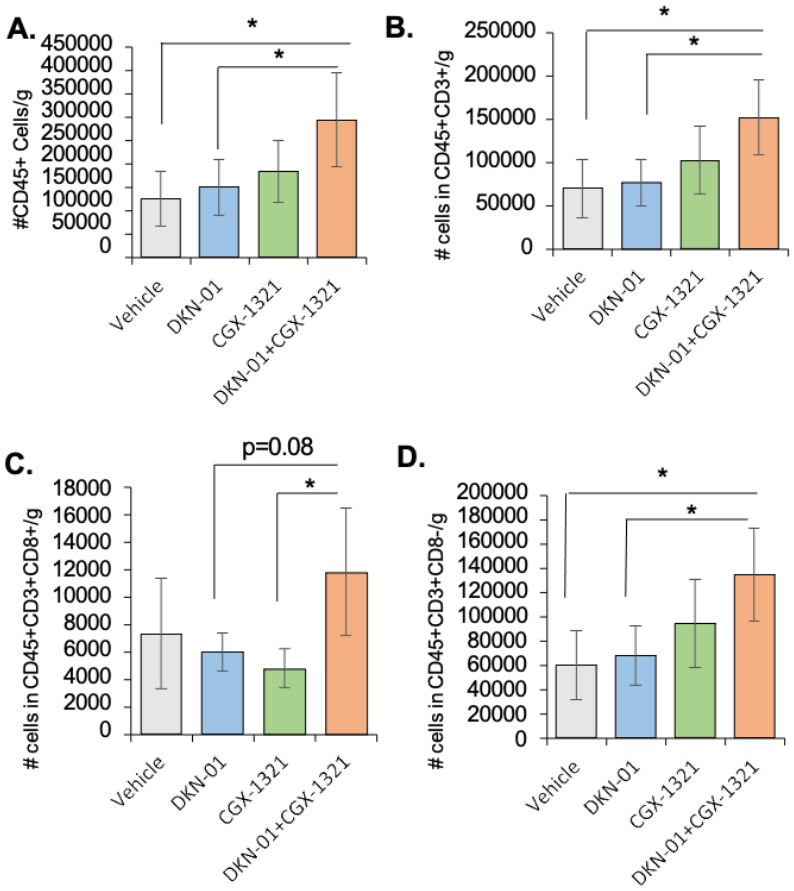


Flow cytometry analysis was performed to measure infiltration of CD8+ T cells into the tumor microenvironment. CD8+ T cells increased in the tumor microenvironment in CD11c-cre x Beta-catenin$^{-/-fl/fl}$ mice with ID8 tumor challenge, a finding that was further exaggerated with CGX-1321 treatment. ***Conclusion: inhibition of beta-catenin signaling in dendritic cells increased CD8+ T cell infiltration, which is enhanced with CGX-1321 treatment.***

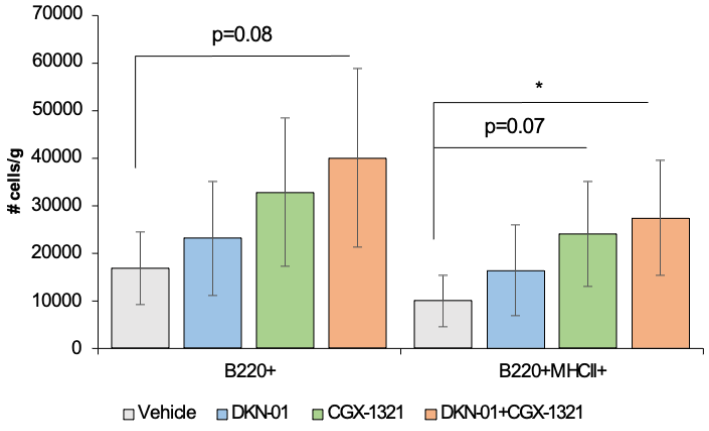


Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model (schema same as above), n=8 per treatment arm. FACS analysis of total (**A**) immune cells (CD45+), (**B**) T cells (CD45+CD3+), (**C**) CD8+ T cells (CD45+CD3+CD8+), and (**D**) presumptive CD4+ T cells (CD45+CD3+CD8-) presence in the omenta of mice. ***Conclusion:***

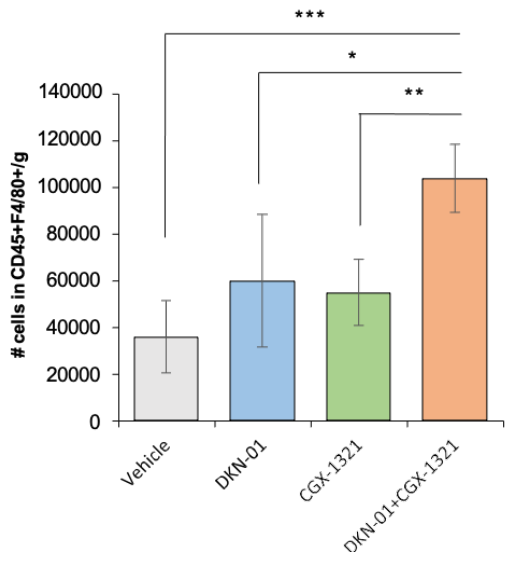
sequential therapy of DKN-01 and CGX-1321 resulted in increased immune infiltration and T cell populations in combo treatment group versus vehicle and monotherapy groups.

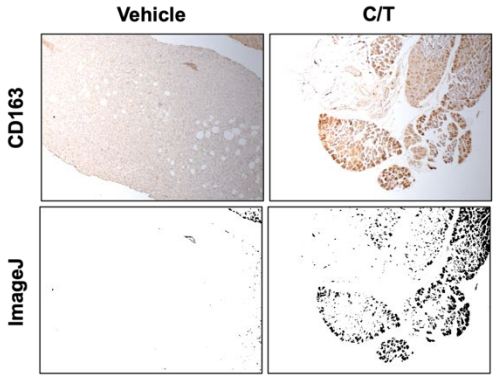


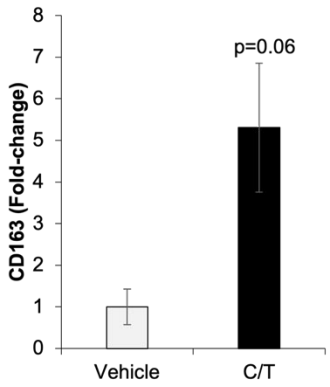
Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model, n=8 per treatment arm. FACS analysis of total B cell (CD45+B220+) and activated B cell (CD45+B220+MHCII+) presence in the omenta of mice. ***Conclusion: sequential therapy of DKN-01 and CGX-1321 resulted in increased total B cell and activated B cells in combo treatment group compared to vehicle and DKN-01 monotherapy groups.***



Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model, n=8 per treatment arm. FACS analysis of total macrophage (CD45+F4/80+) presence in the omenta of mice female, C57BL/6 mice 21-days post tumor injection. ***Conclusion: sequential therapy of DKN-01 and CGX-1321 resulted in increased macrophages in omenta.***



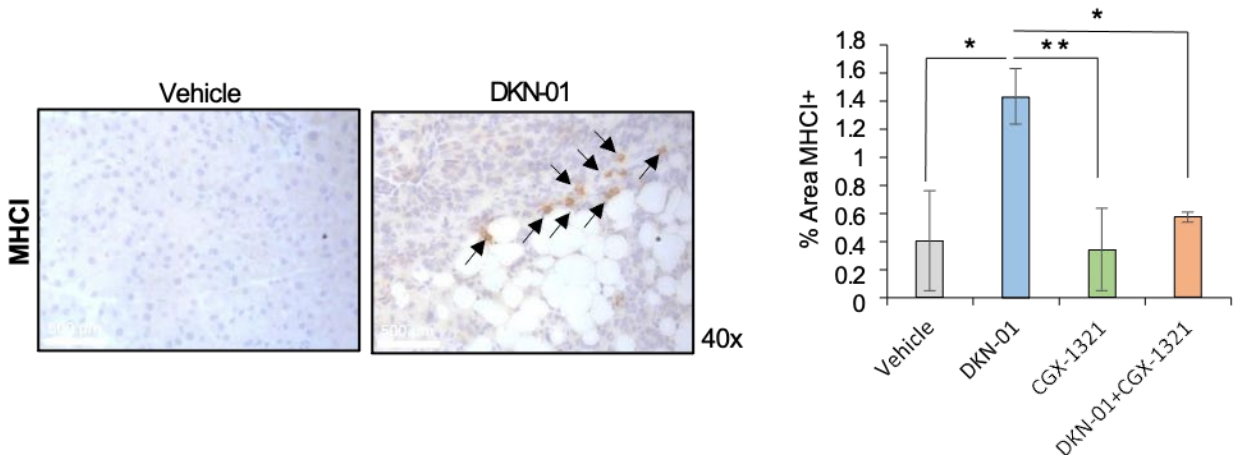




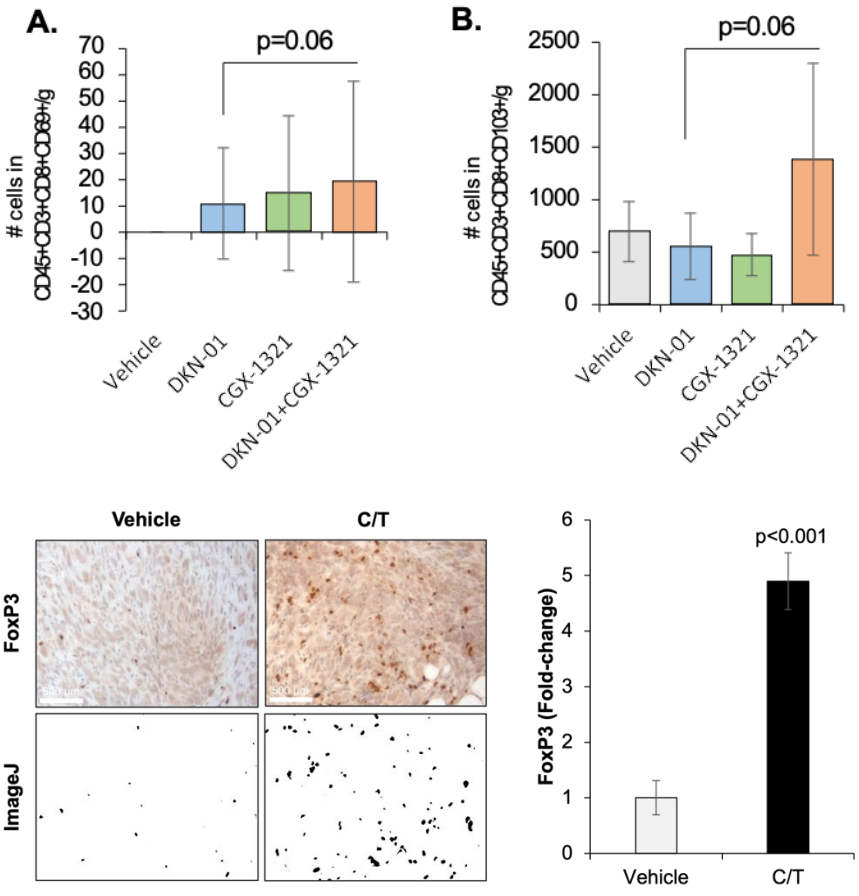
Subtask 4: Monitor T-cell function

////ID8 parental cells were challenged into C57BL/6 mice, and cytokine and inhibitory marker expression from omental tumor TILs after 14 days of treatment was analyzed by flow cytometry. CD4+ T cells (**Figure A - top**) and CD8+ T cells (**Figure A - bottom**), percentage frequency (left) and mean fluorescence intensity (MFI) (right) (n = 5 mice/group, data from one of two independent experiments). Percentage frequency of CD8+ T cells expressing inhibitory markers (**Figure B**) (n = 5 mice/group, data from one of two independent experiments, dots represent individual mice). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ***Conclusion: Wnt inhibition enhances anti-tumor immune response and reduces immune cell exhaustion.***

Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model, n=8 per treatment arm. Immunohistochemistry (IHC) demonstrates of MHCI expression on ID8 cells with DKN-01 administration (*p<0.05). ***Conclusion: DKN-01 treatment in ID8 model results in increased MHCI staining, suggesting this treatment can increase tumor-immune recognition.***



Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model, n=8 per treatment arm. FACS analysis of abundance of activated CD8+ T cells (**A**) CD8+CD69+ and (**B**) CD3+CD8+CD103+ in omenta of mice in response to treatments. ***Conclusion: sequential therapy of DKN-01 and CGX-1321 resulted in increased CD8+ T cell activation compared to monotherapy groups.***

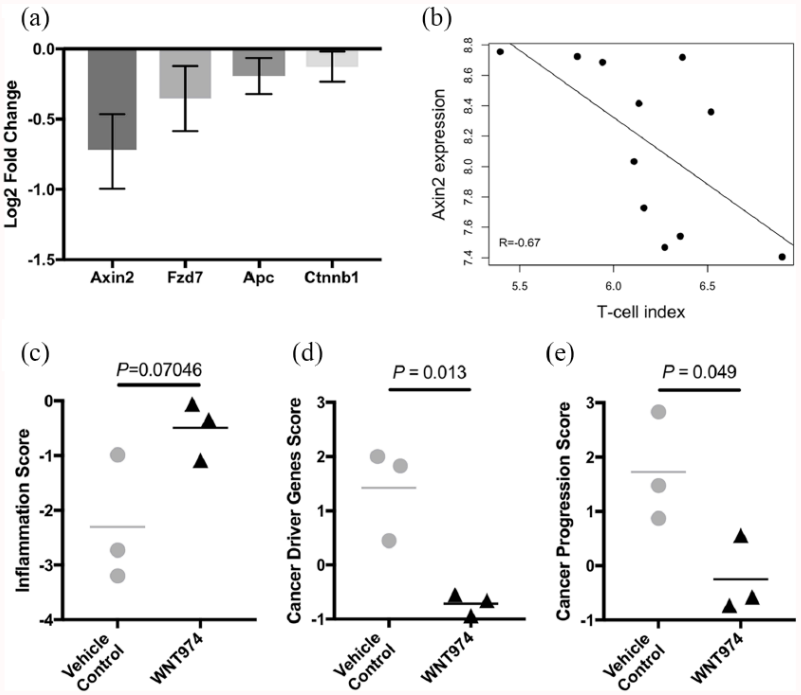


Subtask 5: Monitor gene expression by RNA seq (WNT, immune, HRD signatures)

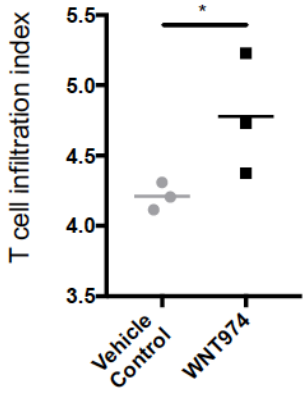
Characterization of Wnt profile in ID8 and ID8p53$^{-/-}$ cell lines. Normalized Wnt related gene expression data for WNT2B, WNT5A, FZD4, and AXIN2 in ID8 (n=6) and ID8p53$^{-/-}$ (n=6) was decreased in the ID8p53$^{-/-}$ cell line compared to the parental ID8 cell line (**Figure A**). Western blot analysis revealed reduced beta-catenin levels in the ID8p53$^{-/-}$ cell line comparted to ID8 cell line (**Figure B**). ***Conclusion: p53 mutation results in decreased Wnt activity in ID8 model.***

////

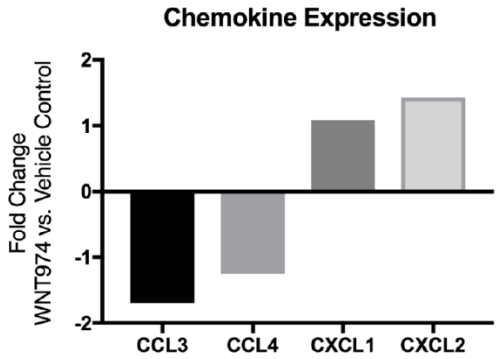
mRNA expression patterns from omental tumors developed from C57BL/6 mice challenged with ID8 cells with 14 days of WNT-974 versus vehicle control. **(A)** Log2 fold change of target genes in the Wnt pathway after treatment with WNT974. **(B)** Gene expression of Axin2 negatively correlates with T cell infiltration. **(C)** NanoString-defined inflammation score. **(D)** NanoString-defined cancer driver genes score. **(E)** NanoString-defined cancer progression score. n = 3 mice/group for each panel. ***Conclusion: Wnt inhibition promotes an anti-tumor gene expression pattern.***



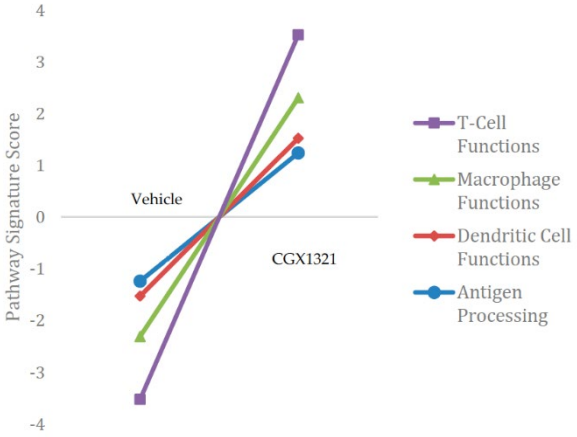
Excised SC tumors from C57BL/6 mice challenged with ID8 cells were analyzed for mRNA expression. Statistical significance was determined using the t-test. T cell infiltration signature was calculated (n=3/group, 2 at day 7, 1 at day 14). * $P < 0.05$. ***Conclusion: A gene signature of T cell infiltration is up-regulated in the WNT-974 treatment arm compared to control.***



Expression of chemokines by omental tumors in C57BL/6 mice challenged with ID8 cells with WNT-974 and vehicle treatment, as measured by Nanostring. ***Conclusion: Wnt inhibition increased CXCL1 and CXCL2 expression while decreasing CCL3 and CCL4 expression. CXCL1 is responsible for recruitment various inflammatory immune cells and CXCL2 promotes inflammatory response.***

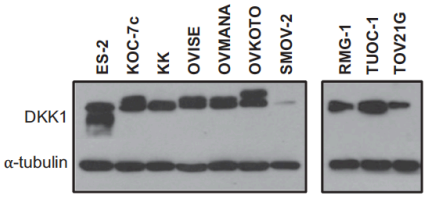


In vivo effect of Wnt signaling inhibition via CGX-1321 in omentum tumor and the microenvironment of ID8 model. ***Conclusion: increase in gene signatures for T cell functions, macrophage functions, dendritic cell functions, and antigen processing with CGX-1321 treatment via NanoString analysis.***



DKK1 is broadly expressed in human ovarian cancer cell lines**. (A)** Western blots for DKK1 (38 kDa) in ovarian cancer cell lines **(B)** hDKK1 protein (pg/ml) secreted into culture supernatant was measured via ELISA. All cell lines represent clear cell ovarian cancer (CCOC), except for TOV112D, which is an endometrioid ovarian cancer cell line. The quantification of secreted DKK1 was performed independently in three experiments. Data represents one representative experiment. ***Conclusion: the human ES2 cell line harbors elevated expression of DKK1.***

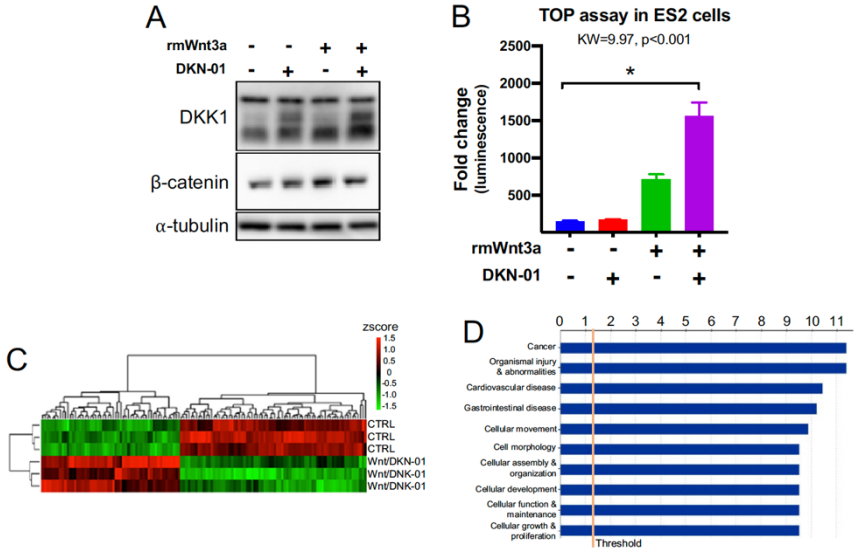
A



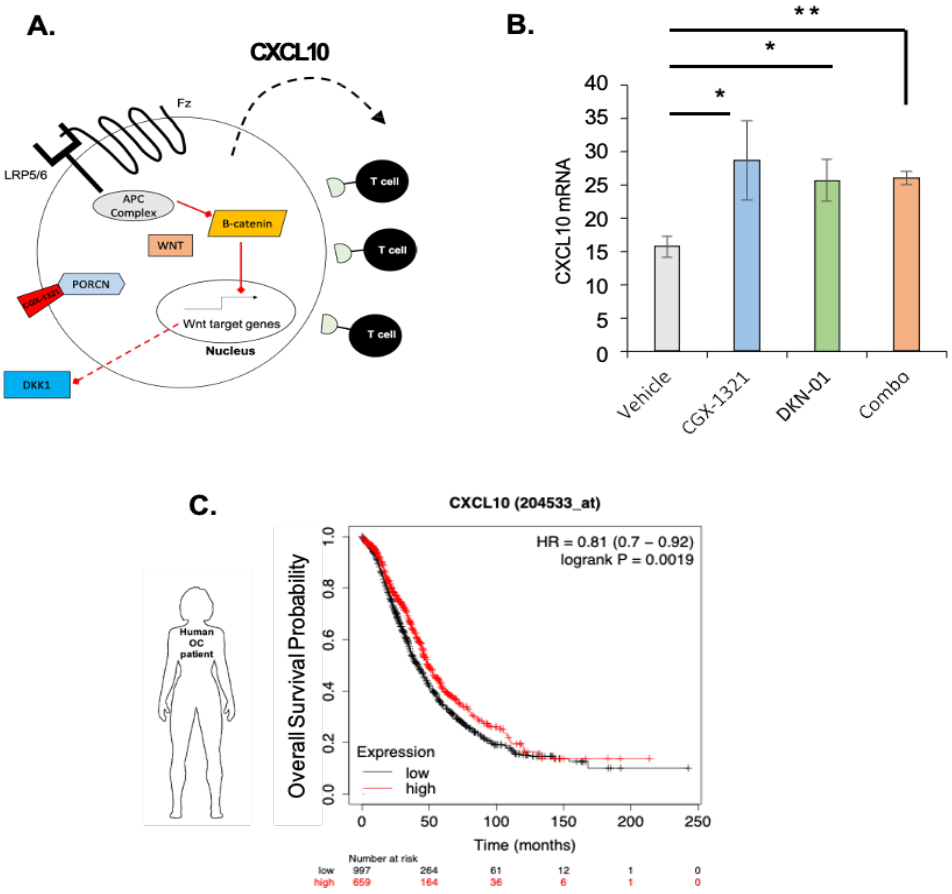
B

	ES2	KOC-7c	OVISE	SMOV2	RMG-1	TUOC1	TOV21G	TOV112D (EOC)
DKK1 (pg/µl)	1984.9	15.7	10.8	14.7	19.4	10.8	1765.1	1447.7

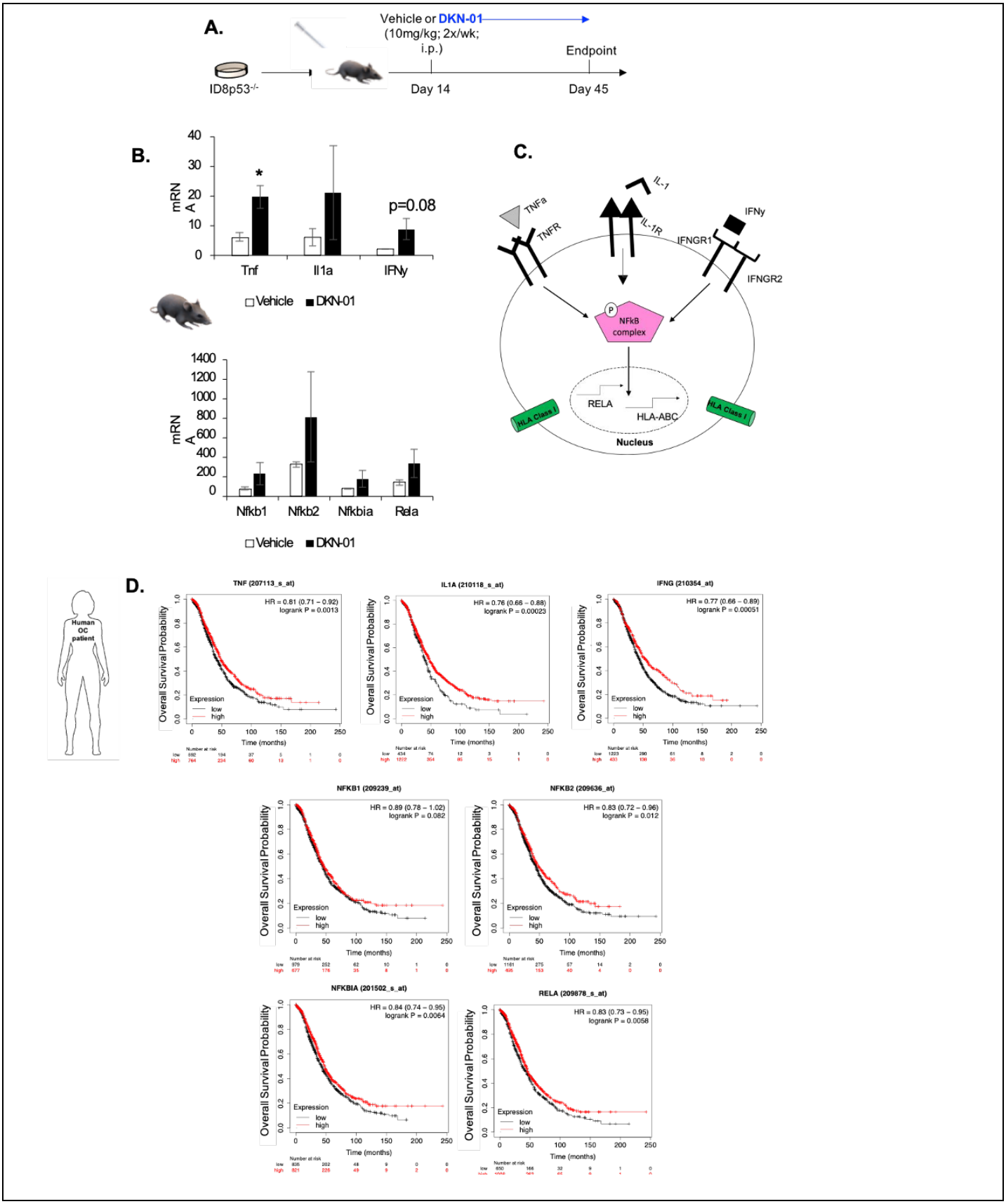
In vitro Wnt pathway stimulation and DKK1 blockade alters biological pathways activity in ES2 ovarian cancer cells. **(A)** Western blot of ES2 cell lysates 24 h after stimulation with rmWnt3a+/− DKN-01. **(B)** Wnt ligand and DKK1 antibody both stimulate the activity of the canonicalWnt pathway when measured by TOP/FOP flash nuclear β-catenin reporter assay with additive effects. **(C)** Histogram of RNAseq fromES2 cells treated for 24h with no treatment vs. combined rmWnt3a/DKN-01. 55 genes were significantly upregulated ($p < 0.01$) and 74 genes were significantly downregulated ($p < 0.01$). **(D)** The top-ten biological functions affected by stimulating ES2 cells with combined rmWnt3a and DKN-01 antibody. ***Conclusion: the human ES2 cell line is sensitive to combined Wnt3a/DKN-01 treatment.***

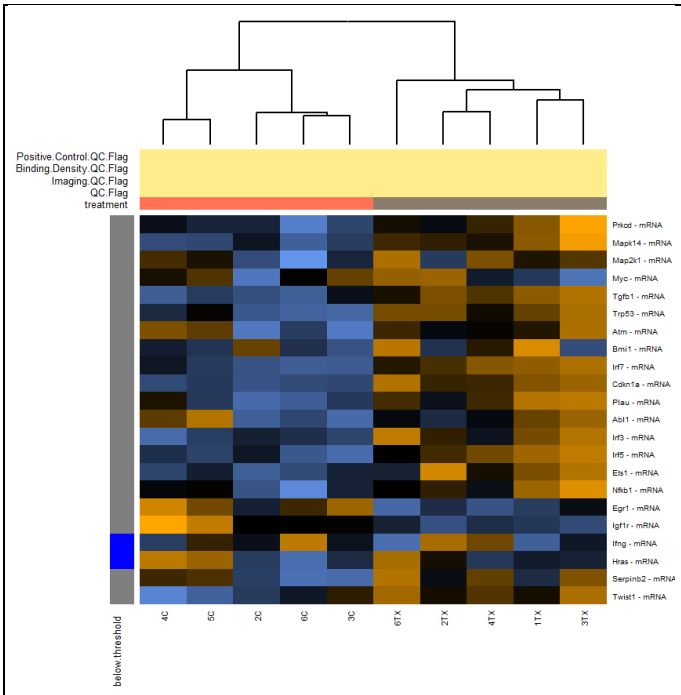


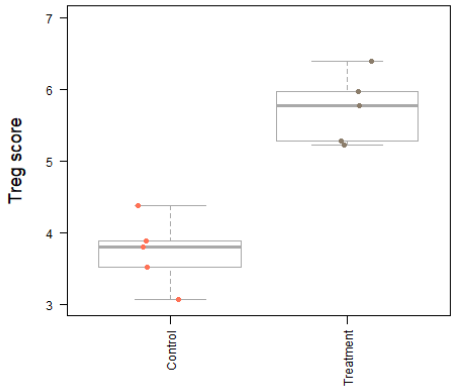
Sequential treatment with DKN-01 (i.p.; 1mg/kg; 2x/week) and CGX-1321 (oral gavage; 10mg/kg; 5x/week) in ID8 model, n=8 per treatment arm. (**A**) Tumor expression of chemotactic factor CXCL10 stimulates T cell infiltration to TME. (**B**) RNAseq data from omenta. (**C**) Kaplan Meier (KM) curve of *CXCL10* expression effects on ovarian cancer (OC) overall survival (OS). ***Conclusion: sequential therapy increases CXCL10 (*p<0.05, **p=0.01) mRNA expression in tumors compared to vehicle. KM curve shows increased OS in OC patients with high expression of CXCL10.***



Schematic of **(A)** treatment timeline with either vehicle (n=2) or DKN-01 (n=3) monotherapy. (**B**) RNAseq data of TNF, IL-1a, IFNy, NFkB, and RELA mRNA expression in response to DKN-01 therapy. (**C**) TNF, IL-1, and IFNy signaling activation of NFkB increases HLA transcription. (**D**) Kaplan-Meier curves of OS in OC patients with high expression of the indicated factors. ***Conclusion: DKN-01 up-regulates signaling pathways related to HLA stabilization in a syngeneic ID8p53-/- murine model. These signaling components correspond to increased OC patient OS.***


A.
Vehicle or DKN-01
(10mg/kg; 2x/wk;
i.p.)
Endpoint
ID8p53-/-
Day 14
Day 45
B.
mRNA
Tnf
Il1a
IFNy
p=0.08
Vehicle
DKN-01
Nfkb1
Nfkb2
Nfkbia
Rela
C.
TNFa
TNFR
IL-1
IL-1R
IFNy
IFNGR1
IFNGR2
NFkB complex
RELA
HLA-ABC
HLA Class I
Nucleus
D.
Human OC patient
TNF (207113_s_at)
HR = 0.81 (0.71 – 0.92)
logrank P = 0.0013
IL1A (210118_s_at)
HR = 0.76 (0.66 – 0.88)
logrank P = 0.00023
IFNG (210354_at)
HR = 0.77 (0.66 – 0.89)
logrank P = 0.00051
NFKB1 (209239_at)
HR = 0.89 (0.78 – 1.02)
logrank P = 0.082
NFKB2 (209636_at)
HR = 0.83 (0.72 – 0.96)
logrank P = 0.012
NFKBIA (201502_s_at)
HR = 0.84 (0.74 – 0.95)
logrank P = 0.0064
RELA (209878_s_at)
HR = 0.83 (0.73 – 0.95)
logrank P = 0.0058
Overall Survival Probability
Expression
low
high
Time (months)
Number at risk





Subtask 6: Extract RNA and use the iRep technique

Goals not met: We need to investigate our findings on the effects of WNT signaling alteration in the ID8 model with homozygous BRCA2 knock-out.

- **What opportunities for training and professional development has the project provided?**

  I completed a Master of Science in Public Health through the School of Public Health in Clinical and Translational Sciences. The program allowed for the development of skills required for clinical research and academic medicine including clinical trial design, ethics, informatics, biostatistics, grant writing, and data collection and management. Through the program I was able to network with mentors and advisors that she will maintain relationships with throughout her career.

  As faculty at UAB, I have had many opportunities to serve both on the institutional and national level. My interest in personalized medicine has led me to a few different roles here at UAB as I have developed the Personalized Medicine project within the Gyn Onc clinic, in which we have the ability to run NGS on patient tumor samples to understand how these results can affect their standard of care. At present, I have acted as the alternate chair for the Personalized Medicine Working Group since 2017. Another opportunity to use this interest has been serving as a member on UAB's Molecular Tumor Board, where I get the chance to review complex cases and make recommendations based on the patient's molecular profile. In addition to these groups, I am currently serving as a member on the Search Committee for Preventative Medicine. I have served as the chair of the Tissue Committee since 2015, and I have continued to be the chair of UAB's Tissue Committee and to optimize a pathway for tissue banking of ovarian cancer patients and other cancer patients at UAB O'Neal Comprehensive Cancer Center. As chair of the Tissue Committee, I work with a multidisciplinary group of people to see through that human tissue collection for basic and translational research is running as smoothly and efficiently as possible to ensure both patient safety and quality research. I have continued to serve at the co-chair of the Gynecologic Oncology Disease Oriented Working Group (DOWG) and the Precision Oncology Working Group (POWG) where I have enhanced my involvement in the scientific and ovarian cancer research communities at UAB. My responsibilities in these groups include reviewing trials for feasibility and assuring that clinical trials in the pipeline are moving efficiently to opening for enrollment. I am also an active member of the Protocol Review Committee as well as three Clinical Trials Committees: Gyn, Breast, and Phase 1. Lastly, I serve as the Project Leader for the Cervical SPORE Biorepository at UAB.

I also serve several different roles in national groups outside of the university. Particularly, I am very active in the Society of Gynecologic Oncology (SGO), NRG Oncology (formerly the Gynecologic Oncology Group (GOG)), as well as the American Association for Cancer Research (AACR). I am currently the co-chair of Translational Science on the NRG Ovarian Committee as well as the Ovarian Committee representative to the NRG Translational Research Committee. For the SGO, I have served for two years now as the Emerging Clinical Trialists Course Co-Director. I have also had the honor of serving on the SGO Program Committee and Steering Committee, as well as the SGO Research and Awards committee. Additionally, I was selected to be the on the program committee for SGO 2020. Lastly, my other national service activities have been as co-chair of the FDA-AACR-SGO Workshop, as well as a previous member on the NCI Research Taskforce in 2019.

I have had the opportunity to collaborate with many scientists both at UAB and around the country by providing samples from my lab, performing key experiments, and supplying my expertise in gynecologic cancers. In addition to the many publications I have served as mentor or collaborator on since joining faculty at UAB, I have also been first author several publications including both reviews and scientific papers (PMIDs 29523763, 30139839, 29843906, 29455465, and 31320488). My foundation in translational research has led me to serve as Co-Investigator or Investigator on several national trials. Those that I am currently serving on are as follows:

1. A Phase 2 Study Evaluating the Efficacy and Safety of DKN-01 as a Monotherapy or in Combination with Paclitaxel in Patients With Recurrent Epithelial Endometrial or Epithelial Ovarian Cancer (**UAB 17105**, NCT03395080). Principal Investigator.
2. Pilot Study of Daily Exemestane in Women with Complex Atypical Hyperplasia of the Endometrium / Endometrial Intraepithelial Neoplasia or Low Grade Endometrial Cancer (**UAB 1788**, NCT03300557). Sub-Investigator.
3. A Phase 1 Trial of M4344 and Niraparib in patients with PARP resistant recurrent ovarian cancer (**UAB 1885**, NCT04149145). Principal Investigator.
4. Cabozantinib Plus Nivolumab and Ipilimumab Women With Recurrent Gynecologic Carcinosarcoma (**UAB 1921**, NCT04149275). Principal Investigator.
5. A Phase 2, Single Arm Study of Atezolizumab + Bevacizumab in Women with Advanced, Recurrent or Persistent Endometrial Cancer (**UAB 18107**, NCT 03526432). Co-Investigator.
6. Phase 1 trial of CB-839 in Combination with Niraparib in platinum resistant BRCA-wild type Ovarian Cancer Patients (**UAB 1801**, NCT03944902). Co-Investigator.

NCT03395080 is a study investigating the Wnt modulator DKN-01 (Leap Therapeutics) alone or in combination with paclitaxel in women with recurrent endometrial and epithelial ovarian cancer. Recently the trial has been expanded to include an arm for women with recurrent carcinosarcomas due to promising responses seen previously in these patients. Two current trials (both Investigator Initiated Trials) include novel therapies for PARP inhibitor resistant ovarian cancer patients – one using the ATR inhibitor M4344 (NCT04149145) and the other using the glutaminase inhibitor CB-839 (NCT03944902). NCT04149275, treatment with cabozantinib along with ipilimumab/nivolumab for gynecologic carcinosarcomas is also an Investigator Initiated Trial.

Dr. Birrer, Dr. Odunsi, and myself had combined lab meetings and bi-montly one-on-one meetings to discuss her career development and research progress. In addition, I had weekly lab meetings with Dr. Sara Cooper from HudsonAlpha and Dr. Troy Randall from UAB to discuss on-going projects. My lab members and myself went to both Dr. Birrer and Dr. Randall's labs to learn molecular biology and immunology techniques that were not previously known.

I trained the gynecologic oncology clinical fellows Drs. Whitney Goldsberry and David Doo on this grant. In addition to these fellows, I have also directly and indirectly mentored several PhD, MD, and MD/PhD students. They had the opportunity to perform basic science research, exploring the role of the Wnt signaling pathway in progression of ovarian cancer. In addition, they each wrote a review and research article pertaining to their projects. They were required to present their findings at weekly lab meetings and once each semester to the Department of OB/GYN – Gynecologic Oncology Division. Furthermore, they each presented their research projects with either a poster or oral presentation at SGO. I was able to attend the AACR general meeting,

ASCO meeting, and SGO meeting. These meetings allowed for me to gain further knowledge and expertise in ovarian cancer and the immune system.

My bi-monthly, one-on-one lab meetings with Dr. Birrer, Dr. Odunsi, and myself discussing my career development and research progress continued in this year of funding. In addition, my weekly lab meetings with Dr. Sara Cooper from HudsonAlpha and Dr. Troy Randall from UAB continued to discuss on-going projects. My lab members and myself have each attended webinars and sought expertise on molecular biology and immunology techniques that were not previously known.

I have continued serving on committees/positions at both the institutional and national levels (*as previously outlined in my 2019 Annual Progress Report*). In addition, I am still fostering collaborations with investigators and physicians both at UAB and other institutions.

Gynecologic oncology clinical fellow, Dr. Jackie Wall was trained on this grant. She had the opportunity to perform basic science research, exploring the role of the Wnt signaling pathway in progression of ovarian cancer. In addition, she wrote a review and research article pertaining to her projects. She was required to present her research findings at weekly lab meetings and once each semester to the Department of OB/GYN – Gynecologic Oncology Division. Furthermore, she presented her research project with either a poster or oral presentation at SGO. Furthermore, I have continued to directly and indirectly mentor several PhD, MD, and MD/PhD students, each with presentations at local and/or national conferences.

My bi-monthly, one-on-one lab meetings with Dr. Birrer, Dr. Odunsi, and myself discussing my career development and research progress continued in this year of funding. In addition, my weekly lab meetings with Dr. Sara Cooper from HudsonAlpha and Dr. Troy Randall from UAB continued to discuss on-going projects (*as previously outlined in my 2020 Annual Progress Report*). Furthermore, I started weekly meetings with Dr. Lyse Norian who is one of my collaborators and another expert in the field of immunology to help with manuscript preparations and grant submissions. My lab members and myself have each attended webinars and sought expertise on virtual platforms that can be used to investigate large datasets (e.g., cBioPortal). In addition, we have optimized two systems, bioreactor and 3-D magnetized spheroids, that we are currently using as models to study patient-derived tissues and their tumor-intrinsic and immune responses to targeted and immune checkpoint blockade (ICB) therapies.

I have continued serving on committees/positions at both the institutional and national levels. In addition, I am still fostering collaborations with investigators and physicians both at UAB and other institutions (*as previously outlined in my 2020 Annual Progress Report*).

Gynecologic oncology clinical fellow, Dr. Jhalak Dholakia was trained on this grant. She had the opportunity to perform basic science research, exploring the role of the WNT signaling pathway in progression of ovarian cancer and how it effects immune response in the tumor microenvironment. In addition, she wrote a review and research article pertaining to her projects. She was required to present her research findings at weekly lab meetings and once each semester to the Department of OB/GYN – Gynecologic Oncology Division. Furthermore, she presented her research project with an oral presentation at SGO. Furthermore, I obtained a postdoctoral research fellow, Dr. Carly Bess Scalise, who has a background in cell and molecular pharmacology and immunology and fellowship training in immunotherapy and clinical trials.

I have continued to mentor several other PhD, MD, and MD/PhD students directly and indirectly, each with presentations at local and/or national conferences.

- **How were the results disseminated to communities of interest?**

The results from these studies were presented at local and national conferences to audiences that are in similar areas of research and/or medicine.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Specific Aim 1
Major Task 1: Obtain RNA sequencing results from 917 patients from GOG 218
Subtask 1: Identify location of data
Subtask 2: Download data in a format that can be transferred to Hudson Alpha Institute for analysis
Proposed experiments: None.
Major Task 2: Categorize the patient data into T cell inflammation subtype, WNT pathway score, HRD status, correlate with survival stratified by treatment
Subtask 1: Create categories based on the ovarian TCGA analysis of "hot" and "cold" tumors
Subtask 2: Analyze the previously collected data (BROCA-HR assay) as it relates to the "hot" and "cold" signature
Subtask 3: Analyze the RNA sequencing data pertinent to HRD status
Subtask 4: Categorize patient data based on WNT pathway gene expression
Proposed Experiments: None.

Specific Aim 2
Major Task 1: Determine whether inhibition or activation of the WNT pathway impacts T cell response
Subtask 1: Breed enough female Tg*MISIIR-Tag-Low* mice (n=40)
Subtask 2: Inject mice with MOVCAR-luc cells
Subtask 3: Treat mice with vehicle control, WNT974, WNT7A (n=10 in each group) with one untreated group
Subtask 4: Sac 5 mice at day 14 and the remainder on day 28 and quantify tumor-specific CD8+ T cells, test whether the T cells are activated, and send whole tumor for RNA seq
Subtask 5: Dissociate fresh tumor tissue and sort-purify cells into CD3/4+ or CD3/8+ T cells
Subtask 6: Extract RNA from sorted cells and use TCR repertoire sequencing
Proposed Experiments: None.
Major Task 2: Create a CRISPR/Cas9-mediated β-catenin knockout model and access T cell response
Subtask 1: Create MOVCAR knockout cell line
Subtask 2: Compare T cell infiltration, proportion of TAMs, proportion of Tregs, presence of CD103+ dendritic cells in the β-catenin knockout model compared to wild-type
Proposed Experiments: None.

Specific Aim 3
Major Task 1: Evaluate the number and types of T cells that are present after treating mutated and wild-type cells
Subtask 1: Implant C57BL/6 mice with ID8 cells that have been modified using CRISPR/Cas9 to lack TRP53 or both TRP53 and BRCA2
Subtask 2: Treat mice with vehicle control, WNT974, WNT7A (n=10 in each group*4 groups/ cell line) with one untreated group.
Subtask 3: Quantify and sort CD8+ T cells, CD4+ T cells, and dendritic cells
Subtask 4: Monitor T-cell function

Subtask 5: Monitor gene expression by RNA seq (WNT, immune, HRD signatures)
Subtask 6: Extract RNA and use the iRep technique
Proposed Experiments: None.

4. **IMPACT:**

   - **What was the impact on the development of the principal discipline(s) of the project?**

     The data obtained from these studies lay the foundation for treatment strategies that will optimize ovarian cancer patient response to immunotherapy and to determine whether WNT signaling inhibition could be beneficial in other treatment settings such as in combination with neoadjuvant chemotherapy (NACT).

   - **What was the impact on other disciplines?**

     The data obtained from these studies lay the foundation for treatment strategies that could enhance cancer patient immune response, and ultimately lead to improved therapeutic success via increased sensitivity to immunotherapy and/or decreased chemoresistance.

   - **What was the impact on technology transfer?**

     Nothing to Report.

   - **What was the impact on society beyond science and technology?**

     I continue to serve on multiple boards and panels that pertain to patient education and patient advocacy.

5. **CHANGES/PROBLEMS:**

   - **Changes in approach and reasons for change**

     In ***Specific Aim 1***, we were unable secure data from GOG 218 trial hence we moved forward with analyzing data from our 18-Study with pre- (treatment naïve) and post-NACT matched patient samples.

     In ***Specific Aim 2***, we used a DKK1 over-expressing ID8 cell line ($ID8^{DKK1}$) as an additional cell line for analyses and comparison. DKK1 is a negative regulator of Wnt signaling, so altering DKK1 function (via DKN-01 monoclonal antibody) will alter Wnt signaling.

   - **Actual or anticipated problems or delays and actions or plans to resolve them**

     COVID-19 outbreak significantly limited in-lab research during the months of July 2020-December 2020. This also impacted in-person training on lab techniques and training. Furthermore, the SGO and ASCO conferences were virtual this year, and this platform limited collaborative conversations with other researchers and clinicians in the field.

   - **Changes that had a significant impact on expenditures**

     During July 1, 2018-June 30, 2019, it came to our attention that the amount of training needed to prepare clinical fellows (with minimal, if any, basic science background) to properly execute proposed experiments was mirrored by a lac in proposed study supply

utilization. During this time, only one research technician was executing experiments, teaching clinical fellows, and analyzing data. This resulted in an excess of unused funds for this progress period. In addition, COVID-19 outbreak during the months of July 2020-December 2020 delayed the hiring of a research technician due to a hiring freeze implemented by UAB. This, in addition to the first year's unused funds, provided us with enough monetary support for an additional research technician that officially joined the lab in May 2021 and has been an irreplaceable asset in driving our research forward.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  Nothing to Report.

- **Significant changes in use or care of human subjects.**

  Nothing to Report.

- **Significant changes in use or care of vertebrate animals.**

  MISIIR-Tag colony was lost, but we acquired new animals to gain back colony at UAB.

- **Significant changes in use of biohazards and/or select agents.**

  Nothing to Report.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

  - **Journal publications.**

    1) Goldsberry, W., *et al.* (June 2019). A review of the role of Wnt in cancer immunomodulation. *Cancers.* 11, 771. DOI: 10.3390/cancers11060771. (No federal support)
    2) Doo, D. Norian, L., and Arend, R. (June 2019). Checkpoint inhibitors in ovarian cancer: A review of preclinical data. *Gyn Onc Reports* 29: 48-54. (No federal support)
    3) Betella, I., Turbitt, J., Szul, T., *et. al*. (June 2020). Wnt signaling modulator DKK1 as an immunotherapeutic target in ovarian cancer. *Gynecol Oncol* 157(3): 765-774. (No)
    4) Wall, J., *et. al.* (May 2020). The anti-DKK1 antibody DKN-01 as an immunomodulatory combination partner for the treatment of cancer. *Expert Opin Investig Drugs.* DOI: 10.1080/13543784.2020.1769065. (No)
    5) Doo D., Meza-Perez S, Londono A. (April 2020). Inhibition of the Wnt/β-catenin pathway enhances anti-tumor immunity in ovarian cancer. *Ther Adv Med Oncol*. DOI: 10.1177/1758835920913798. (Yes; P30 CA013148/CA/NCI NIH HHS/United States)
    6) Goldsberry W., Meza-Perez S., Londoño A., *et al.* (March 2020). Inhibiting WNT Ligand Production for Improved Immune Recognition in the Ovarian Tumor Microenvironment. *Cancers (Basel).* 12(3):766. DOI:10.3390/cancers12030766. (Yes; W81XWH-18-1-0231/U.S. Department of Defense and R21 CA223126/NH/NIH HHS/United States)
    7) Wall JA, Meza-Perez S, Scalise CB, Katre AA, Londono, AI, Turbitt WJ, Randall TD, Norian LA, Arend RC. (November 2020). "Manipulating the Wnt/β-catenin signaling pathway to promote anti-tumor immune infiltration

into the TME to sensitize ovarian cancer to ICB therapy." *Gynecol Oncol*. PMID: 33168307.

8) Arend RC, Fisher AJ, Chou J, Jacobs I, Monk BJ. (January 2021). "Ovarian cancer: new strategies and emerging targets for the treatment of patients with advanced disease." *Crit Rev Oncol Hematol*. PMID: 33427569
9) Dholakia JJ, Scalise CB, Arend RC. (April 2021). "Assessing Preclinical Research Models for Immunotherapy for Gynecologic Malignancies." *Cancers (Basel)*. PMID: 33918476
10) Martinez A, Buckley M, Scalise CB, Katre AA, Dholakia JJ, Crossman D, Birrer MJ, Berry J, Arend RC. (April 2021). "Understanding the effect of mechanical forces on ovarian cancer progression." *Gynecol Oncol*. PMID: 33888338
11) Dholakia J, Scalise CB, Katre AA, Goldsberry WN, Meza-Perez S, Randall TD, Norian LA, Arend RC. (2022) "Sequential modulation of the Wnt/β-catenin signaling pathway enhances tumor-intrinsic MHC I expression and tumor clearance." *Gynecol Oncol*.
12) Martinez, A., Buckley, M.S, Scalise, C.B., Wang, D., Katre, A., Birrer, M.J., Berry, J., and Arend, R.C. (2021). Utilization of a 3-D tissue engineered model to investigate the effects of perfusion on gynecologic cancer biology. *Journal of Tissue Engineering* – under review.

- **Books or other non-periodical, one-time publications.**

  Nothing to Report.

- **Other publications, conference papers, and presentations.**

  - Oral Presentations

    1) IGCS Annual Meeting – Rio de Janeiro, Brazil. Arend RC (September 2019). Immunotherapeutic potential of non-immunotherapy drugs: enhancing immunogenicity of cold tumors. *Oral Presentation.*
    2) SGO Annual Meeting – Virtual. Arend RC, Castro C, Matulonis U, *et. al.* (March 2020). DKN-01 treated patients with recurrent epithelial endometrial (EEC) or ovarian (EOC) cancers which harbor Wnt activating mutations have longer progression free survival and improved clinical benefit. *Oral Presentation.*
    3) Dholakia J, Wall JA, Scalise CB, Katre AA, Arend RC. Towards a 'hot' tumor phenotype: DKN-01 sensitizes the tumor micro-environment via pro-immune cell cytokine release in vitro and ex vivo. *Featured Oral Presentation*, SGO Annual Meeting, Virtual, March 2021.
    4) Arend RC, Castro C, Matulonis UA, Hamilton EP, Gunderson C, Lybarger KS, Goodman HM, Duska L, Mahdi H, ElNaggar AC, Kagey M, Barroilhet LM, Bradley WH, Sachdev J, Sirard C, O'Malley DM, Birrer MJ. Patients with recurrent gynecologic cancers and Wnt activating mutations demonstrated greater clinical benefit when treated with DKN-01 therapy. *Featured Oral Presentation*, SGO Annual Meeting, Virtual, March 2021.

  - Poster Presentations

    1) *AACR Ovarian Conference – Atlanta, GA. Goldsberry W, Wall JA, Meza-Peres S, *et. al*. (September 2019). PORCN Inhibition Prolongs Survival, decreases tumor Burden, and Alters the Immune Microenvironment in Ovarian Cancer. *Poster Presentation.*

2) *ASCO Annual Meeting – Virtual. Wall JA, Katre A, Meza-Perez S, *et. al.* (May 2020). Utilizing porcupine (PORCN) and DKK1 inhibition to improve anti-tumor immunity in a murine model of ovarian cancer. *Poster Presentation.*
3) Arend RC, Castro C, Matulonis UA, Hamilton EP, Gunderson C, Lybarger KS, Goodman HM, Duska L, Mahdi H, ElNaggar AC, Kagey M, Barroilhet LM, Bradley WH, Sachdev J, Sirard C, O'Malley DM, Birrer MJ. Patients with recurrent epithelial endometrial cancers (EEC) and Wnt signaling alterations demonstrated greater clinical benefit when treated with DKN-01 monotherapy. *Poster Presentation*, AACR Virtual Special Conference on Endometrial Cancer: New Biology Driving Research and Treatment, November 2020.
4) Dholakia, J. and Arend, R. Immunotherapeutic potential of non-immunotherapy drugs. University of Alabama at Birmingham O'Neal Cancer Center Young Investigator Symposium, November 2020.

- Abstracts

1) SGO Annual Meeting – Goldsberry, W., *et. al.* (March 2019). Inhibition of PORCN in a p53-/- Knockout Syngeneic Ovarian Cancer Model. *Abstract.*
2) SGO Annual Meeting – Doo, D. *et al.* (March 2019). Inhibition of the Wnt/β-catenin pathway enhances anti-tumor immunity in ovarian cancer. *Abstract.*
3) *SGO Annual Meeting – Virtual. Goldsberry W, Wall JA, Meza-Perez S, *et. al.* (March 2020). Inhibition of PORCN in a p53-/- Knockout Syngeneic Ovarian Cancer Model. *Abstract.*
4) Dholakia J, Arend RC. Modification of intrinsic ovarian cancer tumor factors by Wnt-pathway agents influences macrophage recruitment and activity. *Abstract*, ASCO Annual Meeting, Virtual, June 2021.
5) Martinez A, Buckley M, Berry J, Birrer MJ, Arend RC. Using 3D perfusion bioreactor system to studying cell biology of Ovarian Cancer. *Abstract*, ASCO Annual Meeting, Virtual, June 2021.

- Lectures:

1) "Immunotherapeutic Potential of Non-Immunotherapy Drugs: Turing Cold tumors Hot" *Mayo GYN Seminar Series* – October 30, 2020, Virtual.
2) "Invited Distillant, Focused Plenary VI: Pre-Clinical Innovation - Exploring New Horizons" *SGO Annual Meeting* – March 21, 2021, Virtual.
3) "Other Potential Biomarkers in Ovarian Cancer" *University of Colorado Reproductive Sciences Seminar* – April 6, 2021, Virtual.
4) "Immunotherapeutic Potential of Non-Immunotherapy Drugs: Turing Cold tumors Hot" *University of Colorado OB-Gyn Grand Rounds* – April 7, 2021, Virtual.
5) "Invited Discussant, Gynecologic Cancer Poster Discussion Session: Using Biomarkers to Improve Outcomes in Ovarian Cancer" *ASCO Annual Meeting* – June 4, 2021, Virtual.

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report.

- **Other Products**

  - ***Biospecimen collections:***

    During the course of study, we continued to bank multiple ovarian cancer tissues and matched blood from patients at UAB. These specimens will be used in patient-derived xenografts (PDX) and 3-dimensional (3-D) organoid models to further study the effects of WNT pathway alteration on immune and chemotherapy response.

  - ***Clinical interventions:***

    The results from this study provided evidence for the use of WNT and/or AKT1 inhibitors as a means to enhance ovarian cancer's anti-tumor immune response. Importantly, these interventions could sensitize ovarian cancer to immune checkpoint blockade (ICB) therapy.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	**Dr. Rebecca Arend**
Project Role:	Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-2108-3426
Nearest person month worked:	3
Contribution to Project:	Dr. Arend has conceptualized and designed all the mouse model experiments. She has given expert advice for treatment regimen and harvesting and processing tissues from experiments.
Funding Support:	N/A

Name:	**Dr. Michael Birrer**
Project Role:	Mentor
Researcher Identifier (e.g. ORCID ID):	0000-0001-6464-4225

Nearest person month worked:	1
Contribution to Project:	Dr. Birrer has provided mentorship in my career and guidance in designing experiments and analyzing data. He is also helping in acquisition of data from GOG trials.
Funding Support:	N/A

Name:	**Dr. Kunle Odunsi**
Project Role:	Mentor
Researcher Identifier (e.g. ORCID ID):	0000-0002-4444-7651
Nearest person month worked:	1
Contribution to Project:	Dr. Odunsi has provided guidance in designing experiments, professional guidance and analyzing data.
Funding Support:	N/A

Name:	**Dr. Troy Randall**
Project Role:	Mentor
Researcher Identifier (e.g. ORCID ID):	0000-0003-0643-0311
Nearest person month worked:	1
Contribution to Project:	Dr. Randall provided guidance in experimental design related to immunology.
Funding Support:	N/A

Name:	**Dr. Sara Cooper**
Project Role:	Faculty Investigator at Hudson Alpha Institute for Biotechnology
Researcher Identifier (e.g. ORCID ID):	0000-0002-9627-0309
Nearest person month worked:	9
Contribution to Project:	Dr. Cooper has analyzed RNA sequencing data for this study.

Funding Support:	N/A

Name:	**Dr. David Doo**
Project Role:	Clinical Gynecologic Oncology Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9
Contribution to Project:	Dr. Doo has performed and contributed to *in vitro* and *in vivo* experiments described in the study.
Funding Support:	N/A

Name:	**Dr. Whitney Goldsberry**
Project Role:	Clinical Gynecologic Oncology Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9
Contribution to Project:	Dr. Goldsberry has performed and contributed to *in vitro* and *in vivo* experiments described in the study.
Funding Support:	N/A

Name:	**Ashwini Katre**
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	Ashwini Katre has performed animal experiments described in the study.
Funding Support:	N/A

Name:	**Dr. Jhalak Dholakia**

Project Role:	Clinical Gynecologic Oncology Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9
Contribution to Project:	Dr. Dholakia has performed non-animal and animal experiments described in the study.
Funding Support:	N/A

Name:	**Dr. Lyse Norian**
Project Role:	Mentor
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Norian has provided guidance in designing experiments, professional guidance and analyzing data.
Funding Support:	N/A

Name:	**Dr. Carly Bess Scalise**
Project Role:	Postdoctoral Research Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Dr. Scalise has aided in experimental design and data analysis.
Funding Support:	N/A

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to Report for July 1, 2021-June 30, 2022. However, there was a change in the "Other Mentor" position in 2020 due to fact that the "Designated Mentor," Dr. Michael Birrer, relocated to another institution. Dr. Troy Randall was promoted from his role as a collaborator to "Other Mentor." Dr. Birrer remained as the primary mentor and continued

to participate in activities to develop and aid in sustaining Dr. Arend's independent career in ovarian cancer research. In addition, Dr. Kunle Odunsi was promoted to a collaborator, and continued to also mentor Dr. Arend. Dr. Arend's mentorship team was is an integral component to her career development. She learned molecular biology and immunology techniques through attending research meetings and collaborations.

- **What other organizations were involved as partners?**

Organization Name	**Novartis**
Location of Organization	Basel, Switzerland
Partner's contribution to the project	
Financial support	N/A
In-kind support *(e.g., partner makes software, computers, equipment, etc., available to project staff)*;	Supplied WNT-974 compound for studies
Facilities *(e.g., project staff use the partner's facilities for project activities)*;	N/A
Collaboration *(e.g., partner's staff work with project staff on the project)*;	N/A
Personnel exchanges *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site)*;	N/A
Other	N/A

Organization Name	**HudsonAlpha Institution for Biotechnology**
Location of Organization	Huntsville, AL
Partner's contribution to the project	
Financial support	N/A
In-kind support *(e.g., partner makes software, computers, equipment, etc., available to project staff)*;	N/A
Facilities *(e.g., project staff use the partner's facilities for project activities)*;	N/A
Collaboration *(e.g., partner's staff work with project staff on the project)*;	Analyzed RNA-sequencing data
Personnel exchanges *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site)*;	N/A
Other	N/A

8. Organization Name	LEAP Therapeutics
Location of Organization	Massachusetts
Partner's contribution to the project	[redacted]
Financial support	N/A
In-kind support *(e.g., partner makes software, computers, equipment, etc., available to project staff)*;	Supplied DKN-01 monoclonal antibody for studies.
Facilities *(e.g., project staff use the partner's facilities for project activities)*;	N/A
Collaboration *(e.g., partner's staff work with project staff on the project)*;	N/A
Personnel exchanges *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site)*;	N/A
Other	N/A

Organization Name	Curegenix
Location of Organization	San Francisco Bay Area
Partner's contribution to the project	[redacted]
Financial support	N/A
In-kind support *(e.g., partner makes software, computers, equipment, etc., available to project staff)*;	Supplied CGX-1321 compound for studies
Facilities *(e.g., project staff use the partner's facilities for project activities)*;	N/A
Collaboration *(e.g., partner's staff work with project staff on the project)*;	N/A
Personnel exchanges *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site)*;	N/A
Other	N/A

9. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:**

  Nothing to Report.

- **QUAD CHARTS:**

  Nothing to Report.

10. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***