UNIVERSITY OF CALIFORNIA SAN DIEGO

# Type-I interferon regulates resistance to carboplatin in the human ovarian cancer cell line CAOV3

A thesis submitted in partial satisfaction of the requirements
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in

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by

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The thesis of Hrishi Venkatesh is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2018

# DEDICATION

I dedicate this thesis to my parents, for their love and support.

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Figures 1A and 2A are from the work of Dr. Olivier Harismendy and Dr. Stephen B. Howell. Figures 1-11, in full are currently being prepared for submission for publication of the material. Champa, Devora; Venkatesh, Hrishi; Wenzel, Alexander; Sun, Si; Tsai, Cheng-Yu; Bui, Jack; Howell, Stephen B.; and Harismendy, Olivier. "Evolution and Characterization of Carboplatin Resistance at a single-cell level." The thesis author was a co-author of this material.

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ABSTRACT OF THE THESIS

Type-I interferon Regulates Chemoresistance in the Human
Ovarian cancer cell line CAOV3

by

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Interferons are a group of signaling proteins produced by host cells in response to infection by a variety of pathogens, as well as during cancer. Type-I interferons are

produced by immune and non-immune cells when pathogenic or self-nucleic acid is recognized by cytosolic sensors such as STING. In the context of chemoresistance in cancer, type-I interferons have been predominantly shown to play anti-tumor roles through direct tumor killing and enhancement of anti-tumor immunity. However, recent work by Andy Minn's group hints at a potential pro-tumor role for interferons. In this thesis, we take advantage of the parental and resistant clones of the human ovarian cancer cell line generated by Dr. Oliver Harismendy and Dr. Stephen Howell's group. We were able to reproduce Dr. Harismendy and Dr. Howell's findings showing that the resistant clones had increased percent survival in response to carboplatin, and that the resistant clones had a type-I interferon gene signature. Treatment of the resistant clones with the pharmacological JAK inhibitor Ruxolitinib resulted in a pronounced loss of resistance to carboplatin, particularly in clone 18 that had the strongest type-I interferon gene signature. A type-I interferon neutralizing antibody was also able to reduce resistance in clone 18. Conversely, pre-treatment of the parental clone with either human interferon-b or the human STING agonist G10 induced resistance to carboplatin. We were thus able to demonstrate that type-I interferon signaling is necessary and sufficient to induce resistance to carboplatin in the human ovarian cancer cell line CAOV3.

# I.

# Introduction

**Interferons**

Interferons are a group of signaling proteins produced by host cells in response to infection by a variety of pathogens, and during cancer. They were first studied in viral infections and were named for their ability to interfere with viral replication.[1] Interferons belong to three main classes. The two most well characterized classes are type-I interferons and type-II interferons. Type-I interferons consist of interferon-α and interferon-β, as well as other less-characterized members, and are produced by immune and non-immune cells in response to recognition of pathogen or self-nucleic acid in the cytosol by nucleic acid sensors such as STING and RIG-I[2]. Type-II interferons consist of interferon-γ alone and are produced by immune cells in response to various pro-inflammatory stimuli. Type-III interferons are analogous to type-I interferons, however are only produced by epithelial cells.[2]

**Role of interferons in cancer**

In the context of cancer, both type-I interferons and interferon-γ were thought to predominantly play anti-tumor roles. There is an abundance of literature characterizing the importance for interferons in inducing robust anti-tumor immunity and in controlling tumor growth itself. Interferons have been shown to kill tumor cells directly[1,4] and improve the cytotoxic tumor-killing potential of CD8 T cells and NK cells.[1,3] Interferons have also been shown to be important for regulating cross-presentation in $CD8^+$ dendritic cells[4], and for inducing macrophage polarization into an anti-tumor M1 phenotype[5].

In contrast, the literature characterizing the pro-tumor roles of interferons is scant. There have been reports as early as 2004 indicating that interferon-γ was associated with immune suppression in cancer via induction of PDL1 on tumor cells[7,9]. Recently, cutting-edge work by Andy Minn's group detailed the role of both type-I interferons and interferon-γ in regulating resistance to checkpoint blockade therapy[8]. The paper showed that interferon-induced STAT1

activation regulated a resistance program in tumor cells chiefly driven by the upregulation of various immune-inhibitory ligands such as PDL1, TIM3 and LAG3. While interferon-γ was shown to be necessary and sufficient for resistance, type-I interferons were shown to be necessary but not sufficient to induce 'adaptive resistance' to checkpoint blockade therapy[8]. However, the tumor cell-intrinsic impact of interferons was not studied.

Previous work from our lab has shown that interferon-γ was necessary to promote tumor progression in $RAG^{-/-}$ mice in the inducible MCA-sarcoma tumor model, and that this was associated with a significant increase in the expression of interferon-β by tumor cells[10]. However, it was not determined whether interferon-β had a functional impact on tumor cells.

**Ovarian Cancer**

Ovarian cancer in the seventh most commonly diagnosed cancer among women in the world[12]. The most common sub-type of malignant ovarian cancer is high-grade serous ovarian cancer that arises from epithelial cells lining the fallopian tube in the ovary.[11] It is extremely aggressive and associated with the lowest survival rates.[11] Most patients with high grade ovarian cancers are diagnosed at stage III or later, when the primary tumor has begun spreading into the peritoneum and in some cases even metastasized to the peritoneal lymph nodes[12]. Treatment approaches to ovarian cancer are a combination of surgery and adjuvant chemotherapy[12]. For high-grade serous cancers, the first line of treatment is generally surgical cytoreduction followed by a combination of platinum-based chemotherapy such as cisplatin or carboplatin, and paclitaxel[12]. In some cases, a few cycles of neoadjuvant chemotherapy with carboplatin and paclitaxel is given prior to surgery and post-surgery chemotherapy, especially if the patient is too ill for primary surgery or if the tumor burden is sufficiently large to prevent complete resection[12]. However, over 80% of patients with stage III or stage IV high grade serous cancers relapse[12].

Barring rare cases, recurrent ovarian cancer is generally incurable and current approaches are limited to management of the disease[12].

**Resistance to platinum-based chemotherapy**

Resistance to chemotherapy is a common phenomenon observed in cancer and a pertinent health concern. This phenomenon has been extensively studied in platinum-based chemotherapies since their inception[15,16]. The outcome of chemoresistance is either non-responsiveness to chemotherapy, or an initial response followed by relapse[13]. Resistance to chemotherapy can either be inherited i.e. cancer cells are intrinsically chemoresistant or acquired i.e. cancer cells are altered in a certain way and become resistant[13,14]. Intrinsic resistance is associated with increased activity of certain oncogenes such as MYC and AKT, increased expression of pro-survival factors such as BCL-2, inhibition of DNA-damage response, as well as alteration of certain pathways necessary for normal stem cell function such as WNT signaling[14]. Mechanisms of acquired resistance include transfer of survival factors to sensitive cells via exosomes, changes associated with acquisition of migratory and invasive mesenchymal stem cell phenotype via Epithelial-Mesenchymal Transition, and epigenetic changes such as the STAT1-mediated induction of inhibitory ligands described by Andy Minn's group[8,14].

An abundance of work has characterized the importance of interferons in mitigating chemoresistance. Research has shown that both interferon-γ and tumor-produced type-I interferons synergize with chemotherapy to kill tumor cells via immune activation[1,6,17]. However, potential pro-tumor roles of type-I interferons in the context of chemoresistance have not been sufficiently explored. To study this, we take advantage of parent and carboplatin-resistant clones of the human ovarian cancer cell line CAOV3 developed by Dr. Harismendy and Dr. Howell's

group[18]. RNA sequencing data from Dr. Harismendy and Dr. Howell implicates type-I interferon signaling in regulating chemoresistance in the CAOV3 cell line[18]. Using RT-qPCR, we show that type-I interferons are upregulated in the resistant clones relative to the parent clone. Blocking interferon signaling via a pan-JAK inhibitor, or specific blockade of type-I interferon signaling in the resistant clones abrogated chemoresistance and resulted in a net negative growth rate in the presence of chemotherapy. Pre-treatment of the parent clone with exogenous interferon-β induced resistance to and increased growth rate in the presence of low doses of carboplatin. A similar effect on resistance in the parent clone was seen when a human STING agonist was used. We were thus able to determine that interferon signaling was necessary and sufficient for chemoresistance to carboplatin in the human ovarian cancer cell line CAOV3.

# II.

# Results

**Generation of parental and carboplatin-resistant clones of the ovarian cancer cell line CAOV3**

The thesis originated from work by Dr. Olivier Harismendy and Dr. Stephen Howell's group at UCSD studying resistance to carboplatin in the human ovarian cancer cell line CAOV3[18]. Resistance to platinum-based chemotherapy drugs via repeated exposure to chemotherapy has been well-documented[15,16]. However, heterogeneity between and within cell lines and the lack of selection replicates have made identification of molecular mechanisms of drug resistance challenging. In order to minimize variability in the system, a single-clone approach was adopted to generate the sensitive parental and carboplatin-resistant clones of the ovarian cancer cell line CAOV3 (Figure 1A)[18].

The CAOV3 parental clone was derived from a single cell of the ovarian cancer cell line CAOV3. To generate the resistant clones, cells were seeded at a density of 4 x $10^5$ cells/well in a six-well plate[18]. The cells were then treated with 4 cycles of 5μM carboplatin; each cycle consisting of treatment with carboplatin for 7 days, followed by an approximately 2 week resting period until they resumed growth and reached confluence (Figure 1A)[18]. To generate the resistant clones used in this study, the previous resistant clones were treated with additional cycles of carboplatin at gradually increasing concentrations up to 15μM[18]. In line with Dr. Harismendy and Dr. Howell's data, we observed an increase in percent survival in resistant clones after 96h of carboplatin treatment (Figure 1C); although the degree of resistance we observed was weaker than what was reported by them[18].

**Carboplatin resistance is associated with a type-I interferon gene signature in the Ovarian cancer cell line CAOV3**

To determine the mechanism of resistance, expression of various genes in the parental clone and the 4 resistant clones was analyzed. RNA sequencing data from Dr. Harismendy and Dr. Howell showed that type-I interferon signaling was upregulated in the resistant clones relative to the parental clone, especially in Clone 16 and clone 18 (Figure 2A)[18]. To confirm the RNA sequencing data, the expression of type-I interferons in the parental and the two resistant clones was determined by RT-qPCR. We observed that interferon-α and interferon-β were upregulated in the resistant clones relative to the parental clone. Interestingly, interferon-β was much more strongly upregulated in clone 18 relative to the other clones (Figure 2B). This was associated with a concomitant increase in the expression interferon-induced gene ISG15 in clone 18 and clone 16 relative to the parental clone (Figure 2B). Thus, we validated that the carboplatin resistant clones, in particular clones 16 and 18, had a type-I interferon gene signature.

**Validation of the Ruxolitinib in the CAOV3 cell line**

Although the carboplatin-resistant clones were shown to have a type-I interferon gene signature, it was not clear whether the type-I interferon signaling had a functional consequence. To test the impact of the enhanced type-I interferon signaling on chemoresistance, we used Ruxolitinib: a pharmacologically-approved inhibitor of the JAK-STAT pathway[20,21]. The JAK-STAT pathway lies downstream of the interferon receptor and is necessary for both type-I and type-II interferon signaling[19,20]. Ruxolitinib specifically inhibits JAK1 and JAK2[20].

To validate that Ruxolitinib inhibits type-I interferon signaling in the CAOV3 cell line, we treated the parental clone with 1000 units of human interferon-β for 24 hours (Figure 3A).

Half of the wells treated with interferon-β were pre-treated with Ruxolitinib for 24 hours, following which the expression of the interferon-induced gene ISG15 was determined by RT-qPCR. We observed that Ruxolitinib was able to completely repress the Interferon-β mediated induction of ISG15 in the parental clone (Figure 3A).

Although Ruxolitinib was shown to strongly repress type-I interferon signaling, it was possible that potential cytotoxic effects of Ruxolitinib could be responsible for the decrease in expression of ISG15 seen in the Ruxolitinib-treated parental clone: selective killing of cells that upregulated ISG15 expression in response to Interferon-β treatment could explain the RT-qPCR data. Thus, we decided to test the effects of various concentrations of Ruxolitinib on the parental and resistant clones. To simulate the exposure to Ruxolitinib in subsequent dose response assays, we seeded the parental and resistant clones at a density of $2 \times 10^3$ cells/well. The cells were then treated with the indicated concentrations of Ruxolitinib for 5 days, following which the number of live cells in each well was counted using a hemocytometer. We observed that at concentrations up to 10μM, Ruxolitinib alone had no significant effect on the survival of the parental and resistant clones (Figure 3B). However, we observed a significant reduction in the number of cells in the presence of 20μM Ruxolitinib (Figure 3B).

When the parental clone was treated with 5μM Ruxolitinib, the Interferon-β mediated induction of ISG15 was completely abrogated. Furthermore, we showed that at this concentration, Ruxolitinib alone had no significant impact on survival in the parental or the resistant clones. Thus, for future experiments, Ruxolitinib was used at a concentration of 5μM.

**Ruxolitinib reduces resistance to carboplatin in the resistant clones**

Dr. Harismendy and Dr. Howell had previously shown that the resistant clones had a slower growth rate than the parental clone[18]. Based on the previous survival assay, we seeded 2 x $10^3$ cells of the parental clone and the resistant clones in a 96-well plate. The resistant clones were either pre-treated with 5μM of Ruxolitinib or the equivalent amount of DMSO 24h prior to treatment with the indicated doses of carboplatin for 96 hours (Figure 4A). The number of cells in untreated and carboplatin-treated wells was then counted for each clone and percent survival relative to untreated cells was determined for each clone. We observed that Ruxolitinib weakly reduced percent survival in clone 6 and had no impact on clone 14 (Figure 4B). Ruxolitinib reduced percent survival in clone 16, especially at 30μM or higher concentrations of carboplatin. In clone 18, Ruxolitinib completely abrogated chemoresistance with percent survival values similar to the parental clone being observed. Interestingly, Ruxolitinib seemed to weakly increase percent survival in the parent clone in response to carboplatin (Figure 4C). Thus, Ruxolitinib reduced percent survival to carboplatin in the resistant clones, in particular to clones 16 and 18.

While percent survival normalizes for the number of untreated cells of each clone, it does not take into account the difference in the growth rate between the clones. A recent paper described a protocol to accurately measure drug sensitivity while taking into account differences in proliferation[22]. The authors propose a method that takes into account the Growth Rate of the cells before and after chemotherapy treatment[22]. The method the authors employ is described in Figure 5A. We seeded the parent and resistant clones in 96-well plates and pre-treated with Ruxolitinib as described in the previous experiment. However, this time, we also included 2 "control wells" per clone per pre-treatment condition. Thus, there were 4 "control wells" per parent/resistant clone: 2 treated with DMSO and 2 treated with Ruxolitinib. The next day prior to

carboplatin treatment, the number of cells in the "control wells" were determined using a hemocytometer (Figure 5A). 96 hours after carboplatin treatment, the number of cells in the remaining "treatment wells" were counted and Normalized Growth Rate was determined by the formula shown in Figure 5A[22]. Similar to percent survival, we observed that Ruxolitinib had a very weak effect on the growth rate of clone 6 and no effect on clone 14 (Figure 5B). We were able to see a strong reduction in growth rate in clone 16 at 30μM or higher concentrations of carboplatin. However, clone 18 showed an extremely strong reduction in growth rate in response to Ruxolitinib. In fact, Ruxolitinib was able to induce a net negative growth rate in clone 18 cells treated with carboplatin. This effect was seen even in low doses of carboplatin (Figure 5B). As with percent survival, Ruxolitinib seemed to weakly increase the growth rate of the parental clone treated with carboplatin (Figure 5C). Thus, we showed that Ruxolitinib strongly reduced growth rate in response to carboplatin in clone 18 and to some extent, in clone 16.

**Growth curves of parental and resistant clones**

When calculating relative growth rate, a crucial assumption made is that the untreated cells are in an exponential growth phase[22]. To confirm that the parental and resistant clones are in exponential phase, we decided to generate growth curves for the clones at 3 seeding densities: $5 \times 10^2$ cells/well, $1 \times 10^3$ cells/well and $2 \times 10^3$ cells/well (Figure 6A). Each of the clones were seeded at each of the densities in triplicate. The number of cells of each clone was counted 24, 48, 72 and 96h post-seeding. The growth curves of the parental and resistant clones showed that the parental clone grew the quickest (Figure 6B). Clone 6 grew slightly slower than the parent (Figure 6C). However, clone 14, clone 16 and clone 18 grew significantly slower than the parental clone (Figures 6C and 6D), with clone 18 growing the slowest. All of the clones were in exponential phase 96h post seeding.

**Ruxolitinib reduces resistance to lower concentrations of carboplatin in the resistant clones**

Although significant differences in survival and growth rate were observed in clone 18 and to some extent in clone 16, we observed extremely high cell death even at the lowest concentration of carboplatin used. This meant that our growth curves did not show a linear decline that is crucial for interpretation of the results. We thus decided to use a lower concentration range of carboplatin to give us more reliable data.

Based on the growth curves generated in Figure 6, the parental and resistant clones were seeded at the following densities- parental clone and clone 6: 2 x $10^3$ cells/well, clone 14: 2.5 x $10^3$ cells/well, clone 16 and clone 18: 3 x $10^3$ cells/well. The cells were pre-treated with either 5μM Ruxolitinib or equivalent amount of DMSO. As with the previous experiment, 2 "control wells" were included per pre-treatment condition. The next day, the number of live cells in the "control wells" were determined using a hemocytometer. The remaining wells were treated with the indicated concentrations of carboplatin for 96 hours, following which the number of live cells in the carboplatin-treated and untreated wells were determined as described previously. Percent survival and relative growth rate were then calculated as described in Figures 4 and 5 respectively. Interestingly, we observed that Ruxolitinib had no effect on growth rate or survival in clone 6 and weakly reduced growth rate and survival in clone 14 (Figure 7B and 7C). This was contrary to what we observed with the previous concentration range (Figure 4B and 5B). We also observed that the JAK inhibitor strongly reduced survival and growth rate in clone 16 and clone 18 (Figures 7D and 7E). The effect on clone 16 was also different in this concentration range (Figure 7D, Figures 4B and 5B); however, the reduction of resistance in clone 18 was consistently observed in both concentration ranges (Figures 7D and 7E).

We thus determined that Ruxolitinib consistently reduced resistance to carboplatin in clone 18, and inconsistently in clone 14 and clone 16.

**Type-I interferon receptor neutralizing antibody weakly reduces survival to carboplatin in clone 18**

The fact that we observed the strongest effects in clone 18 correlates well with the RNA sequencing as well as RT-qPCR data showing that Clone 18 has the strongest type-I interferon signature. However, inhibition of the JAK-STAT pathway affects both type-I interferon and interferon-γ signaling. To specifically block type-I interferon signaling, we used a neutralizing antibody against the type-I interferon receptor (IFNAR). We first decided to test the efficacy of neutralizing antibody. To do so, we treated the parental clone with 100 units of human interferon-β for 48 hours (Figure 5A). Half of the wells treated with interferon-β were pre-treated with the IFNAR neutralizing antibody for 24 hours, following which the expression of the interferon-induced gene ISG15 was determined by RT-qPCR. We observed that the neutralizing antibody was able to significantly repress interferon-β induced gene ISG15; however, the expression of ISG15 after IFNAR blockade remained significantly higher than the untreated cells (Figure 8A). We determined that the IFNAR neutralizing antibody was able to neutralize interferon-β signaling upto 75%.

To test the effect of the IFNAR neutralizing antibody on regulating chemoresistance, the resistant clones 18 was pre-treated either with PBS or with 5μg/mL of the neutralizing antibody 24h prior to treatment with indicated doses of carboplatin for 96 hours (Figure 8B). Clone 18 was chosen as it showed the strongest response to the JAK inhibitor. The cells were again treated with the neutralizing antibody 48 hours after carboplatin was added. Percent survival was

determined as described earlier (Figure 8B). The IFNAR neutralizing antibody was able to reduce percent survival in response to carboplatin in clone 18 at all doses, albeit to a much lesser extent than the JAK inhibitor (Figure 8C). However, this could be explained by our observation that the antibody isn't able to completely repress type-I interferon signaling.

We thus showed that type-I interferon signaling was necessary for regulating chemoresistance, in particular in the carboplatin-resistant clone 18.

**Exogenous interferon-β is sufficient to induce resistance to carboplatin in CAOV3 cell line**

Having showed that tumor-intrinsic type-I interferon signaling is necessary for inducing resistance to carboplatin treatment in the human ovarian cancer cell line CAOV3, we decided to test whether exogenous type-I interferon was sufficient to induce resistance to chemotherapy. The parental clone was seeded in a 6-well plate at a density of $1 \times 10^5$ cells/well, and pre-treated either with PBS or 1000 units of human interferon-β for 7 days. The cells were passaged every 3 days. The untreated and interferon pre-treated cells were then seeded at a density of $1 \times 10^3$ cells/well in a 96-well plate. 3 control wells were seeded each for PBS/ interferon-β pre-treated cells. After 24 hours, these control wells were counted prior to treatment with the indicated concentrations of carboplatin for 96 hours (Figure 9A). The number of cells in the "treatment wells" were then counted, and percent survival and Normalized Growth Rate were calculated as described before (Figure 9A). Pre-treatment of the parent clone with interferon-β increased percent survival and relative growth rate in the parent clone at concentrations of carboplatin lower than 30μM (Figure 9B and 9C). Thus, exogenous interferon-β was able to induce resistance to concentrations of carboplatin below 30μM.

**Tumor-produced type-I interferon is sufficient to induce resistance to low-dose carboplatin in CAOV3 cell line**

We now decided to test whether tumor-produced type-I interferon was sufficient to induce resistance to carboplatin. To specifically induce type-I interferon with minimal off-target effects, we decided to take advantage of a STING agonist known as G10, that was recently characterized for its ability to specifically activate STING and induce type-I interferon in human cells[23]. We weren't able to use the well-characterized mouse STING agonist DMXAA due to its inability to bind to and activate human STING[24,25]. We thus decided to confirm that G10 could induce expression of type-I interferon in the CAOV3 cell line. We seeded the CAOV3 parental clone into 6-well plates at a density of 1 x $10^5$ cells/well and treated them either with DMSO or with the indicated doses of G10 for 24 hours, followed by RT-qPCR. We observed that G10 was able to weakly induce expression of interferon-α, which was associated with a strong upregulation of the interferon-induced gene ISG15 (Figure 10A).

We then decided to test whether G10 pre-treatment could induce resistance to carboplatin in the parental clone of the CAOV3 cell line. The parental clone was pre-treated with the indicated doses of G10 in a 6-well plate for 7 days, similar to the pre-treatment with interferon-β described previously. The untreated and G10 pre-treated cells were then seeded at a density of 1 x $10^3$ cells/well in a 96-well plate. After 24 hours, the cells were treated with the indicated dose of carboplatin for 96 hours, following which percent survival was determined as described previously (Figure 10B). Based on the results of the resistance assay with interferon-β pre-treated cells, we chose to test resistance at 3 doses below 60μM (Figure 10B). We observed that G10 pre-treated cells had higher percent survival to carboplatin compared to the DMSO pre-

treated cells at all of the doses tested (Figure 10C). We thus confirmed that tumor-produced type-I interferon was sufficient to induce resistance to low-dose carboplatin treatment.

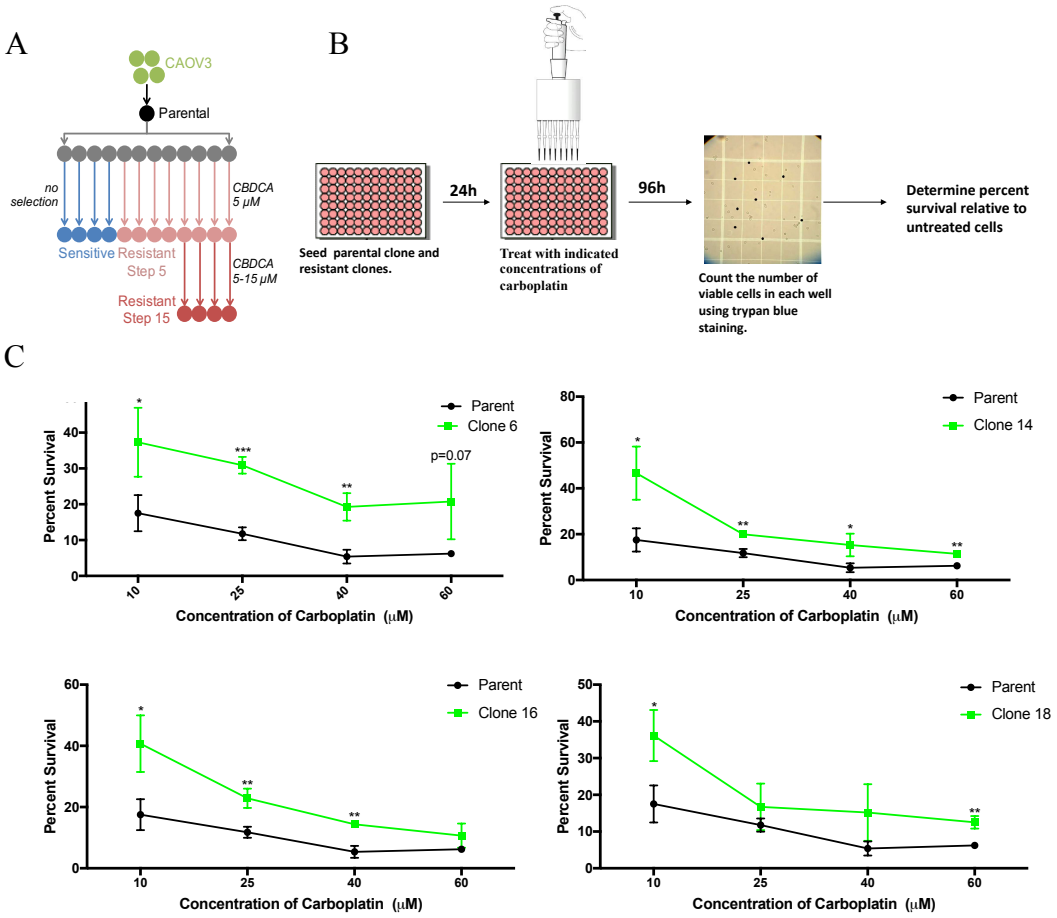
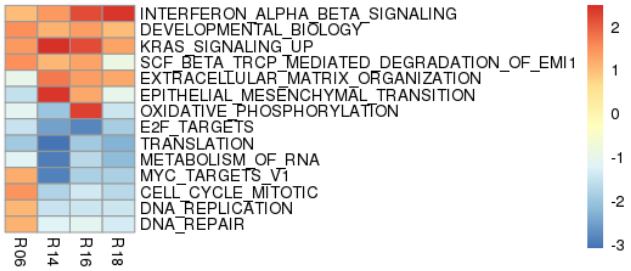



**Figure 1: Generation of parental and carboplatin-resistant clones of CAOV3 cell line**

A. Schematic showing how the parental clone and carboplatin-resistant clone were generated. Resistant clones 6, 14, 16 and 18 are generated at the end of step 15.
B. Survival assay protocol.
C. Percent survival of parental and carboplatin-resistant clones treated with the indicated concentrations of carboplatin. The error bars represent standard deviation.

A




B

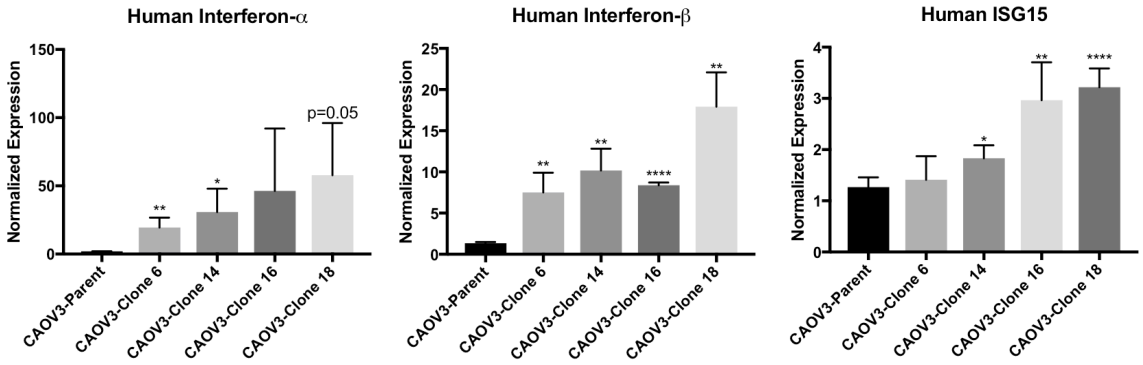
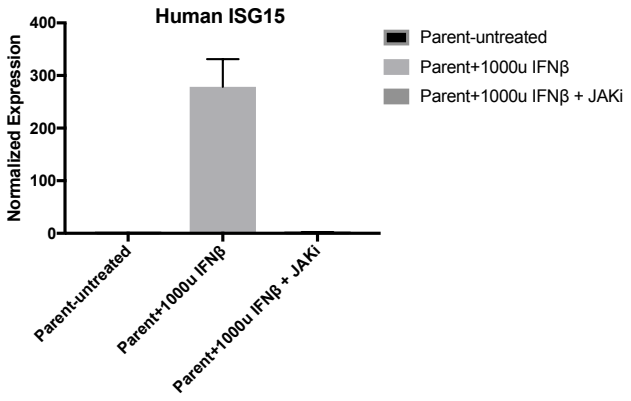



**Figure 2: Carboplatin resistance is associated with a type-I interferon gene signature in the CAOV3 cell line**

A. RNA sequencing data from Dr. Harismendy comparing the parental clone and the carboplatin-resistant clone

B. Expression of interferon-α and interferon-β, as well as the interferon-induced gene ISG15 in the parental clone and resistant clones. The error bars represent standard deviation.

A




B

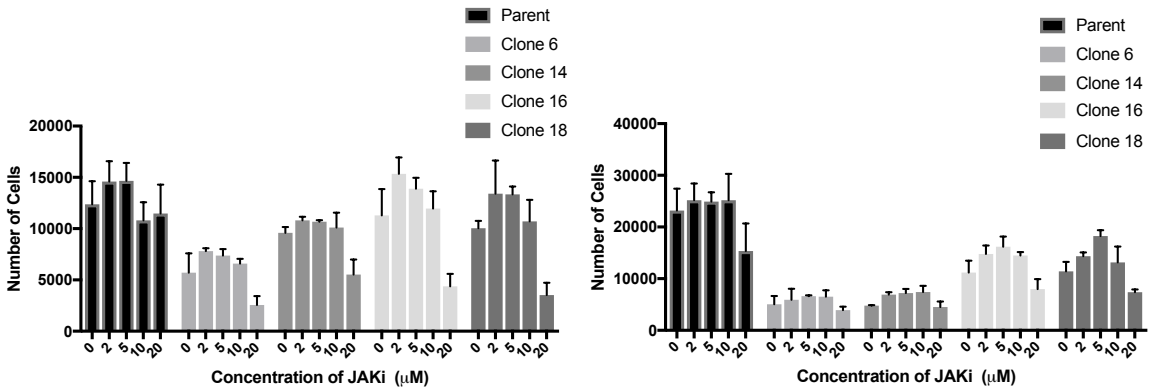
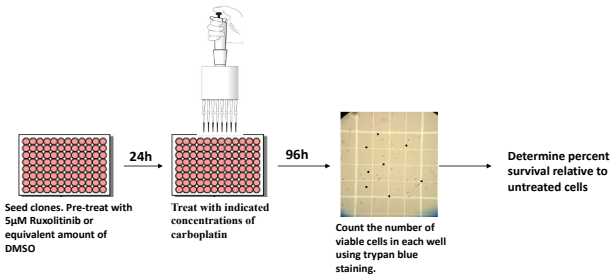



**Figure 3: Validation of the JAK1/2 inhibitor Ruxolitinib in the CAOV3 cell line**

A. Expression of the interferon-induced gene ISG15 in the parental clone alone or treated with indicated amount of interferon-β with and without pre-treatment with 5μM Ruxolitinib for an hour. The error bar represent standard deviation.

B. Number of cells of the parental and resistant clone treated with the indicated concentrations of Ruxolitinib for 5 days. Each graph represents an independent experiment. The error bars represent standard deviation.

A

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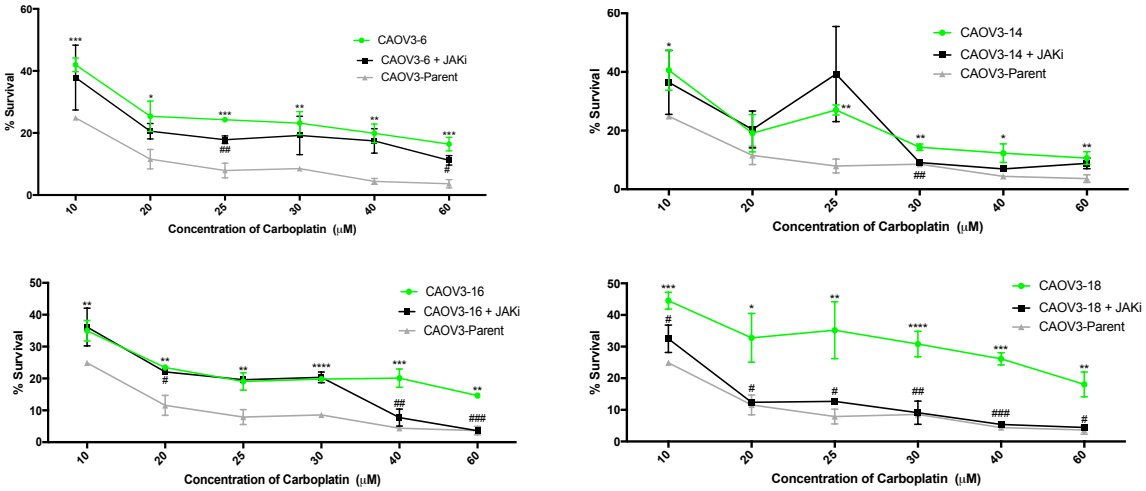



C

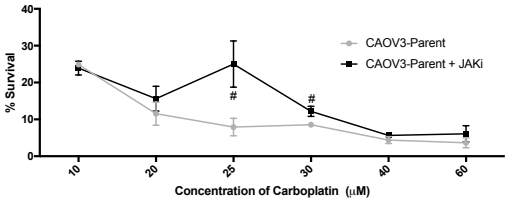



**Figure 4: The JAK 1/2 inhibitor Ruxolitinib reduces percent survival of resistant clones, but not the parental clone in response to carboplatin**

A. Protocol for survival assay including pre-treatment with Ruxolitinib
B. Percent survival of resistant clones with and without pre-treatment with Ruxolitinib. Asterisk (*) is for comparison between parental and resistant clone. Hashtag (#) is for comparison between the resistant clone with and without Ruxolitinib pre-treatment. The error bars represent standard deviation.
C. Percent survival of parental clone with and without Ruxolitinib pre-treatment. The error bars represent standard deviation.

**(Note: The curve for the "CAOV3-Parent" clone is included in all the plots for comparison purposes)**

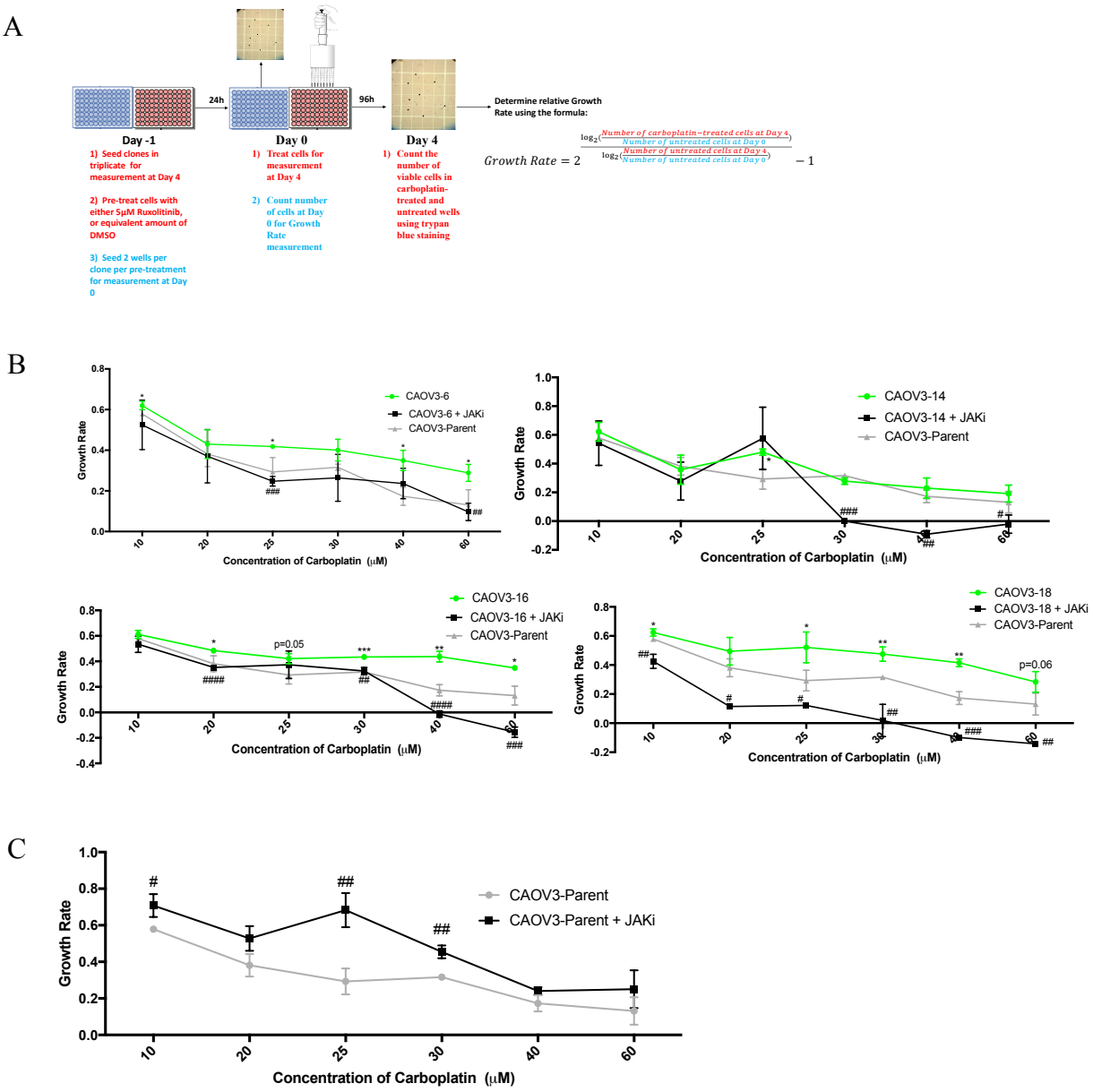



**Figure 5: Ruxolitinib reduces growth rate of resistant clones in response to carboplatin**

A. Protocol for growth rate assay with pre-treatment using JAK inhibitor Ruxolitinib, including the formula to calculate Growth Rate.

B. Normalized Growth Rate of resistant clones with and without pre-treatment with Ruxolitinib. Asterisk (*) is for comparison between parental and resistant clone. Hashtag (#) is for comparison between the resistant clone with and without Ruxolitinib pre-treatment. The error bars represent standard deviation.

C. Normalized Growth Rate of the parental clone with and without Ruxolitinib pre-treatment. The error bars represent standard deviation.

**(Note: The curve for the "CAOV3-Parent" clone is included in all the plots for comparison purposes)**

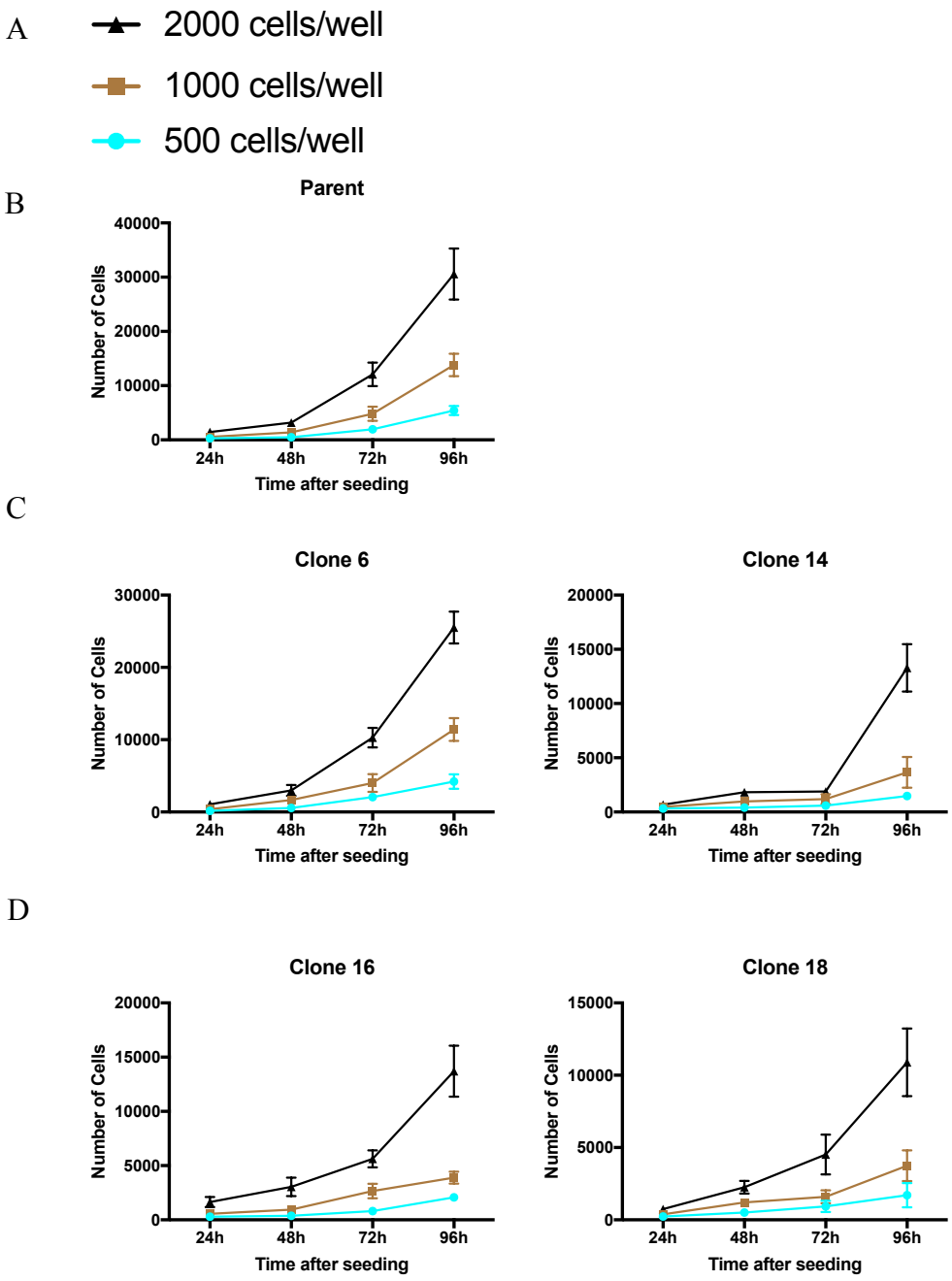



**Figure 6: Growth Curves of parental and resistant clones**

A. Seeding densities used for generating growth curves
B. Growth curve of the parental clone
C. Growth Curve of clone 6 and clone 14
D. Growth Curve of clone 16 and clone 18

The error bars represent standard deviation.

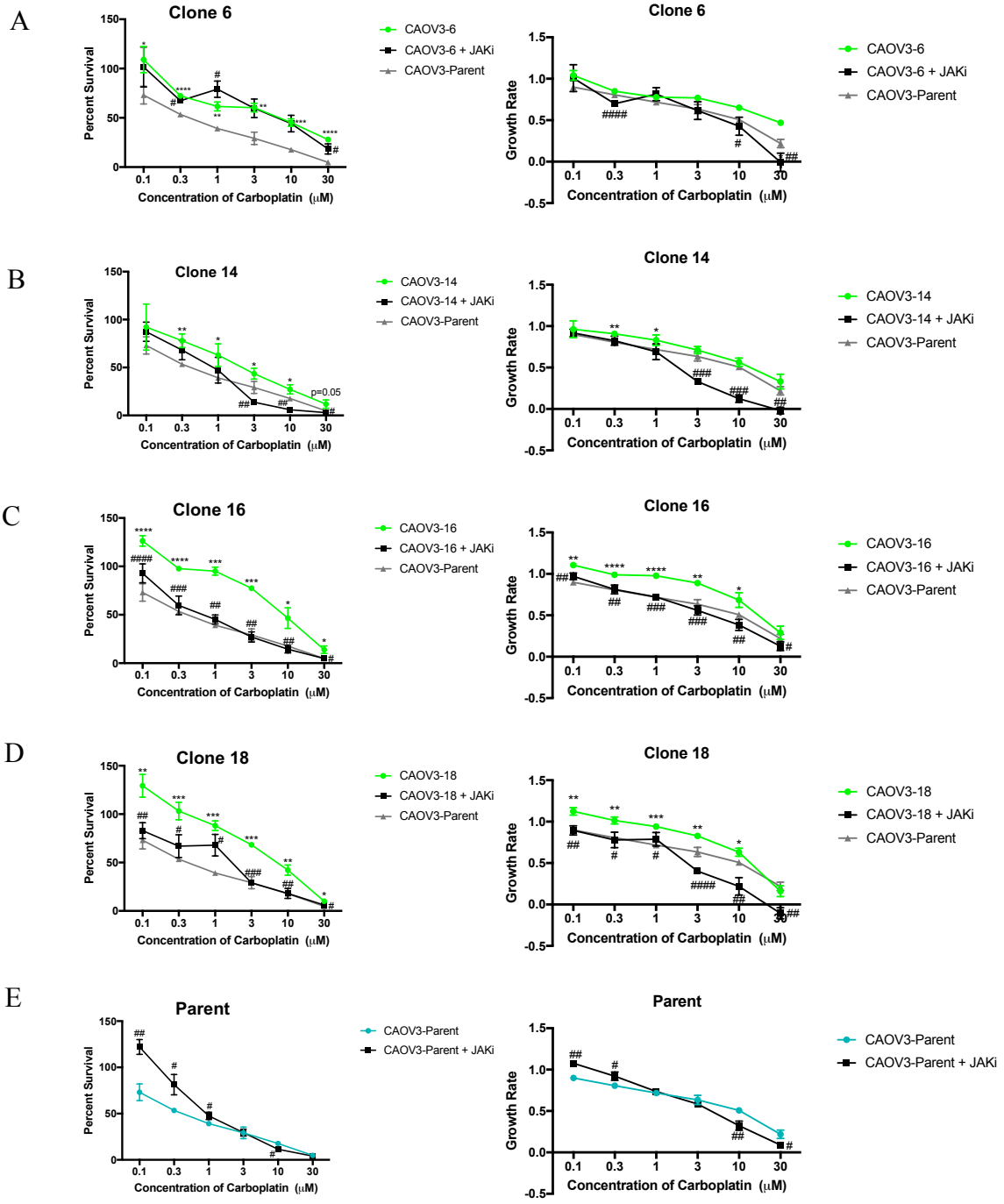



**Figure 7: Ruxolitinib reduces growth rate and percent survival of resistant clones in the presence of lower concentrations of carboplatin**

Percent Survival and Normalized Growth Rate of the resistant clones (Figures A-D) and the parental clone (Figure E) with and without JAKi pre-treatment in the presence of lower concentrations of carboplatin. Asterisk (*) is for comparison between parental and resistant clone. Hashtag (#) is for comparison between the parental/resistant clone with and without Ruxolitinib pre-treatment. The error bars represent standard deviation. **(Note: The curve for the "CAOV3-Parent" clone is included in all the plots for comparison purposes)**

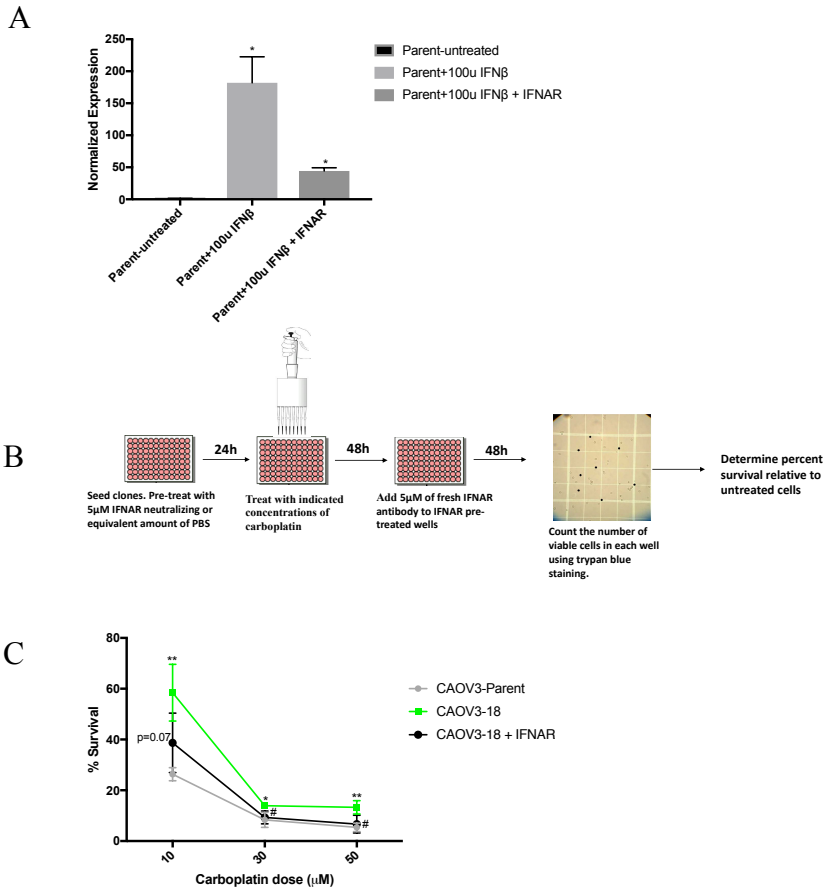
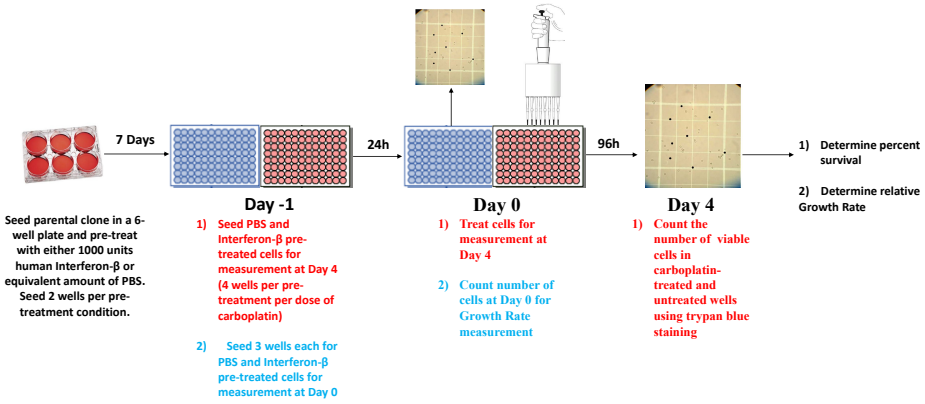


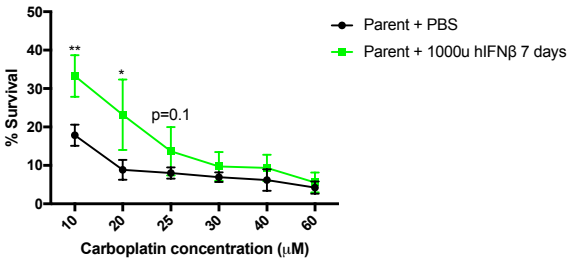

**Figure 8: Type-I interferon receptor (IFNAR) neutralizing antibody weakly reduces percent survival of resistant clone 18 in response to carboplatin**

A. Expression of the interferon-induced gene ISG15 in the parental clone alone or treated with indicated amount of interferon-β with and without pre-treatment with the IFNAR neutralizing antibody for an hour. The error bars represent standard deviation.
B. Protocol for survival assay with the IFNAR neutralizing antibody pre-treatment
C. Percent survival of parental, and resistant clone 18 with and without pre-treatment with the IFNAR neutralizing antibody. Error bars represent Standard Deviation. Asterisk (*) is for comparison between parental and resistant clone 18. Hashtag (#) is for comparison between clone 18 with and without IFNAR pre-treatment. The error bars represent standard deviation.

A




B




C

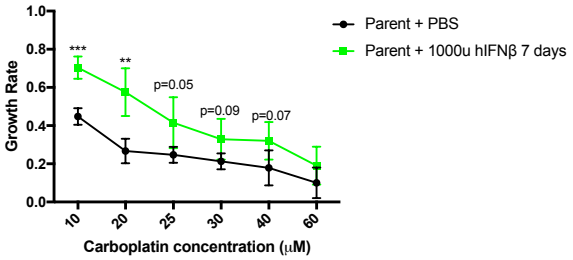
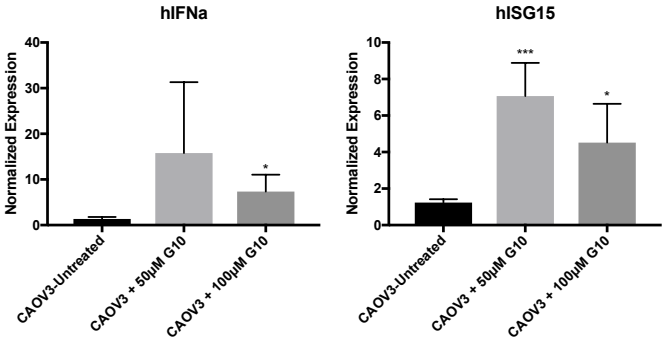


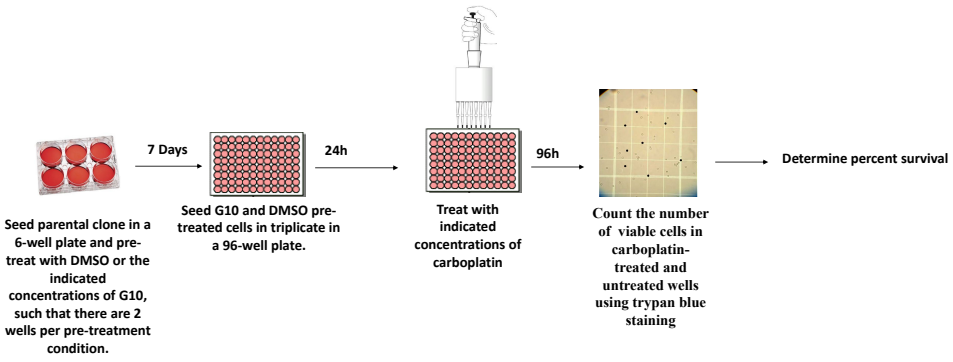

**Figure 9: Exogenous type-I interferon is sufficient to induce resistance to carboplatin in parental clone**

A. Protocol for simultaneous survival assay and growth rate assay with PBS and interferon-β pre-treated parental clone, including the formula to calculate Growth Rate.
B. Percent survival of parental clone pre-treated with either PBS or 1000 units of human interferon-β for 7 days. The error bars represent standard deviation.
C. Normalized Growth Rate of parental clone pre-treated with either PBS or 1000 units of human interferon-β for 7 days. The error bars represent standard deviation.

A




B




C

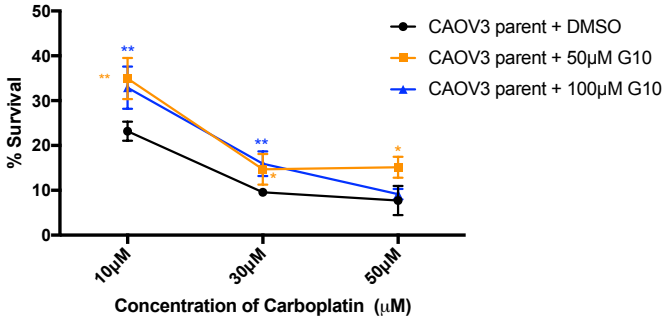



**Figure 10: The human STING agonist G10 is sufficient to induce resistance to carboplatin in the parental clone**

A. Expression of Interferon-α and the interferon-induced gene ISG15 in parental clone pre-treated with the indicated concentrations of the human STING agonist G10 for 24 hours. The error bars represent standard deviation.
B. Protocol for survival assay with the human STING agonist G10
C. Percent survival of parental, and resistant clone 18 with and without pre-treatment with the STING agonist G10. Error bars represent Standard Deviation. Asterisk (*) is for comparison between parental and resistant clone 18. Hashtag (#) is for comparison between clone 18 with and without G10 pre-treatment. The error bars represent standard deviation.

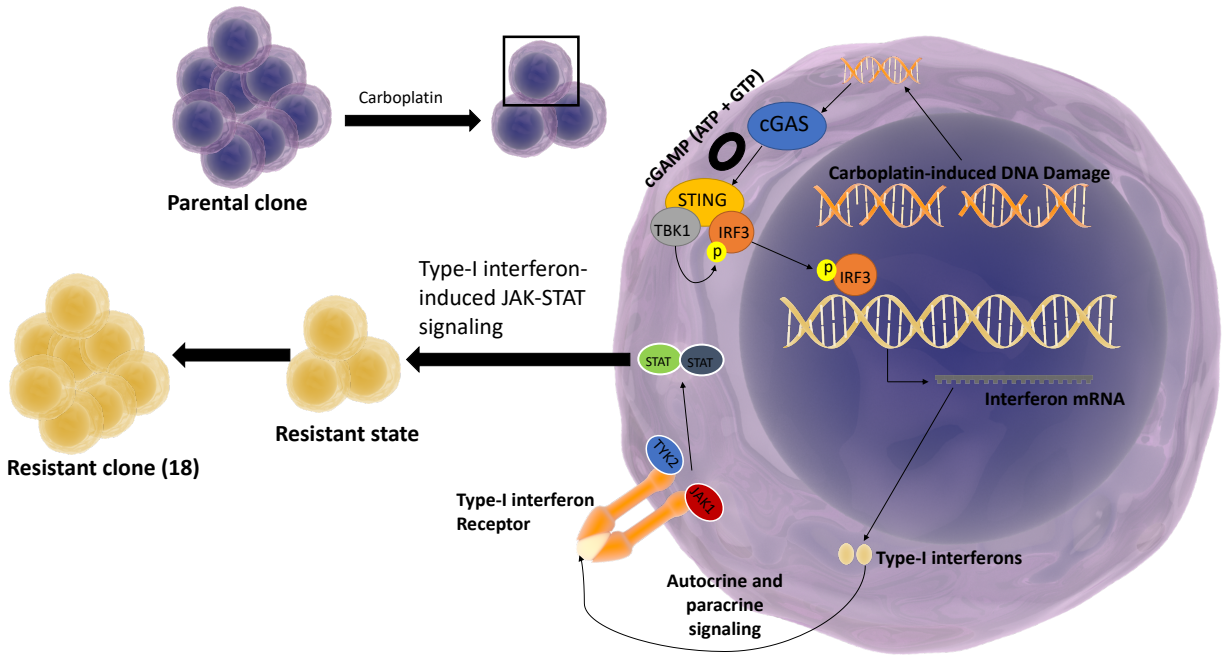



**Figure 11: Proposed model of type-I interferon-regulated resistance**

**Acknowledgement**

Figures 1A and 2A are from the work of Dr. Olivier Harismendy and Dr. Stephen B. Howell. Figures 1-11, in full are currently being prepared for submission for publication of the material. Champa, Devora; Venkatesh, Hrishi; Wenzel, Alexander; Sun, Si; Tsai, Cheng-Yu; Bui, Jack; Howell, Stephen B.; and Harismendy, Olivier. "Evolution and Characterization of Carboplatin Resistance at a single-cell level."

# III.

# Discussion

In this thesis, we characterize the biological consequence of type-I interferon signaling in regulating resistance to carboplatin in the human ovarian cancer cell line CAOV3. Dr. Harismendy and Dr. Howell had previously used a single clone approach to generate parental and carboplatin-resistant clones of the CAOV3 cell line in order to minimize variability while studying mechanisms of carboplatin-induced resistance[18]. RNA sequencing data from Dr. Harismendy and Dr. Howell had indicated that type-I interferon signaling was upregulated in the resistant clones relative to the parent clone[18]. We were able to validate these findings by RT-qPCR and show that expression of interferon-α and interferon-β were upregulated in the resistant clones relative to the parent clone. This was associated with a concomitant upregulation in the interferon-induced gene ISG15. Clone 18 showed the strongest type-I interferon gene signature. Treatment of the resistant clones with the JAK inhibitor Ruxolitinib reduced percent survival to as well as growth rate in response to carboplatin treatment. The reduction in resistance was seen inconsistently in clone 14 and clone 16. However, pre-treatment of clone 18 with Ruxolitinib consistently resulted in a strong reduction in percent survival as well as growth rate in the presence of carboplatin. This correlated well with the RNA sequencing data and our RT-qPCR data showing clone 18 to have the strongest type-I interferon gene signature. Carboplatin did seem to weakly induce resistance in the parent, however the effect was extremely small. We also showed that Ruxolitinib alone had no cytotoxic effects on the parental and resistant clones. Treatment of resistant clone 18 with a type-I interferon neutralizing antibody weakly reduced percent survival in response to carboplatin treatment. We thus showed that type-I interferon signaling was necessary for resistance to carboplatin in the CAOV3 cell line, especially in clone 18.

Andy Minn's paper had shown that type-I interferon signaling was necessary, but not sufficient for resistance to checkpoint blockade therapy[8]. However, in our model system, pre-treatment of the parental clone with exogenous interferon-β increased percent survival and growth rate in response to 30μM or lower concentrations of carboplatin. A newly-characterized human STING agonist G10 was shown to upregulate type-I interferon expression in the parent clone. Pre-treatment of the parent clone with G10 for 7 days increased percent survival to carboplatin, although its effect on growth rate wasn't characterized. Interestingly, a significant effect was seen in concentrations of carboplatin as high as 50μM. We were thus also able to demonstrate that type-I interferon was sufficient to induce resistance to carboplatin in the parent clone of the human ovarian cancer cell line CAOV3.

**Improving the Growth Rate assay**

The purpose of generating clonal populations of sensitive and resistant cells was to minimize noise in the process of deriving mechanistic insights[18]. In contrast to the observations made by Dr. Harismendy[18], we observed a loss of resistance in the clones at later passages. Using early passages of the clones thus became crucial. Importantly, the growth rate of the clones tended to vary between batches of the same clone. Determination of relative growth rate to correct for this difference in proliferation was crucial in interpreting the results. Furthermore, using a lower concentration range of carboplatin led to generation of survival and growth curves that were more reliable. The experiments with interferon-β and G10 pre-treatment need to be repeated with the lower concentration range. This could yield more significant results, especially in the case of interferon-β pre-treatment where resistance to lower concentrations of carboplatin was much stronger.

**Mechanism of type-I interferon induction and interferon-induced resistance**

Although we showed that type-I interferons were upregulated in the resistant clones, the mechanism of induction was not described. Previous reports have shown that type-I interferons are induced in tumor cells via sensing of cytosolic DNA by the nucleic acid sensor STING[26]. Indeed, this was the basis behind which the STING agonist G10 was used in Figure 7. However, this has not been confirmed in our model system. A western blot to detect activation of signaling components downstream of STING such as TBK1 as well as IRF3 in the parental clone and the resistant clones could provide evidence that the induction of type-I interferon is associated with STING activation. Genetic or siRNA-mediated knockdown of STING in clone 18 would be challenging, owing to the passage-dependent loss of resistance observed. An alternative approach would be the use of a recently-characterized novel STING antagonist carbonyl cyanide 3-chlorophenylhydrazone (CCCP)[35] to test whether STING antagonism is sufficient to reduce survival and growth rate in the resistant clones, especially in clone 18. Loss of resistance on CCCP pre-treatment would be strong evidence of STING-mediated type-I interferon induction.

Additionally, the mechanism of type-I interferon induced resistance has not been determined. Dr. Harismendy has preliminary evidence of a Cancer Stem Cell signature in the resistant clones (data not shown)[18]. Determining whether exogenous interferon-β or a human STING agonist like G10 can increase the stemness of the parental clone is an important future direction. To test this, the parent clone can be pre-treated with interferon-β or G10 as described earlier. Then, stemness can be assessed by various assays such as single or multi-cell sphere formation assay[27] as well as a wound scratch assay[28].

**Understanding response of resistant clones to Ruxolitinib**

In this study, we made the interesting observation that clone 18 was the only clone where Ruxolitinib was able to consistently reduce resistance. However, the reason for this phenomenon is unknown. The RNA sequencing data in Figure 2 shows that clone 18 not only had the strongest type-I interferon gene signature, but also is the only clone where type-I interferon alone is upregulated to a significant extent. Thus, it is likely that other pathways contribute to resistance in the other clones while type-I interferon signaling significantly contributes to resistance in clone 18, which could explain our observations. RNA sequencing on the resistance clones treated with and without Ruxolitinib could yield mechanistic insights into why and how Ruxolitinib reduces resistance to carboplatin in clone 18 alone.

**Broadening the scope of in-vitro studies**

The current study exclusively focuses on one human ovarian cancer cell line-CAOV3. To fully understand the role of type-I interferons in resistance to carboplatin in ovarian cancer, the in-vitro studies need to be repeated with other ovarian cancer cell lines. This could begin with studying the ability of exogenous type-I interferon and/or a human STING agonist such as G10 to induce resistance in other human ovarian cancer cell lines. Resistant and parent clones could then be generated for other cell lines, and the role of type-I interferons in regulating resistance in those cell lines could be analyzed. Alternatively, the previous experiments involving pre-treatment with exogenous type-I interferon/a human STING agonist could also include a third condition involving co-treatment with a JAK inhibitor to confirm both necessity and sufficiency. It is likely that type-I interferon will be relevant in some cell lines but not in others and may even reduce resistance in some cell lines. Then, understanding the features of the cell lines that

contribute to the differences in response could yield insights that could help predict the effect of type-I interferon signaling on chemoresistance in patients with ovarian cancer.

As these observations were made in clones of a cell line, testing whether interferon correlates with resistance to platinum-based chemotherapies in patients with ovarian cancer is a crucial next step to evaluate the clinical significance of the preliminary in-vitro data. Analyzing The Cancer Genome Atlas (TCGA) data to see whether type-I interferon signaling correlates with survival of ovarian cancer patients receiving platinum-based chemotherapies could be a good starting point. In ovarian cancer patients, surgery post relapse is done in some cases[12]. Testing whether type-I interferon signaling is upregulated in tumor samples from such patients relative to a bulk tumor population (expression from bulk tumor population via TCGA data) would yield great insight into whether the pro-tumor resistance regulating properties of type-I interferon are significant in patients.

**In-vivo xenograft studies and implications for clinical trials**

To study the role of type-I interferon in chemoresistance in a more physiological setting, it is important to develop an in-vivo xenograft model using immune deficient $RAG^{-/-}$ mice[29]. This has been challenging for us, as the CAOV3 cell line doesn't form tumors easily in mice via sub-cutaneous injection[30]. Although an intraperitoneal injection could be used, tumor measurements would require engineering a reporter gene into the parent and resistant clones which could alter their behavior. An alternative is to use severely immunodeficient mice that lack both an innate and adaptive immune system, such as $RAG2^{-/-}\gamma_c^{-/-}$ mice[31]. If and when a xenograft model is made, it can be used to analyze whether the parent and resistant clones have a difference in in-vivo tumor forming capacity and whether the resistance to carboplatin can be observed in-vivo. If resistance is seen in-vivo, then the effect of the JAK inhibitor on the

resistant clones in-vivo can be determined. This would be extremely clinically relevant, as JAK inhibitors are being considered as a strategy to fight cancer[32]. In fact, a clinical trial is currently studying combination therapies involving a novel JAK inhibitor and other chemotherapies (NCT01929941). The above in-vivo experiments will yield valuable insights on the potential of JAK inhibitors to mitigate chemoresistance in tumors. The above experiments could also be repeated with the novel STING antagonist CCCP[35]: most research is focused on activating the STING pathway in tumors[33]; thus, data that inhibition of STING is able to reduce resistance in-vivo would be extremely significant.

The in-vivo xenograft model could also be used to characterize the ability of STING agonists to induce resistance to chemotherapy in a physiological setting. As indicated earlier, STING agonists are being actively explored as an anti-tumor therapeutic strategy, due to the ability of the STING pathway to induce apoptosis in cancer cells[33] as well as due to the importance of STING-dependent type-I interferon signaling in promoting anti-tumor immunity[34]. Recently, the human STING agonist MIW815 is in Phase-I clinical trials along with a PD-1 checkpoint inhibitor (NCT03172936). Our in-vitro data hints at a paradoxical pro-tumor role for these STING agonists at the level of the tumor cell. In-vivo data will yield a more comprehensive picture of the pro-tumor potential of STING agonists in the context of chemotherapy, which will be an important consideration in clinical trials.

# IV.

# Materials and Methods

**Cell Culture**

The human ovarian cancer cell line CAOV3, and carboplatin-resistant clones of the same were a generous gift from Dr. Olivier Harismendy at the University of California, San Diego. The cells were grown in Roswell Park Memorial Institute Medium (RPMI-1640 medium) containing 100μg/μL Penicillin, 100μg/μL Streptomycin and supplemented with 10% (v/v) Fetal Bovine Serum.

**Reagents**

Carboplatin (10mg/mL saline solution) was a generous gift from the Moore Cancer Center pharmacy at University of California, San Diego.

Ruxolitinib was acquired from LC laboratories and reconstituted in DMSO at a concentration of 50mM. The human type-I interferon Receptor (IFNAR) neutralizing antibody (Clone MMHAR-2) was acquired from PBL assay science in the form of a 0.5mg/mL stock solution in Phosphate Buffer Saline (PBS) containing 0.1% Bovine Serum Albumin (BSA).

The human STING agonist G10 was acquired from Bio-techne and was reconstituted at a concentration of 50mM in DMSO

**Quantitative Reverse Transcription Polymerase Chain Reaction**

Parental or resistant clones of CAOV3 were seeded in 6 well plates at a concentration of 1 x $10^5$ cells/well. After the cells adhered to the plate, they were treated with the indicated chemotherapy drug, exogenous Interferon-β or STING agonist for the indicated time, if applicable. RNA was then isolated using TRIzol® reagent (Invitrogen) according to the Manufacturer's instructions. Total RNA yield was quantified using Nanodrop and 1μg of RNA was used for cDNA synthesis. cDNA synthesis was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions using Veriti 96-well Thermal Cycler (Applied Biosystems). For quantitative real-time PCR amplification of interferon and interferon-induced genes, the cDNA sample was denatured at 95°C for 10 minutes, followed by 34 cycles of denaturation at 95°C for 1 minute, primer annealing at 60°C for 30 s, and primer extension at 72°C for 1 minute. Upon completion of the cycling steps, a final extension at 72°C for 5 min was done. Each experiment was repeated at least twice. The primer sequences are provided in Table 1 below. Expression data were normalized to the geometric mean of housekeeping gene 18s to control the variability in expression levels and were analyzed using the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen[19].

**Table 1:** Primer sequences of indicated genes used for qPCR

| Gene | Primer |
|---|---|
| Human 18s_ FWD | CGC CGC TAG AGG TGA AAT TCT |
| Human 18s_ REV | CGA ACC TCC GAC TTT CGT TCT |
| Human Interferon-α_FWD | GTG AGG AAA TAC TTC CAA AGA ATC AC |
| Human Interferon-α_REV | TCT CAT GAT TTC TGC TCT GAC AA |
| Human Interferon-β_FWD | AAA CTC ATG AGC AGT CTG CA |
| Human Interferon-β_REV | AGG AGA TCT TCA GTT TCG GAG G |
| Human ISG15_FWD | GAG AGG CAG CGA ACT CAT CT |
| Human ISG15_REV | CTT CAG CTC TGA CAC CGA CA |

**Ruxolitinib (JAK1/2 inhibitor) sensitivity assay**

The parental and carboplatin-resistant clones were seeded in triplicate in 96-well plates at a density of 2 x $10^3$ cells/well and pre-treated with the indicated concentrations of Ruxolitinib for 5 days. The cells were detached using 50μL 0.25% Trypsin-EDTA for 10 minutes and diluted 1:2 in trypan blue to exclude dead cells. The number of live cells for each clone at each concentration of Ruxolitinib was then counted manually using a hemocytometer.

**In-vitro resistance assay for the CAOV3 cell line**

The parental and carboplatin-resistant clones of CAOV3 were seeded in a 96-well plate at a density of 1 x $10^3$ cells/well, unless otherwise indicated. The plate was seeded such that there were 4 replicates of parent/resistant clone per dose of chemotherapy. The next day, the cells were treated with the indicated dose of Carboplatin for 96 hours. The cells were detached using 50μL 0.25% Trypsin-EDTA for 10 minutes and diluted 1:2 in trypan blue to exclude dead cells. The number of live cells was then counted manually using a hemocytometer. The number of live cells in each carboplatin-treated well was normalized to the geometric mean of untreated cells to determine percent survival.

In experiments using the JAK inhibitor Ruxolitinib, the resistant clones were seeded in triplicate in 96-well plates at a density of 2 x $10^3$ cells/well, unless otherwise indicated. The cells were treated with 5μM Ruxolitinib or equivalent amount of DMSO, such that the DMSO was less than 1% of the total volume in the well. The next day, the cells were treated with the indicated dose of carboplatin for 96 hours, following which percent survival was determined as indicated above.

In experiments using the human interferon neutralizing antibody, the parental clone and clone 18 were seeded in triplicate in 96-well plates at a density of 2 x $10^3$ cells/well. The next day, the cells were treated with the indicated dose of carboplatin for 96 hours. Clone 18 was treated with 5μg/mL of the anti-human IFNAR neutralizing antibody or equivalent amount of PBS the day before carboplatin treatment, as well as 2 days after carboplatin treatment. Percent survival was determined as indicated previously.

**Growth curve assay**

The parental and resistant clones of the CAOV3 cell line were seeded in triplicate at the indicated seeding densities in 96-well plates such that there were 3 wells per clone per time point (24,48, 72 and 96 hours after seeding). At the indicated time points, the cells were detached using 0.25% Tryspin-EDTA and diluted 1:2 in trypan blue to exclude dead cells. The number of live cells in each well was then determined via manual counting using a Hemocytometer. The growth curves were generated by plotting the average number of cells per well at each time point for the clones.

**In-vitro growth rate assay**

The parental or resistant clones were seeded in triplicate into 96-well plates at a density of 2 x $10^3$ cells/well, unless otherwise indicated. The cells were treated with 5μM Ruxolitinib or equivalent amount of DMSO, such that the DMSO was less than 1% of the total volume in the well. Separately, 6 wells each of DMSO and Ruxolitinib treated cells per clone were seeded in parallel as *"control wells."* The next day ("Day 0"), the number of viable cells in the *"control wells"* were counted using a hemocytometer, as described previously. The remaining cells were treated the same day with the indicated concentrations of carboplatin for 96 hours, following which the number of viable cells in the untreated and carboplatin-treated cells was determined ("Day 4") using a hemocytometer as described previously. Growth Rate was then determined using the formula:

$$\textbf{Growth Rate} = 2^{\frac{\log_2\left(\frac{\text{Number of cells in treated wells at Day 4}}{\text{Number of cells in control wells at Day 0}}\right)}{\log_2\left(\frac{\text{Number of cells in untreated wells at Day 4}}{\text{Number of cells in control wells at Day 0}}\right)}} - 1$$

In experiments studying the impact of exogenous interferon-β on growth rate, the parent clone was seeded in duplicate in a 96-well plate and was either pre-treated with 1000 units of human interferon-β or PBS for 7 days. The cells were split every 3 days. Then, the PBS and interferon-β pre-treated cells were seeded in a 96-well plate at a density of 1 x $10^3$ cells/well, with 4 biological replicates per carboplatin dose per pre-treatment condition. Additionally, 3 wells each of PBS or human interferon-β-treated cells were seeded, and those wells were counted the next day (Day 0). The remaining cells were treated on the same day with the indicated concentrations of carboplatin for 96 hours, following which growth rate was determined as above

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