The Role of NFAT3 in Ovarian Cancer Quiescence and Chemotherapy Resistance

by

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Abstract

The Nuclear Factor of Activated T-Cells (NFAT) transcription factor family has been implicated in various physiological and pathological functions, including stress responses, immune activation, and stem cell quiescence. It has been shown that the slow-cycling nature of quiescent cells can be protective during chemotherapy. We found that the transcription factor NFAT3 is overexpressed in ovarian cancer stem-like cells and have created two constitutively active NFAT3 (cNFAT3) models to study its impact in ovarian cancer biology. *In silico* TCGA analysis has shown that NFAT3 dysregulation is correlated with poorer overall survival as well as significant decreases in gene sets associated with ribosomal structure, translation, and oxidative metabolism. We have observed significantly decreased cell division and BrdU incorporation in cNFAT3 cells, as well as a 10-30% decrease in cell size, total RNA, and ribosomal structural proteins without any effect on viability, apoptosis, or senescence. This is highly consistent with a quiescent state. In addition, cNFAT3 expression increased chemotherapy resistance to cisplatin, while co-treatment with cisplatin and VIVIT, an NFAT inhibitor, decreased survival during chemotherapy.

While investigating the mechanisms of NFAT3's effects on cell division and growth, we observed significant CDK6 downregulation in response to NFAT3. Given that tumor growth is vastly retarded or even inhibited by cNFAT3 *in vivo*, we investigated CDK4/6 inhibition as a maintenance therapy for ovarian cancer. TCGA mining and mutational analysis showed that CDK4/6 inhibition is a rational therapy for most ovarian cancer patients. The CDK4/6 inhibitor LEE-011 led to significant cell cycle arrest and senescence in sensitive ovarian cancer lines and delayed tumor growth by 55% *in vivo*. In particular, LEE-011 significantly delayed tumor growth in platinum-resistant tumors, which is particularly useful as these patients have significantly poorer prognoses. We have also observed significant synergy between cisplatin and LEE-011, including an impaired ability to return to normal cell cycling after chemotherapy, and suggest that this is a rational combination therapy for use in patients with recurrent or platinum-resistant ovarian cancer.

Chapter 1: Introduction

Section 1: Clinical problems in ovarian cancer

Ovarian cancer is the fifth most common cancer in American women and the most lethal gynecological cancer in the USA, with a 5-year survival rate under 50% [1]. Multiple factors contribute to this poor prognosis. There are no effective population-level screening tests for ovarian cancer, so early detection of the disease is uncommon; only 14.7% of patients are diagnosed with local, circumscribed tumors [2]. In addition, ovarian cancer is usually asymptomatic or causes nonspecific symptoms such as bloating and abdominal discomfort; these are often attributed to other causes, delaying diagnosis [3]. As a result, most patients (66%) are diagnosed with late-stage disease, when the cancer has already spread either around the peritoneum and abdominal cavity (Stage III) or to distant organs (Stage IV) [4].

First-line therapy for ovarian cancer typically consists of cytoreductive surgery when possible, along with adjuvant or neoadjuvant combination therapy with a platinum agent and a taxane (typically carboplatin and paclitaxel) [5, 6]. 30% of patients are initially refractory to this therapy, which confers a very poor prognosis. The other 70% of patients typically respond well

to the platinum/taxane combination; however, most of these patients eventually relapse and succumb to their disease [7]. Patients are classified as platinum-sensitive if they relapse more than six months after the completion of platinum-based chemotherapy; typical prognosis after a platinum-sensitive recurrence ranges from 2-3 years. Platinum-sensitive patients tend to respond to additional rounds of platinum and will therefore undergo additional platinum chemotherapy until they experience a relapse less than six months after the end of treatment, at which point they are classified as platinum-resistant. Platinum-resistant patients receive second-line chemotherapy options, such as doxorubicin, topotecan, gemcitabine, and others [8]; prognosis after a platinum-resistant relapse ranges from 12-18 months. In total, about 70% of patients who respond to initial therapy will relapse within two years, and most of them will eventually pass away after multiple rounds of chemotherapy followed by progressively shorter remissions [7]. Therefore, current management of recurrent ovarian cancer primarily involves controlling the disease and associated symptoms while maintaining quality of life [9]. Effective maintenance therapy that lengthens remissions, increasing progression-free and overall survival, would represent a significant improvement in treatment for this group. Unfortunately, there are few effective non-cytotoxic therapies that prolong overall survival in ovarian cancer. The most promising option currently available is Bevacizumab (Avastin), which is an anti-angiogenesis drug that has shown promise in other cancers. Avastin increased progression-free survival in relapsed ovarian cancer by 4 months [10] and 3.3 months [11] when combined with other chemotherapeutic agents. However, improvements in overall survival were minimal in these trials [10-12], and this improvement in progression-free survival, while statistically significant, was small.

Taken together, the statistics on survival and recurrence suggest two major and related clinical issues in ovarian cancer: chemotherapy resistance and recurrent disease. Studying chemotherapy resistance is essential to preventing recurrent disease, which is usually caused by residual cells that have survived through chemotherapy. One potential mechanism that could explain this is the existence of slow-cycling or quiescent, chemotherapy-resistant cancer stem-like cells which are able to survive through therapy and then repopulate tumors after the withdrawal of cytotoxic drugs.

Section 2: Cancer Stem-like Cells (CSLC)

Though cancers were initially thought to be comprised of a uniform tumor cell population, it is now well accepted that most tumors actually contain a heterogeneous population of cancer cells [13-15]. Decades-old studies show that only a small proportion of cancer cells can grow colonies in soft agar (a measure of tumorigenicity) [16] or initiate tumors *in vivo* [17], suggesting that not all cells have the same tumorigenic potential. Two competing theories attempt to explain this observation. In the stochastic model, all cancer cells are capable of initiating tumors or metastases, but only a small, random proportion manage to accumulate the mutations necessary to exit the primary tumor, intravasate into the bloodstream or lymphatics, extravasate successfully, and initiate a new tumor. The cancer stem cell model instead postulates the existence of a distinct subset of tumor cells with stem-like properties, specific markers, and significant tumorigenic potential that is not shared by other tumor cells [18-20]. In this model, the cancer stem cell is analogous to the adult stem cell in mature tissue, where a small proportion of stem cells renew the differentiated cells and

respond to injury and the bulk of the tissue is largely made up of differentiated cells. This concept is highly attractive due to its ability to explain many clinical problems in the field of oncology. A slow-cycling and chemotherapy resistant cancer stem cell may survive through chemotherapy and then, potentially sensing the lack of other tumor cells, proliferate to give rise to more bulk tumor cells. Such feedback loops between non-stem and stem-like cells have already been described [21]. In addition, slow-cycling quiescent or dormant tumor cells may migrate far from the original tumor and eventually resume cycling, creating late distant metastases. The following sections provide a brief review of evidence on the cancer stem cell hypothesis and quiescence, chemotherapy resistance, and relapse/metastasis in cancer stem cells before focusing on stem-like cells in ovarian cancer.

Section 2.1: Evidence for the Cancer Stem Cell (CSC) Hypothesis

The first cancer cells with stem-like properties were discovered in 1994 in acute myeloid leukemia (AML) by John Dick [22]. While significant controversy still surrounds the cancer stem cell theory [23], there are numerous reports of cancer stem-like cells in multiple types of cancers, including leukemia [22, 24], breast cancer [25], colon cancer [26], pancreatic cancer [27], glioblastoma [28] , and ovarian cancer [29-32]. These reports tend to describe the isolation of a small population of tumor cells, usually through flow cytometry, that are more effective in *in vitro* and *in vivo* tumor initiating assays than bulk tumor cells. The first cancer stem-like cells (CSLCs) in solid tumors were discovered in breast cancer and were marked by a $CD44^{high}/CD24^{-/low}$ phenotype. As few as 100 $CD44^{high}/CD24^{-/low}$ CSLCs could initiate heterogeneous tumors *in*

vivo that mimicked the cell composition of the original tumors, while their bulk tumor counterparts failed to initiate tumors even with thousands of cells [25]. Similar studies using fluorescence-activated cell sorting (FACS) analysis to identify CSLCs by surface markers or enzymatic properties have been conducted in most of the tumor types above.

In addition to these prospective analyses, other studies have used lineage tracing models to show that a small population of slow-dividing, multipotent cells tends to drive the growth of heterogeneous tumors, while the majority of tumor cells cannot [33-35]. For example, Driessens et al. labeled individual tumor cells in squamous skin cancer and observed their growth. This report showed that these tumors were composed of two distinct subsets: a population that cycled twice a day and had limited proliferative potential, and a slower-cycling population that gave rise to the more differentiated cells that occupied most of the tumor. This study provided the first direct evidence for a CSLC-driven growth pattern in undisturbed tumors [35]. Another study which used long-term labeling found that this slow-cycling CSLC population appeared to drive regrowth of glioblastoma cells after chemotherapy, and that therapies specifically targeting CSLCs significantly improved outcomes in a mouse model [34]. In general, these studies are consistent with observational reports indicating that the presence of CSLCs is associated with poorer prognosis and increased disease stage, grade, and/or recurrence risk [36, 37].

Section 2.2: Controversies

There is significant controversy surrounding the cancer stem cell hypothesis. Many of the prominent concerns involve the methodologies used to study CSLCs. Associations have often been made between CSLC markers and disease stage, grade, or prognosis based on prospective analysis of known or suspected stem cell markers (usually performed through fluorescence-activated cell sorting (FACS)). FACS sorting of CSLC from non-CSLC has also been used to separate these stem-like cells for *in vitro* experimentation and *in vivo* xenografts. However, FACS has a well-known contamination rate of around 1%. Even a tiny number of putative stem cells added into a non-stem cell pool may proliferate and make it appear that differentiated cells have de-differentiated, invalidating a more hierarchical stem cell model. Conversely, the addition of a tiny number of differentiated cells to a stem cell pool may falsely give the appearance that the stem cells can regenerate the hierarchy. Therefore, this error rate is a constant source of criticism, especially since the definite assay used to define CSLCs, the *in vivo* tumor xenograft assay, typically depends on FACS-sorting of cells prior to injection.

Moreover, prospective FACS-sorting does not take into account the potential for stem cell plasticity. In the classic hierarchical model, a cell with stem-like markers remains a stem cell until it divides, and a cell with more differentiated markers is not a stem cell and will not acquire stem cell properties or markers; in this model, “dedifferentiation” does not occur. However, this is not always the case with stem-like cells in cancer. For instance, Dr. Weinberg’s lab has observed spontaneous conversion of non-stem cells to stem cells in breast cancer, using accepted breast cancer stem cell markers [38]. In addition, the induction of epithelial-mesenchymal transition (EMT) has been shown to induce cancer stem cell characteristics in

breast cancer epithelial cells [39]. If CSLCs are to be targeted for therapeutic purposes and used as prognostic markers, a stable phenotype is clearly an advantage, though not an absolute requirement. FACS analysis provides a snapshot of cellular markers in time and cannot address this question. Direct observation of stem-like cells has been one way to overcome this accuracy limitation. Our lab, for example, has observed several hundred CSC divisions in single-cell microfluidics chips, which allows for direct visual confirmation that each cell expresses the appropriate markers as well as direct tracking of each division [21]. Lineage tracing methods that track cells in their native tumor environment also provide some of the stronger evidence supporting the CSC hypothesis, as described above.

The second major methodological challenge to the cancer stem cell hypothesis comes from the *in vivo* xenograft model, which is ultimately the gold standard for defining cancer stem cells. An elegant study in melanoma has shown that the proportion of cancer stem cells is largely dependent on the assay system chosen [40]. This study found that melanoma models in the NOD/SCID mouse, which most studies of CSC tumorigenesis have used, produced CSC frequencies of 0.1-.001%, which are consistent with the hypothesis of a rare tumor-initiating parent cell. However, when the same cells were transplanted into a more immunocompromised mouse, the NOD/SCID/IL2Rγ model, up to 25% of cells from melanoma cell lines and patients formed tumors; this large frequency is not particularly consistent with the current CSC model. Of course, every assay system comes with limitations, and the data generated need to be considered in light of those limitations. These limitations on FACS-sorting and the assays currently used to define cancer stem cells are nontrivial, and accurate definitions and characterizations of these cancer stem cells will assist in the development of

relevant therapeutics. However, the evidence supporting tumor heterogeneity and the existence of specific subsets of highly efficient tumor initiating cells is significant. Given the association of these cells with aggressive disease and poor prognosis, agents targeting CSC are likely to be rational therapeutics regardless of stem cell plasticity and hierarchical behavior (or the lack thereof), particularly when used in combination with traditional therapies that kill bulk tumor cells.

Section 2.3: Quiescence in Cancer Stem-like Cells

Quiescence is a property of stem cells and is one factor that may contribute to chemotherapy resistance. However, the study of quiescence is generally difficult and poses specific issues in cancer research. Due to the nature of most assays and culture conditions for cancer cell lines, a small number of quiescent, slow-cycling cells are almost inevitably lost in a much larger number of rapidly proliferating bulk tumor cells. Therefore, the specific assays that are used significantly impact the classification and quantification of quiescent cells.

Numerous studies have shown slower cycling rates in stem-like cells than in non-stem cells. Notably, the CD24+ cells mentioned above in breast cancer do not appear to cycle differently from their CD24- counterparts [25], though the evidence presented in that report only shows that the two cell types have similar cell cycle phase diagrams; it does not directly analyze cycling rate. However, CD24+ stem-like cells in ovarian cancer are enriched in the S phase when compared with their CD24- counterparts [41]. Our lab has noted a slower cycling rate in primary ALDH+ cells than in ALDH- cells when their divisions are observed in single-cell microfluidics chips [21]. Other recent studies on quiescence in cancer stem cells provide mixed

evidence. While there is clear evidence for quiescent cells in cancer, it is not always clear that these are cancer stem cells. For example, one group found a clear JARID1B-positive population in melanoma with division times >4 weeks which gave rise to rapidly proliferating cells. Without JARID1B+ cells, cell growth curves and tumor xenografts plateaued, presumably due to the lack of stem-like cells to renew the tumor. However, JARID1B expression was not stable within individual cells and JARID1B-negative cells could give rise to JARID1B-positive cells. In addition, JARID1B expression did not correlate with known CSC markers [42]. A similar result was shown in medulloblastoma; in this model, quiescent $Sox2^+$ cells gave rise to a more rapidly dividing population that made up most of the tumor. In addition, the $Sox2^+$ population increased after treatment, indicating that these cells had survived through chemotherapy and might drive relapse [43]. In another study which used label retention to identify slow-cycling cells, pancreatic CSC markers such as CD24, CD133, and ALDH only partially overlapped with a slow-cycling label-retaining population, but the slow-cycling population was chemotherapy resistant and able to recreate heterogeneous tumor populations with all of the above CSLC markers [44]. Clearly, stem-like cells with undiscovered markers may exist and overlap with quiescent or chemoresistant cells; in addition, the potential for plasticity between stem and non-stem states has already been discussed above. However, these studies do call into question the evidence for chemoresistant, quiescent CSLCs. This also raises the question of whether label-retaining cells can interchange with CSLCs and vice versa. Overall, while slow-cycling and chemoresistant tumor cells have been identified, the claims surrounding quiescent cancer stem cells require significantly more study along with better methodology.

Section 2.4: Chemotherapy Resistance in Cancer Stem-like Cells

Chemotherapy resistance is a second major characteristic attributed to cancer stem cells, as it is thought that they can survive through therapy and may be partially responsible for causing recurrent disease. Mechanisms of chemotherapy resistance are varied and may involve quiescence, expression of active detoxification genes, or both; however, it is well documented that CSLC are correlated with chemotherapy resistance. Many studies of various stem cell markers in various cancers have provided evidence of improved CSLC survival during chemo- and radiotherapy. For example, a higher proportion of glioma CD133+ stem cells survive through radiotherapy when compared to CD133- cells [45]. Studies in breast cancer have shown that the presence of stem cells correlates with response to cytotoxic chemotherapy and to the targeted therapy trastuzumab [46]. A particularly elegant study in ovarian cancer investigated a set of cancer stem cell markers in primary tumors, tumor remnants after initial therapy, and recurrent disease. Though this involved a relatively small sample set, this group found that stem cell markers were enriched in the tumor remnants after primary chemotherapy, indicating that the stem-like cells had better survival rates during therapy or that they were able to proliferate despite therapy [47]. Samples collected from recurrent tumors generally had similar compositions to those of the primary tumors. These reports are representative of a large body of literature correlating CSLCs with chemotherapy resistance.

There has been some debate on whether cancer stem cell markers are simply markers or whether they play an active role in the stem cell phenotype. Both ALDH and CD133 have been implicated in direct chemotherapy resistance pathways. Although different ALDH isoforms have different specific activities, there are reports of ALDH isoforms actively

metabolizing and detoxifying cyclophosphamide [48], and ALDH1 inhibition specifically reduced both chemoresistance and radioresistance in breast cancer stem cells [49]. At least in these cases, ALDH appears to function not only as a marker of stemness but as an essential part of the stem cell phenotype that allows better survival through chemotherapy. CD133 also appears to play a functional role in chemoresistance at least in some cancer types; ectopic CD133 overexpression was associated with a two to four-fold decrease in apoptosis in response to doxorubicin and camptothecin in glioma cells. In addition, CD133 overexpression led to increased expression of P-glycoprotein, a well-known drug efflux pump [50]. In other tumor types, differential expression of FLIP [51] or the Akt survival pathway [52] was observed in CD133+ vs. CD133- cells and was correlated with a lack of apoptosis in response to chemotherapeutic drugs.

In addition to the detoxification effects mentioned above, the slower cell cycle associated with these stem-like cells has also been implicated in chemotherapy resistance. Several groups have demonstrated that therapies that kill rapidly proliferating bulk tumor cells do not kill more slowly proliferating cells, indicating that a quiescent or slow-cycling state may protect cells from chemotherapy [44, 53, 54]. In fact, forcing cells to enter the cell cycle in order to target them more effectively has been considered as a therapeutic option in some leukemias [55]. This is not unique to leukemia; a slow-cycling population of quiescent, gemcitabine-resistant stem cells in lung cancer were successfully targeted to inhibit tumor xenograft growth during therapy [56].

Section 2.5: Stem Cells and Stem-like Cells in Ovarian Cancer

There are multiple putative markers for ovarian cancer stem-like cells, including CD44, CD117, ALDH, and CD133 [57]. The Buckanovich lab and others have used cell lines and primary tumors to identify a class of stem-like cells that coexpress ALDH and CD133 [29, 30]. This marker combination has been used in oral cancer to identify the risk of malignant transformation [58] and in breast cancer to predict poor prognosis [59]. Both ALDH and CD133 are also individually well-documented CSLC and prognostic markers in multiple tumor types [60-63] and in ovarian cancer [31, 32, 64]. In ovarian cancer, an increase in the percentage of ALDH+ cells is associated with poor response to therapy and platinum resistance, and the percentage of ALDH+ cells is an independent marker of poor prognosis [65]. In addition, ALDH expression in cells isolated from ascites is correlated with significantly lower progression-free survival [66]. Despite occasional conflicting studies [67], ALDH generally appears to be correlated with aggressive disease and poor prognosis and was associated with reduced survival in a recent meta-analysis [68]. Meta-analysis has also identified CD133 as a stem-like cell marker in ovarian cancer that is correlated with poor prognosis [69].

To characterize these ALDH+/CD133+ cells, the Buckanovich lab isolated and cultured single cells on microfluidics chips [70] to visualize the division and differentiation process. ALDH+/CD133+ cells divide symmetrically to create two ALDH+/CD133+cells or asymmetrically to produce one ALDH+/CD133+ cell and one ALDH-/CD133+ or ALDH+/CD133- transit amplifying cell (TAC). TACs then divide to self-renew or create ALDH-/CD133- cells, which form the bulk of the tumor (Figure 1.1). Therefore, this data suggests the existence of a functional hierarchy that is similar to those found in adult stem cells in mature organs. Our lab has

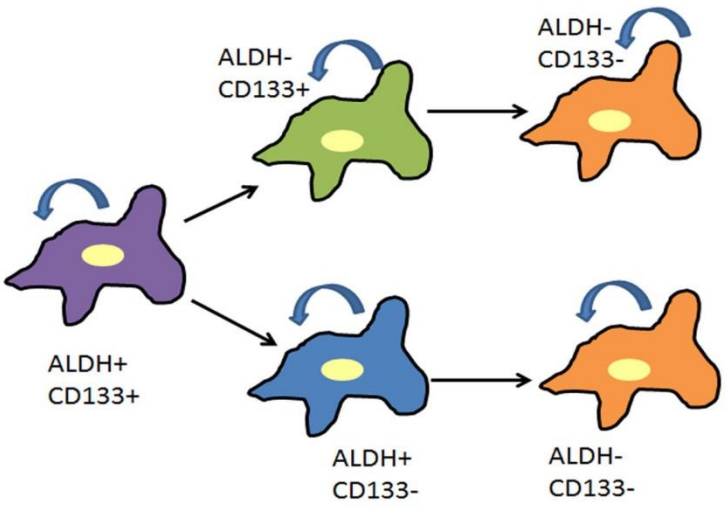

Figure 1.1. The cancer stem-like cell hierarchy. ALDH+/CD133+ cancer stem-like cells can either self renew or divide asymmetrically to produce one ALDH+/CD133+ cell and either one ALDH+/CD133- cell or one ALDH-/CD133+ cell. These intermediate cells. in turn, can either divide symmetrically to self-renew or give rise to ALDH-/CD133- daughter cells, which make up the bulk of the tumor.

observed only one potential “de-differentiation” event in single-cell culture, in which an ALDH- cell appeared to give rise to an ALDH+ cell.

These stem cell-like characteristics persist in functional assays. ALDH+/CD133+ cells efficiently form tumor spheres *in vitro* and have a high tumor initiation capacity *in vivo*. While ALDH-/CD133- cells require 1,000-50,000 cells to initiate tumors in mice, only 11 ALDH+/CD133+ cells are required to grow xenograft tumors; in addition, tumors initiated by ALDH+/CD133+ cells contain ALDH+/CD133+, ALDH+/CD133-, ALDH-/CD133+, and ALDH-/CD133- cells, indicating that these stem-like cells are capable of regenerating multiple populations. An important limitation of this experimentation, as discussed above, includes the FACS error rate; even an error rate of 1% may allow more differentiated cells to be injected along with the stem cells, creating a heterogeneous tumor. However, the significant tumorigenic potential of the ALDH+/CD133+ cells is consistent with clinical data indicating that the presence of ALDH+/CD133+ cells predicts poorer prognosis in ovarian cancer patients [29, 47]. Based on their ability to divide symmetrically or asymmetrically and form heterogeneously populated tumors, our lab has classified ALDH+/CD133+ cells as ovarian CSC.

Previously, our lab performed gene expression array analysis on FACS-isolated ALDH+/CD133+, ALDH+/CD133-, ALDH-/CD133+, and ALDH-/CD133- cells to identify differentially expressed factors that could account for the unique properties of CSC. We found that the transcription factor NFAT3 is specifically expressed in ALDH+/CD133+ cells at significantly higher levels than in more differentiated cells, both in cell lines and in patients. Given the known roles of NFAT proteins in cell fate determination and stem cell quiescence, we decided to further investigate NFAT3 in ovarian CSLC.

Section 3: NFAT Structure, Regulation, and Transcriptional Function

Section 3.1: NFAT Family Structure and Regulation

The Nuclear Factor of Activated T-cells (NFAT) transcription factor family is comprised of the core NFAT transcription factors NFAT 1-4 [71], which are collectively referred to here as NFAT. NFAT1 was initially discovered in the immune system [72], followed closely by NFAT2 [73], NFAT3, and NFAT4 [74]. While NFAT1, NFAT2, and NFAT4 are all expressed in the adult immune system, NFAT3 is not [71]; as NFAT proteins play fundamental roles in the adult immune system, the relative lack of immune NFAT3 expression is an important factor when considering the feasibility of NFAT3 targeting in ovarian cancer.

NFAT proteins share 65% sequence homology and share a specific domain structure, which is characterized by an N-terminal transcriptional activation domain, followed by a calcineurin binding and regulatory domain, a Rel-homology region (RHR), and a C-terminal splice variant domain (Figure 1.2) [74]. The N-terminal transactivation domain is responsible for specific DNA binding, as is the Rel homology region. The RHR is similar to the well-known Rel/NFκB proteins and is also the site of NFAT's interactions with Jun and Fos, along with other transcriptional cofactors. Due to this significant homology, NFAT proteins share many redundant functions, though it is clear that each family member has some unique activities as well. This is particularly evident in studies of NFAT knockout mice. For example, NFAT1-knockout mice display hyperproliferation of immune T- and B-cells and moderate splenomegaly

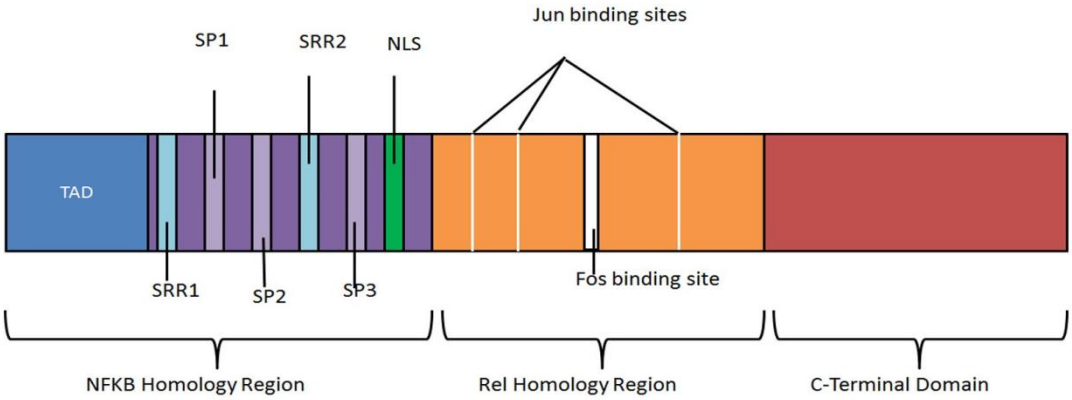

Figure 1.2. NFAT Structure. The NFAT family all share the same basic structural domains, which include a transcriptional activation domain (TAD), a Rel homology region, a C-terminal domain, and a calcineurin regulatory domain. The regulatory domain is comprised of multiple serine-rich regions (SRR) and serine-proline (SP) regions, which are typically phosphorylated in the cytoplasm. This occludes the nearby nuclear localization sequence (NLS), which is exposed after calcineurin dephosphorylates the SRR and SP regions. Exposure of the NLS allows nuclear translocation.

[75], while NFAT4-knockout mice show impaired thymic development and a moderately increased percentage of T- and B-cells outside the thymus [76]. However, double-knockout NFAT1/NFAT4 mice display massive lymphoproliferation and T- and B-cell hyperactivation, as well as cytokine levels that rose up to 75X those of the controls [77]. The interplay between NFAT3 and NFAT4 is even clearer in cardiac development; NFAT3-null mice have minimal phenotypes while NFAT4-null mice show a moderate inability to undergo physiologic cardiac hypertrophy [78]; however, NFAT3/NFAT4-null mice are embryonic lethal due to a massive failure of angiogenesis and cardiovascular development [79]. This indicates a significant level of homology and redundancy while also suggesting unique functions for each NFAT family member. This redundancy poses a technical challenge for researchers attempting to isolate the function of any particular NFAT.

The regulation of NFAT transcription factors adds another layer of complexity. All core NFAT proteins are primarily post-translationally regulated through phosphorylation and dephosphorylation of the regulatory region (Fig. 1.2). This domain is comprised of a calcineurin binding site, multiple serine-rich and serine-proline-rich domains, and a nuclear localization sequence [80]. When the serine-rich domains are phosphorylated, the nearby nuclear localization sequence is hidden, sequestering NFAT in the cytoplasm. Because its only known activity is as a transcription factor, this is effectively complete inhibition. When the phosphatase calcineurin becomes activated by intracellular calcium elevations, it dephosphorylates NFAT, exposing the nuclear localization signal and allowing NFAT to translocate to the nucleus and begin transcription [81, 82]. Calcineurin maintains NFAT dephosphorylation in the cytoplasm [83] and its actions are balanced by nuclear kinases that

phosphorylate NFAT, causing translocation back to the cytoplasm. GSK3B [84] and CK1 [85] are constitutive kinases that phosphorylate the SPxx motifs in the regulatory region, while JNK [86] and p38 [87] are inducible kinases that phosphorylate NFAT specifically at the SP sequences at the beginning of SRR1 (Fig. 1.2).

This calcineurin-dependent regulation is exploited by the immunosuppressants Cyclosporine A (CsA) and FK506, which inhibit calcineurin activity and therefore NFAT translocation [88, 89]. CsA is not a completely specific NFAT inhibitor; it inhibits NF-κB and AP1 by two- to four-fold while producing 100-1000-fold inhibition of NFAT nuclear translocation [90]. CsA has been used in transplant patients to prevent rejection for close to half a century; its record for reasonably safe long-term use and its relative specificity make it an accessible drug for study of the NFAT pathway. However, these immunosuppressants have undesirable side effects; therefore, this work has used a more specific agent, VIVIT, to study NFAT inhibition [91]. VIVIT binds to the calcineurin docking site on NFAT, thereby blocking the calcineurin-NFAT interaction; because calcineurin is unaffected, VIVIT carries far fewer side effects. A bioavailable version of this peptide has been produced for use *in vivo* [92]. Unfortunately, VIVIT (like CsA) is not able to discriminate between NFAT family members; it binds to NFAT 1-4 indiscriminately and is a general NFAT inhibitor rather than a specific one [91]. Due to the better cell viability and lack of side effects compared to cyclosporine (unpublished data), we have used VIVIT as the primary NFAT inhibitor in the following studies.

Section 3.2: Regulation of Transcription by NFAT Proteins

Like the upstream regulation of NFAT family members, NFAT-mediated regulation of transcription is complex and often requires multiple factors. The core NFAT family members are all designated as members of the extended NFκB-like family due to the presence of a common Rel homology region (RHR; Fig. 1.2), which serves as both the DNA binding domain and an important component of the dimerization domain [93]. In part due to the RHR, there is a great deal of versatility in the ways that NFAT proteins can bind to DNA; NFAT proteins have been shown to bind with a large variety of cofactors, including AP-1 [83], GATA4 [94], and IRF-4 [95]. NFAT proteins can also bind DNA as NFAT homodimers [96] or they can pair with other members of the NFAT family to create NFAT heterodimers that bind to DNA. Each of these pairings tends to bind to DNA at different consensus sites. Monomeric NFAT1 binding has been observed at 5'-GGAAA-3' sites [97]. NFAT homodimers and heterodimers tend to have similar structure, due to the significant homology within this family; these dimers typically consist of the C-terminal portion of the RHR serving as the binding interface between the two proteins and the N-terminal portion of the RHR serving as the DNA-binding interface. These dimers tend to bind at consensus sequences that resemble κB-like elements, 5'-GAGGAAAATTTG-3' [96], due to the apposition of the RHR domains of the two NFAT proteins. The consensus site for NFAT-AP-1 complexes is quite different; these sites tend to be a true composite of NFAT (GGAAA) and AP-1 sites (TGTTTCA) [98, 99] and suggest cooperative assembly of the complex, rather than binding of an NFAT-AP-1 dimer [93].

Biologically, this variety in consensus sites and recruitment of different NFAT proteins and partners to different promoter sites has significant implications. The presence of an NFAT

transcription factor does not necessarily imply transcription of downstream genes; this transcription likely depends on the presence or absence of other cellular factors. As different promoter consensus sequences recruit NFAT1 and AP-1 [99] or NFAT1 homodimers [96], two entirely different gene sets may be transcribed the same NFAT family member, depending on other cellular cofactors. In fact, NFAT1 can both positively and negatively regulate *myc*, depending on the other cofactors in the cell and the promoter binding site [100]. This complexity and combinatorial nature is characteristic of the NFAT family and significantly contributes to the wide and overlapping variety of functions discussed below.

Section 4: Physiological and pathological functions of NFAT proteins

NFAT1 was originally discovered as an essential factor for T-cell activation in the immune system [101]. Early work on the NFAT family focused on the roles of NFAT proteins in immune activation and their identity as targets of cyclosporine A and FK506 [102], which had been used as immunosuppressants for decades before the discovery of NFAT. Immune activation is the defining activity of the NFAT family; NFAT1, NFAT2, and NFAT4 play key roles in thymic development, T-cell differentiation and activation through IL-2 signaling, and T-cell anergy [103]. However, NFAT proteins also play significant roles in various other physiological functions, particularly in the regulation of cell cycling and differentiation, stress responses, and cancer.

Section 4.1: NFAT Proteins and Cell Cycle Regulation

Both NFAT1 and NFAT2 have well-characterized roles in the cell cycle, though there are no known roles for NFAT3 and NFAT4 [104]. In general, NFAT1 appears to downregulate cell cycle progression, while NFAT2 tends to increase it. NFAT1$^{-/-}$ mice show significant hyperproliferation of B and T- cells when compared with wild type mice. This same analysis shows a significantly higher proportion of cycling NFAT1$^{-/-}$ T-cells in culture, consistent with the lymphoproliferative phenotype. This cycling phenotype was correlated with overexpression of cyclins A2, B1, and E [105]. Cyclin A2 is active in the S and G2M phases [106], while cyclin B1 is responsible for chromosome condensation in early mitosis [107] and cyclin E primarily regulates transition through the G1 phase [108]. NFAT1 has also been shown to decrease cell cycling in hair follicle stem cells, where it induces a G1 block by downregulating CDK4 transcription [109]. Therefore, it appears that NFAT1 functions as a master regulator of cell cycle progression that affects many phases through transcriptional regulation of cyclins and CDKs.

Unlike NFAT1$^{-/-}$ mice, NFAT2$^{-/-}$ mice show reduced thymocytes and impaired peripheral lymphoproliferation [110]. While NFAT1 downregulates cyclin A1 transcription [111], NFAT2 increases it in the peripheral vasculature, leading to proliferation [112]. NFAT2 can also increase transcription of cyclin D1 [113] , which typically acts in the G1 phase, indicating that NFAT2 also regulates progression through multiple cell cycle phases. NFAT2 also increases proliferation and promotes inflammation-induced tumorigenesis in pancreatic cancer through STAT3 [114]. The disparate actions of NFAT1 and NFAT2 serve to highlight the diverse actions of this family, as well as the far-reaching nature of its role in cell cycle regulation.

Section 4.2: NFAT Proteins and Stress Response

Many NFAT family functions can be loosely grouped as "stress response" activities, which include responses to chemotherapeutic/radiation-induced stress, mechanical stress, and hypoxia.

NFAT proteins play both pro- and anti-apoptotic roles in response to stress. NFAT3 plays a protective role in the response of cardiomyocytes to radiation [115]. NFAT1 induces DDIAS, an apoptosis suppressor, and this transcriptional activation is implicated in cisplatin resistance in lung cancer [116]. However, NFAT proteins also have well-documented pro-apoptotic effects in response to chemo- and radiotherapy. NFAT3 mediates FasL expression and apoptosis in retinal ganglion cells after light-induced damage [117] and in neurons after methamphetamine-induced damage [118]. It also plays a pro-apoptotic role by inducing caspase after intracerebral hemorrhage [119]. Importantly, NFAT3 also plays a pro-apoptotic role in the kidney in response to carboplatin [120] and is required for doxorubicin-mediated apoptosis in glioma [121]. Therefore, the balance of pro- and anti-apoptotic activity seems to vary significantly with the cell type and the specific NFAT cofactors available in the cell. In addition to responding to chemotherapeutic stress, NFAT proteins can also respond to mechanical stressors. NFAT3 is induced by volume overload in cardiac myocytes and may promote differentiation [122]. In fact, multiple reports implicate multiple NFAT family members in responses to mechanical stress in the heart [123] and in response to bladder outlet obstruction [124] through GATA4 [125], which is a known NFAT transcriptional partner.

NFAT proteins are also involved in the response to hypoxia. NFAT3 is clearly induced by hypoxia in the pulmonary vasculature; the hypoxic state leads to expression of endothelin-1, which binds to its receptor and activates calcium flux, which eventually activates calcineurin and then NFAT3 [126]. Interestingly, this expression protects pulmonary artery vascular cells during hypoxic conditions [127]. NFAT3 is also enriched in the brain during hypoxia, and contributes to maintenance of a neural stem cell state and increases neuronal proliferation and self-renewal under these conditions [128]. This response is likely mediated at least partly by NFAT-induced transcription of hypoxia inducible factor 1 (HIF1), which has been documented in multiple contexts [129, 130]. Particularly in the context of survival within a hypoxic cancer stem niche during chemotherapy treatment, the protective and adaptive effects of NFAT3 may play a key role in stem cell maintenance.

Section 4.3: NFAT proteins and Stem Cell Fate

NFAT proteins play various roles in determining stem cell fate. They can serve as regulators of differentiation in neural stem cells [131], Schwann cells [132], and intestinal stem cells [133]. NFAT1 regulates both neural stem cell proliferation and differentiation through Wnt expression [134]. However, Wnt-induced NFAT1 expression can also contribute to the maintenance of a quiescent state in hematopoietic stem cells [135]. This diversity of function is in keeping with the complexity of the NFAT family.

One of the more interesting physiological roles of NFATs is in the hair follicle stem cell. NFAT1 is specifically expressed in the hair follicle stem cells, but not in the more rapidly-dividing

epithelial cells in the follicle. In response to BMP4 signaling, NFAT1 translocates to the nucleus and represses transcription of CDK4, which is required for transition from the G1 to the S phase of the cell cycle. This creates a G1 cell cycle arrest, resulting in a quiescent state in the hair follicle stem cell. In fact, NFAT1 is required for quiescence in these stem cells [109]. This is particularly significant due to the well-known effects of chemotherapy on hair growth. Most cytotoxic chemotherapies target rapidly dividing cells, and therefore can have side effects such as diarrhea (due to degradation of the rapidly dividing gut epithelium) and hair loss (due to death of the rapidly dividing cells in most of the hair follicle.) However, following chemotherapy, patients generally grow back their hair, indicating that even though the NFAT-negative rapidly dividing epithelial cells in the hair follicle die, the stem cells are clearly capable of surviving through chemotherapy and repopulating functional follicles. This situation is potentially analogous to these NFAT3-high quiescent CSLC surviving through chemotherapy and then repopulating tumors in ovarian cancer. Therefore, we have investigated mechanisms of quiescence, cell cycle regulation, and chemotherapy resistance in addition to NFAT's other potential roles in cancer.

4.4: NFAT Proteins in Cancer Progression

As is the case with other functions, different NFAT family members mediate multiple aspects of tumorigenesis and cancer progression, and all four core NFAT family members have been implicated in tumor initiation or progression. These roles primarily include cell proliferation and transformation, migration and invasion, and angiogenesis.

NFAT2 generally functions as a tumor promoter; constitutively active NFAT2 expression leads to a transformed phenotype that involves serum independence, a lack of contact inhibition, and increased tumorigenesis in nude mice [136]. In fact, nude mice expressing a constitutively active NFAT2 tend to develop skin and ovarian tumors, which require continued and constitutive NFAT2 expression for tumor growth and progression [137]. There also appear to be significantly increased cytokine levels in the $NFAT2^{high}$ tumors, suggesting the creation of an inflammatory microenvironment. Constitutively active NFAT2 has been implicated in tumor progression and increased tumor growth in diffuse large B-cell lymphoma [138] and chronic lymphocytic leukemia [139].

By contrast, NFAT1 has more variable roles, as it induces cell cycle arrest and apoptosis and inhibits Ras-induced cell transformation in NIH 3T3 cells [140], but increases expression of the MDM2 oncogene in breast cancer [141]. NFAT3 also plays both pro- and anti-tumorigenic roles in cell transformation. In 3T3L1 mouse embryonic fibroblasts, ectopic NFAT3 activity suppresses Ras-induced cell transformation and focus formation; however, in Cl41 epidermal cells, NFAT3 knockdown actually promotes cell transformation [142, 143]. This is in keeping with the NFAT-mediated transcriptional activity described above, which depends heavily on other cellular cofactors.

In addition to their role in tumor initiation and cancer cell proliferation, NFAT proteins have been implicated in invasion and metastasis. The most prominent of these effects occurs through NFAT1-mediated COX2 induction in breast cancer. NFAT1 induces transcription of COX2 and upregulation of PGE2; increased COX2 in breast cancer cells leads to increased invasion in a matrigel assay, while NFAT1 inhibition with siRNA or cyclosporine decreases

invasion [144]. Another NFAT-mediated invasion pathway in breast cancer involves transcription of autotaxin, which mediates the formation of lysophosphatidic acid (LPA) and was found to account for 80% of the motogenicity of tumor cell conditioned media [145]. NFAT proteins can also promote invasion through expression of matrix metalloproteinases (MMPs), which are well-characterized basement membrane proteases that promote invasion [146]. In the glioblastoma multiforme U87cell line, treatment with cyclosporine decreases invasive phenotypes, which are correlated with NFAT1-induced COX2, MMP7, and MMP9 expression [147]. NFAT1 expression is also required for an invasive phenotype in osteosarcoma that is driven by expression of MMP2 [148]. Interestingly, NFAT3 plays the opposite role, decreasing migration and invasion in estrogen receptor-positive breast cancer by cooperating with ER [149].

Taken together, there is clearly a significant role for NFAT proteins in general and NFAT3 specifically in differentiation, proliferation, stress response, and cancer, though activities clearly vary by cell type and context. This is particularly significant in the ovarian cancer model because tumor stem cells are presumed to live in a hypoxic niche [150]; therefore, NFAT-mediated regulation of the cell cycle and stemlike characteristics combined with a protective response of NFAT3 to hypoxia could play a key role in maintaining the cancer stem cell phenotype.

Section 5: Cell Cycle Regulation and Manipulation

Section 5.1: The Cell Cycle and G0 Phase

The cell cycle in eukaryotic cells is traditionally comprised of the G1, S, G2, and M phases. Cells classically grow in the G1 phase, synthesize DNA in the S phase, grow more in the G2 phase, and then undergo mitosis and cytokinesis in the M phase (Figure 1.3). The progression through the cell cycle is highly regulated by cyclins and cyclin-dependent kinases (CDKs); cyclins fluctuate in concentration with each phase of the cell cycle, while CDK levels tend to remain stable, although their activity depends on the fluctuating cyclins [151]. In addition, cell cycle progression from phase to phase is dependent on checkpoints, which essentially ensure that cells have completed certain processes, such as DNA replication, before progressing through the cell cycle [152]. Failure to complete certain processes leads to arrest at certain checkpoints; for example, DNA damage or stalling of replication forks activates the damage response proteins ATM and ATR, which in turn phosphorylate Chk1 and Chk2. This cascade eventually inhibits various cyclins and CDKs, inhibiting progression through the cell cycle (reviewed in [153]) and eventually leading to apoptosis.

A large number of chemotherapeutic agents therefore target various phases of the cell cycle in order to arrest progression and activate internal apoptosis programs. However, these agents tend to target actively dividing cells in the G1, S, G2, and M phases; cells that have exited the cycle and are considered to be in the G0 phase tend to be less affected by these drugs. While the G0 phase is not particularly well characterized, it encompasses cells that have exited the cell cycle and are not actively dividing, but are also not dying. The best known examples of cells

in the G0 phase include cardiac muscle cells and neurons, which are formed during embryonic development and then usually do not divide again throughout the lifespan of the organism. However, the G0 phase encompasses at least three phenotypes. First, neurons and cardiac myocytes are terminally differentiated tissues that are clearly highly metabolically active, but nonetheless do not divide. Second, there are cells that have exited the cell cycle but are capable of re-entering the cell cycle as needed; these are typically thought of as quiescent cells. The third phenotype within the G0 phase is the senescent phenotype, which consists of cells which have permanently exited the cell cycle and are incapable of dividing again. Though there are markers for each phenotype and some regulatory mechanisms driving senescence and quiescence have been characterized, there is still a great deal of uncertainty regarding the G0 phase and the accurate characterization and classification of these cells.

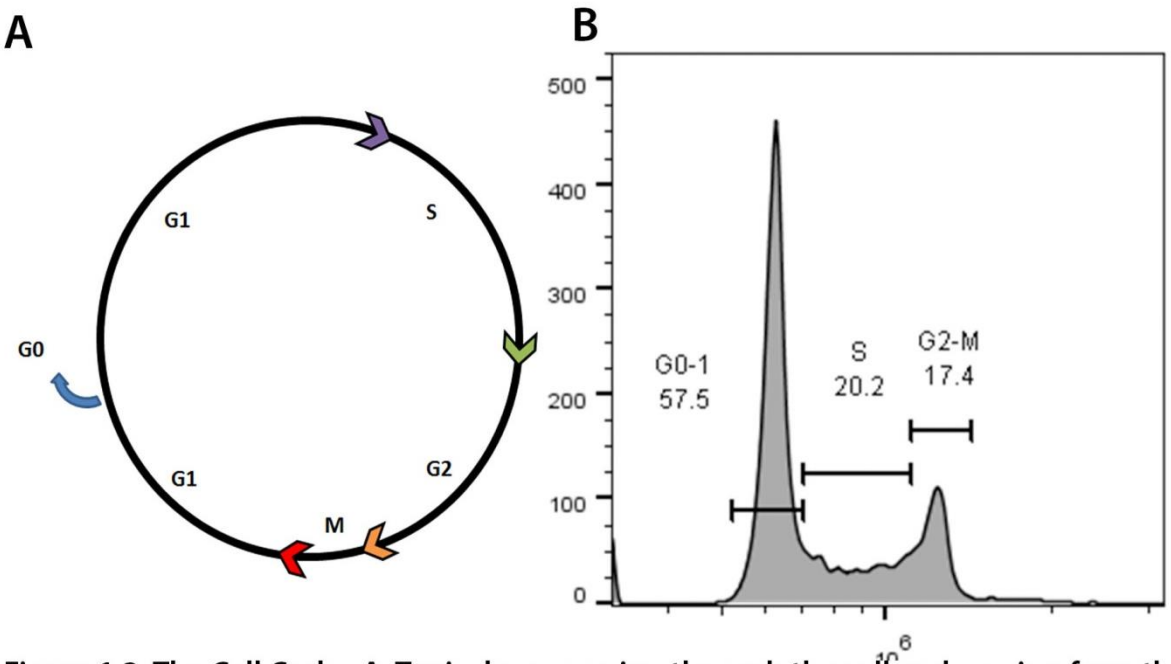

Figure 1.3. The Cell Cycle. A. Typical progression through the cell cycle, going from the G1 (growth) phase into the S (DNA Synthesis) phase, and then progressing into G2 (additional growth) and M (mitosis). The G0 phase represents exit from the cell cycle. B. This is a typical cell cycle phase diagram, which separates cells by DNA content. The G0/G1 peak, on the left, represents cells with 2N DNA. The intermediate central region represents the S phase, where cells have v G1 ying DNA content based on the progression of DNA replication. The G2/M peak represents cells with 4N DNA, which have completed DNA synthesis and are ready to divide. In order to differentiate G0 from G1 and G2 from M phase, further assays are required.

Section 5.2: Senescence

Senescence can be a normal physiological response to shortening telomeres or a pathological one in response to noxious stimuli, such as chemotherapy. The mechanisms that control senescence are unclear and appear to vary considerably between different cell types. In DNA damage-induced senescence, which can occur due to telomere shortening or chemotherapy, the damage sensor ataxia telangiectasia mutated (ATM) travels to the sites of damage and recruits p53 and its target, p21. P21 then prevents CDK2-mediated Rb inactivation, which essentially stops the cells from entering the S phase [154]. A second major senescence pathway begins with the tumor suppressor $p16^{Ink4a}$, which inhibits CDK4 and CDK6 from inactivating Rb, thereby leading to failure to transition from G1 phase into S phase [155, 156]. Notably, though senescence is primarily characterized as a G0 state or as "exit" from the cell cycle, some reports that characterize senescence actually involve prolonged arrests in the G1 or G2 phases [157, 158]. This is another characteristic that makes these cells difficult to classify accurately.

The most common assay for senescence involves staining for senescence associated beta galactosidase (SAβG) [159]. Though it was originally thought that this lysosomal enzyme was responsible for cellular degradation, SAβG is found in non-lysosomal areas and is actually not always required for senescence; one study found that when its enzymatic activity was disrupted, fibroblasts still underwent replicative senescence [160]. In addition, non-senescent cells can stain positive for SAβG [161], which makes this marker relatively nonspecific. However, it is widely used to mark senescent cells, typically in combination with other senescence markers. Senescence-associated heterochromatic foci are intra-nuclear inclusions

that represent condensations of heterochromatin that develop when a cell has permanently inactivated certain regions of DNA; however, they appear in some cell lines and not in others and, like SAβG, are not required for replicative senescence [162]. A third way to identify senescent cells is through the expression of a senescence-associated secretory profile, which is relatively well characterized. This profile includes many inflammatory cytokines such as GM-CSF, IL-6, and others [163]. It is thought that senescent cells in cancer may provoke an inflammatory phenotype by secreting immunological cytokines to recruit the immune system to clear damaged cells. As none of these methods is particularly sensitive or specific, combinations of methods are typically used to more accurately identify senescent cells.

Section 5.3: Quiescence

Like senescence, quiescence is not particularly well defined, but is typically referred to as reversible cell cycle exit. However, this is not easy to confirm, since it is technically difficult to distinguish very slow-cycling or arrested cells from cells that have irreversibly decided not to cycle again; this difficulty is compounded by the limitations of the senescence identification methodologies discussed above. Compared to cycling cells, quiescent cells typically have three major traits: altered cell cycle gene expression, downregulated metabolism, and upregulated survival mechanisms/stress-response genes. Quiescent hematopoietic stem cells and hair follicle stem cells have downregulated cyclins A2, B1, and E2, which control progression through the cell cycle [164, 165]. Quiescence can also be regulated by the p53/p21 axis, which helps to control entry into the cycle at G1 [166]. In addition to cell cycle regulation, quiescent

cells can also show decreased metabolism, reflected by downregulation of cytochrome C [164]. Slow-cycling hematopoietic stem cells also tend to express stress-response genes; for example, HIF1α is upregulated in hematopoietic stem cells in the niche to help cells survive under hypoxic conditions and is necessary for quiescence in hematopoietic stem cells [167]. In addition, various members of the FOXO transcription factor family are upregulated in in HSCs, which tend to exit from the quiescent state when FOXO transcription is inhibited [168]. However, none of these are always present in quiescent cells, and they do not necessarily distinguish quiescent from senescent cells.

There are two major flow cytometry-based assays that attempt to quantify quiescent cells. The first relies on expression of the cell cycle marker ki-67, which marks all cells within the cycling phases (eg. G1, S, G2, and M), but not those in the G0 phase [169]. This makes it an excellent and widely used marker for proliferation. In fact, ki-67 is a commonly used histological marker for clinical assessment of patient tumor samples and is used to quantify the aggressiveness of the tumor, based on the proportion of cells that are dividing. Cells are also co-stained with the nuclear dye DAPI, which is able to distinguish 2N (pre-S phase) from 4 N (mitotic) cells. The combination of 2N DNA and lack of ki67 expression is thought to mark quiescent cells, as they do not have the increased DNA content associated with cycling and are not in any of the cycling phases [170]. Therefore, this method classifies quiescence based on position on the cell cycle and expression of proliferation-related markers.

A second staining method to quantify quiescence uses Hoechst 33442, a DNA dye, in combination with Pyronin Y, an RNA dye. This method depends on the fact that quiescent cells are thought to downregulate their ribosomal content, due to decreased protein synthesis in the

resting state. Because ribosomes make up such a high percentage of the cell's total RNA content, total cellular RNA decreases significantly due to ribosomal downregulation. Cells can therefore be separated by cell cycle phase based on the DNA content as measured by Hoechst dye, and cells with 2N DNA content and measurably lower RNA content are considered to be quiescent [171]. Other quiescence assays tend to depend on label retention of BrdU or tritiated thymidine [172] or, more recently, expression of histones conjugated to GFP [173]. However, as mentioned above, there is no universally accepted quiescence phenotype, which makes it difficult to definitively identify quiescent cells and distinguish them from senescent cells.

Section 5.4: Cell Cycle Control and Maintenance Therapies in Ovarian Cancer

Ovarian cancer is particularly difficult to treat, due to a high recurrence rate combined with a lack of targeted or maintenance therapies for patients between rounds of cytotoxic chemotherapy. 95% of ovarian cancer patients carry mutations in p53, and about 15-20% have mutations in the BRCA genes. However, there are no other major mutations that characterize subsets of ovarian cancer. Therefore, existing targeted therapies are infrequently used in ovarian cancer, and the lack of specific genetic mutations makes it difficult to develop additional targeted agents. Current maintenance therapy options, as discussed in Section 1, have never been shown to prolong overall survival; therefore, the development of better-targeted therapies that can be used for maintenance after or between cytotoxic chemotherapies may significantly improve prognosis.

The lack of large subsets of ovarian cancer patients with significant mutations suggests that therapies that target broader pathways may be required to improve survival in ovarian cancer. CDK4/6 inhibitors, which target cell cycle progression, could be a reasonable option. CDK4 and CDK6 are required for cell cycle progression from the G1 to the S phase; in their absence, cells fail to divide and simply arrest in the G1 phase. Therefore, this could be an ideal therapy to prolong remissions in patients with minimal residual disease. Although there have been many studies on CDK4/6 inhibitors *in vitro*, the only clinical trial published on CDK4/6 inhibition to date studied breast cancer. In this trial, a combination of an aromatase inhibitor (Letrozole) and a CDK4/6 inhibitor (Palbociclib) doubled progression free survival from 10 to 20 months [174]. We have therefore investigated CDK4/6 inhibitors in ovarian cancer as a potential maintenance therapeutic.

Section 6: Contributions of this Thesis Work

This body of work takes two approaches to improving outcomes in ovarian cancer, both of which address different aspects recurrent disease, which is a major clinical problem in this type of cancer. The first portion, which deals with the transcription factor NFAT3 and its effects in ovarian cancer stem-like cells, attempts to investigate the mechanisms behind quiescence and chemotherapy resistance, which we believe are essential for disease relapse. The working hypothesis for this project is that NFAT3 slows the cell cycle in cancer-stem like cells, thereby protecting them from cytotoxic carboplatin-taxane chemotherapy; this therapy primarily targets rapidly dividing cells, and the slow-cycling state may allow CSLC to remain unscathed

and later proliferate to create new tumors and cause relapse. This hypothesis was based on preliminary findings showing that NFAT3 both slowed cell growth significantly and promoted chemotherapy resistance. Through *in vitro* and *in vivo* studies, we have identified that NFAT3 leads cells to remain in a quiescent state and is a protective factor during cisplatin chemotherapy. We have also identified that NFAT3 overexpression tends to decrease transcription of CDK6, which is normally required for the G1-S phase cell cycle transition. This disruption in the cell cycle may help lead to a quiescent phenotype. Despite the associations between quiescence and chemotherapy resistance, cytostatic maintenance therapies that slow tumor growth and block cell cycle progression can often prolong survival in patients who are not candidates for curative therapy. This subset of patients comprises a significant portion of those with ovarian cancer; therefore, the second portion of this thesis work investigates CDK4/6 inhibition as a maintenance therapy in ovarian cancer to improve therapeutic options for women with relapsed ovarian cancer. We have investigated the CDK4/6 inhibitor LEE-011 (Ribociclib, Novartis) and found that it is effective in ovarian cancer through a variety of *in vitro* and *in vivo* studies. In addition, we have observed a novel chemosensitizing effect of LEE-011 in ovarian cancer when this drug is used in combination with cisplatin. Dr. Buckanovich has initiated a clinical trial of this drug, which is the first of its kind in ovarian cancer, to improve therapeutic options for current ovarian cancer patients.

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Chapter 2: NFAT3 Promotes Quiescence and Chemotherapy Resistance in Ovarian Cancer

Abstract

Background: The NFAT transcription factor family regulates many critical biologic functions of normal cells including survival, cell cycling, and, notably, stem cell quiescence. NFAT proteins have recently been implicated in multiple roles in cancer progression, including angiogenesis and malignant transformation. However, the role of NFAT proteins as regulators of cancer cell quiescence is unstudied, and quiescence in cancer cells has been linked with therapeutic resistance. Due to the previously described roles of NFAT proteins as regulators of quiescence and the potential role that cancer cell quiescence plays in chemoresistance, we have characterized the role of NFAT3 in the regulation of ovarian cancer cellular quiescence and chemotherapy resistance.

Methods: qRT-PCR was performed to analyze NFAT3 expression in ovarian CSC and bulk tumor cells. *In silico* gene set enrichment analysis of ovarian cancer patient samples from the TCGA database was performed to identify global impacts of NFAT3 on gene expression. We created

ovarian cancer cell lines expressing constitutively nuclear/active (cNFAT3) or inducible constitutively active (IcNFAT3) NFAT3. Using these cell lines and the NFAT inhibitor VIVIT, we evaluated the impact of NFAT3 activation or inhibition on cancer cell proliferation, cell-cycle, and chemotherapy response *in vitro* and *in vivo*.

Results: NFAT3 is preferentially expressed in ALDH+CD133+ ovarian cancer stem-like cells relative to bulk tumor cells. Analysis of NFAT3 expression in the TCGA dataset showed that NFAT3 activity was correlated with decreases in gene sets for ribosomal structural proteins, translation, and metabolism. Expression cNFAT3 in ovarian cancer cells resulted in 3.4-fold decreased cell division rates, 2- fold decreased BrdU incorporation, a 10% decrease in cell size, and a 25% decrease in total cellular RNA. Despite this decrease in proliferation parameters, cNFAT3 did not impact cell viability, senescence or apoptosis, suggesting the induction of a quiescent state. cNFAT3 expression in tumor cells *in vivo* significantly retarded tumor growth and IcNFAT3 induction arrested tumor growth *in vivo*. Indeed, tumor proliferation was only observed upon loss of cNFAT expression. Suggesting a role for quiescence in chemotherapy resistance, cisplatin treatment of ovarian cancer cells was associated with nuclear translocation of NFAT3, and cNFAT3 cells demonstrated resistance to chemotherapy. Furthermore, NFAT inhibition with VIVIT significantly decreased survival during cisplatin chemotherapy *in vitro*.
Summary: NFAT3 activation is associated with a quiescent state characterized by decreased proliferation, decreased size, and decreased total RNA. Constitute activation of NFAT3 arrests tumor proliferation and is associated with resistance to chemotherapy. NFAT3 represents a therapeutic target to both overcome chemotherapy resistance in quiescent cancer cells and to restrict the growth of therapy-resistant disease.

Background

Ovarian cancer is the fifth most common cancer in American women as well as the most lethal gynecological cancer [1]. This is partly because most women are diagnosed with later stage disease, due to vague early symptoms and a lack of sensitive and specific screening tests [2]. One of the most important clinical factors for prognosis is recurrence; although 70% of patients initially achieve a complete response to first-line chemotherapy, 70% of those patients eventually relapse and succumb to their disease [7]. Therefore, identifying mechanisms of chemotherapy resistance is essential for preventing relapse and improving prognosis in ovarian cancer. One current theory regarding relapse involves the cancer stem cell hypothesis, which suggests that a small fraction of stem-like tumor cells can survive through chemotherapy and then proliferate, repopulating tumors [175]. While significant controversy revolves around these cancer stem-like cells (CSLC), experimental evidence indicates an important biologic role for CSLC. We previously reported a subset of ovarian CSLC that are classified by expression of the markers ALDH and CD133. ALDH+/CD133+ cells were effective in *in vitro* and *in vivo* tumorigenesis assays and their presence correlated with poor prognosis in patients [29]. Single cell analysis of CSLC divisions showed multipotent potential; ALDH+/CD133+ cells could divide symmetrically to self-renew or asymmetrically to produce more differentiated cells [21]. Importantly, increases in the CSLC pool resulted in significant increases in chemotherapy resistance and tumor initiation capacity. Thus, identifying factors which regulate the stem cell pool could significantly impact tumor growth, recurrence, and chemotherapy resistance.

The Nuclear Factor of Activated T-Cells (NFAT) family of transcription factors, comprised of NFAT1-4, has been implicated in the regulation of tissue stem cells. NFAT transcription

factors were initially identified in the immune system as key regulators of T-cell activation and proliferation [73, 74]. These proteins play many roles in cancer, including regulation of angiogenesis [176], cell transformation [177, 178], and response to chemotherapy [120, 121]. They also play key physiological roles, including cell cycle regulation [112, 113] and promotion of stem cell quiescence in the hair follicle [109]. Quiescence is commonly ascribed to stem cells and has been shown to be a protective factor during chemotherapy [56]; because chemotherapy typically targets rapidly dividing cells, slower-cycling cells are often spared. A striking example of this is in the hair follicle, where the transcriptional regulator NFAT1 leads to a quiescent phenotype through CDK4 downregulation only in the stem cells, but not in the more rapidly proliferating cells [109]. While many chemotherapies induce alopecia due to the death of rapidly dividing follicular cells, the slow-cycling stem cells survive through chemotherapy and regenerate functional follicles afterwards, as indicated by the fact that essentially all patients regrow hair after chemotherapy is discontinued. It is possible that NFAT1, which is specifically expressed only in the stem cells, mediates quiescence and may allow these stem cells to survive through chemotherapy and repopulate hair follicles afterwards. This situation is potentially analogous to NFAT3 in ovarian cancer, which is expressed significantly more in the ALDH+/CD133+ stem-like population and may play a role in the survival and chemotherapy resistance of these cells.

We have therefore investigated the effects of NFAT3 in promoting quiescence and chemotherapy resistance in ovarian cancer. We have shown *in silico* data indicating that NFAT3 is correlated with downregulation of gene sets related to ribosomal structure, translation, and oxidative metabolism. We have also shown data from two constitutively active NFAT models

indicating that NFAT3 expression results in the induction of a quiescent state characterized by decreased proliferation, smaller size, and decreased total RNA. In addition, constitutive NFAT3 expression promotes survival during cisplatin chemotherapy, while NFAT inhibition with VIVIT sensitizes ovarian cancer cells to cisplatin. Finally, we have shown that constitutive NFAT3 expression profoundly retards tumor growth *in vivo* and can arrest tumor growth when induced in a murine xenograft model.

Materials and Methods

Cell Culture

The A2780 cell line was obtained from Dr. Susan Murphy at Duke University. ID8 and Hey1 lines were obtained from Dr. Rebecca Liu at University of Michigan. All cells were cultured in RPMI-1640 media with 10% FBS, 1% Penicillin/Streptomycin at 37°F and 5% CO2.

Gene Set enrichment analysis (GSEA)

In order to explore the potential functions of NFAT3, we screened RNA-seq data from 261 serous ovarian carcinomas from the TCGA dataset. Spearman correlation was performed to compare expression of NFAT3 to the expression of all other genes in the Ensmbl genome database (55,840 genes). A p-value cutoff of 1e-8 was applied to generate a list of the most correlated genes. This list was then correlated with established gene sets to identify functional sets that correlated with NFAT3 expression.

Constructs

Our constructs have been created with constitutively nuclear NFAT3. NFAT proteins are regulated by dephosphorylation through phosphatases, which exposes a nuclear localization sequence leading to nuclear translocation. Because the only known function of NFAT proteins is transcription, the phospho-mutants used are constitutively nuclear and therefore constitutively active. A constitutively nuclear NFAT3-YFP fusion (cNFAT3) with the phospho-regulatory domain deleted or a YFP-only control (Ctrl-YFP) were cloned into a pGIPZ lentiviral vector and transduced into the A2780 and ID8 ovarian cancer cell lines [179]. A second, phospho-specific mutant constitutively active NFAT3 [180] was also cloned into the doxycycline-inducible Tet-One expression system (Clontech) to create an inducible and constitutive NFAT3 (IcNFAT3) in the Hey1 ovarian cancer line. This was paired with an inducible luciferase control (ILuc) to control for overexpression. Details regarding the structure and validation of all constructs are presented in the results section.

Cell Cycle Analysis

Ctrl-YFP, cNFAT3, ILuc, and IcNFAT3 cell lines were grown in 6-well plates in triplicate for 72 hours either with or without doxycycline and then harvested, fixed dropwise in 70% ethanol, and incubated with 0.1 ug/mL RNAse for 1h at 37°F. 1 ug/mL PI was added and then cells were analyzed on the BD Accuri flow cytometer. The percentage of cells in each phase was gated and quantified and compared with a Student's t-test.

Senescence Analysis

Ctrl-YFP, ILuc, cNFAT3, and IcNFAT3 cells were grown in in 6 well dishes in triplicate for 72 hours before staining for senescence-associated β-galactosidase with the Senescence β-galactosidase Assay Kit (Cell Signaling) according to the manufacturer's instructions. The percentage of senescent cells in each line was counted on an Olympus BX57 microscope and quantified.

Apoptosis Analysis

For TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) staining, A2780 ctrl-YFP or cNFAT3 cells were grown for 72 hours on coverslips and then fixed with 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X and then stained for TUNEL with a Roche Apoptosis Detection and TUNEL Staining kit along with a positive DNAse control, as described in the manufacturer's protocol. Images were obtained on an Olympus BX57 microscope, and the percentage of TUNEL-positive cells was quantified and compared with a student's t-test.

For apoptosis detection via annexin staining, Hey1 ILuc and IcNFAT3 cells were grown in 6 well dishes with or without doxycycline for 72 hrs. Then, they were stained with the BD Annexin-V FITC apoptosis kit according to the manufacturer's instructions and at least 10,000 events analyzed on the Mo Flo Astrios flow cytometer (BD Biosciences). The percentage of Annexin V^+, PI^+, Annexin V^+/PI^+, and Annexin V^-/PI^- cells was quantified.

Hoechst/Pyronin Quiescence Analysis

ILuc and IcNFAT3 cells were grown in in 6 well dishes in triplicate for 72 hours. Then, they were fixed dropwise in 70% ethanol and incubated at -20 ◦C for at least 30 minutes. Cells were washed with PBS and then incubated with 3uM Hoechst 33442 (Sigma) and 2ug/mL Pyronin Y (Sigma) for 30 minutes at 37◦C before analysis of at least 10,000 events on the MoFlo Astrios flow cytometer (BD Biosciences).

Immunofluorescence

Hey1, COV318, and COV362 ovarian cancer cell lines were grown on glass coverslips and treated with various concentrations of cisplatin based on their respective IC50s. Then, coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X. Cells were blocked with 10% horse serum and incubated with 1:50 mouse anti NFAT3 antibody (Novus Biologicals) in 5% horse serum for 2 hours. After washing three times with PBS for five minutes, cells were incubated with an Alexa Fluor 488 anti-mouse secondary antibody, mounted with DAPI mounting medium (Vector Labs), and then imaged on an Olympus BX41 microscope.

Size Analysis

The flow cytometry forward-scatter parameter was used as a proxy to calculate cell size in ctrl-YFP vs. cNFAT3 cells and ILuc vs. IcNFAT3 cells. Forward-scatter histograms were overlaid

in triplicate and their means were compared with a student's t-test to identify size differences between groups. As confirmation, images were taken of ctrl-YFP and cNFAT3 A2780 cells with an Olympus BX57 microscope and analyzed with ImageJ using the Draw> Select Area tools. The average cell area was calculated and analyzed.

In vivo xenografts

NOD/SCID/IL2R^{KO} or nude mice were injected with 500,000 ctrl-YFP or cNFAT3 cells or 300,000 ILuc or IcNFAT3 cells for tumor xenograft experiments. Animals were maintained at 12-hour light/dark cycles under SPF conditions with free access to food and water. For induction, 2 mg/mL doxycycline was administered in the water along with 5% sucrose to mask its bitter taste. Tumors were monitored once a week initially and twice a week after tumors reached 1000 mm^3, and animals were sacrificed at protocol endpoints. All experiments were conducted in accordance with the animal care and use committee from the University of Michigan.

Results

NFAT3 is differentially expressed in cancer stem-like cells

Our lab previously identified a subset of ovarian cancer stem-like cells (CSLC) marked by expression of ALDH and CD133 [29]. Array analysis of these CSLC showed that NFAT3 is more highly expressed in the stem-like cells than in the bulk tumor cells (data not shown). We have confirmed this overexpression in FACS-sorted patient-derived ALDH+/CD133+ cells with qRT-

PCR in multiple ovarian cancer patients (Fig. 2.1 A-C). While the magnitude of the differential effect varied between patients from 4-fold ($p<0.001$) to nearly 200-fold ($p<0.05$), patient-derived ALDH+/CD133+ cells generally demonstrated more NFAT3 expression than ALDH-/CD133- cells (Figure 2.1A-C). To identify cellular processes influenced by NFAT3 expression, we performed *in silico* gene set enrichment analysis (GSEA) on 261 ovarian cancer patients in the TCGA database [181]. NFAT3 expression was strongly inversely correlated with gene sets associated with ribosomal structural proteins, translation, and metabolism (Figure 2.1D-F, Figures 2.2-2.3). As this set of processes involves fundamental cellular functions required for growth and proliferation, we investigated the impact of NFAT3 on cell proliferation.

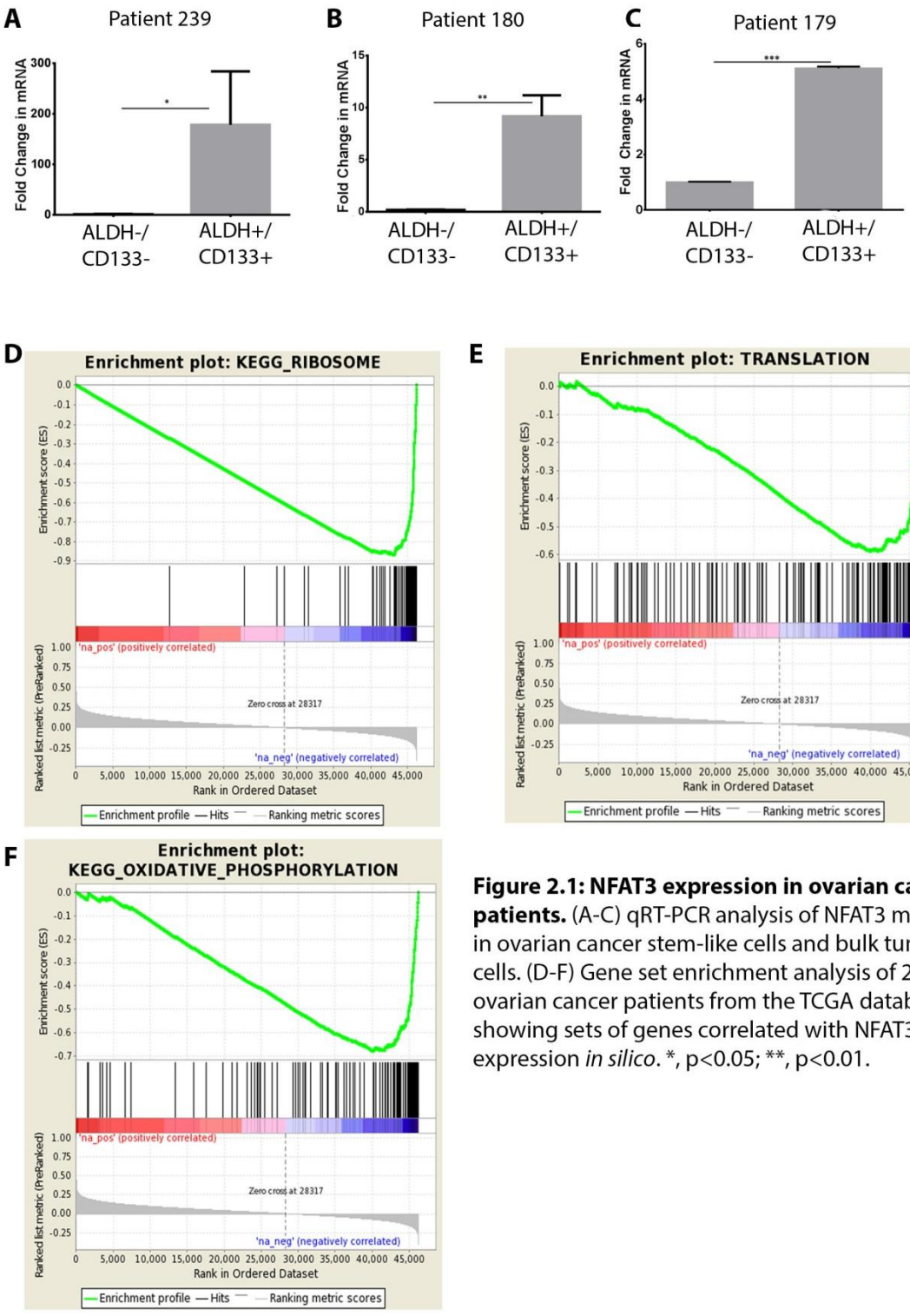


Figure 2.1: NFAT3 expression in ovarian cancer patients. (A-C) qRT-PCR analysis of NFAT3 mRNA in ovarian cancer stem-like cells and bulk tumor cells. (D-F) Gene set enrichment analysis of 261 ovarian cancer patients from the TCGA database showing sets of genes correlated with NFAT3 expression *in silico*. *, $p<0.05$; **, $p<0.01$.

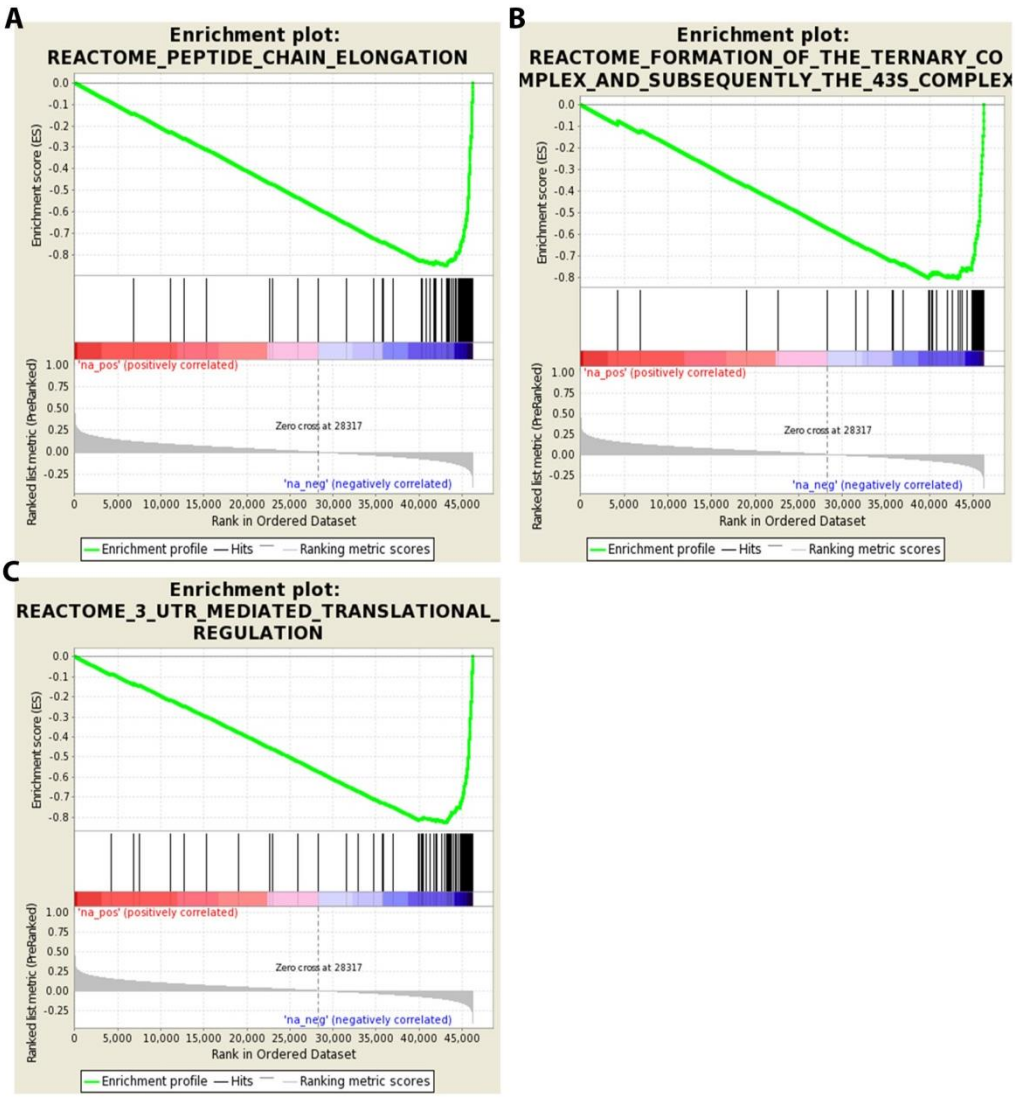


Figure 2.2: Translation-related gene set enrichment analysis of 261 ovarian cancer patients from the TCGA database showing correlations between NFAT3 expression and gene sets related to translation (A-C).

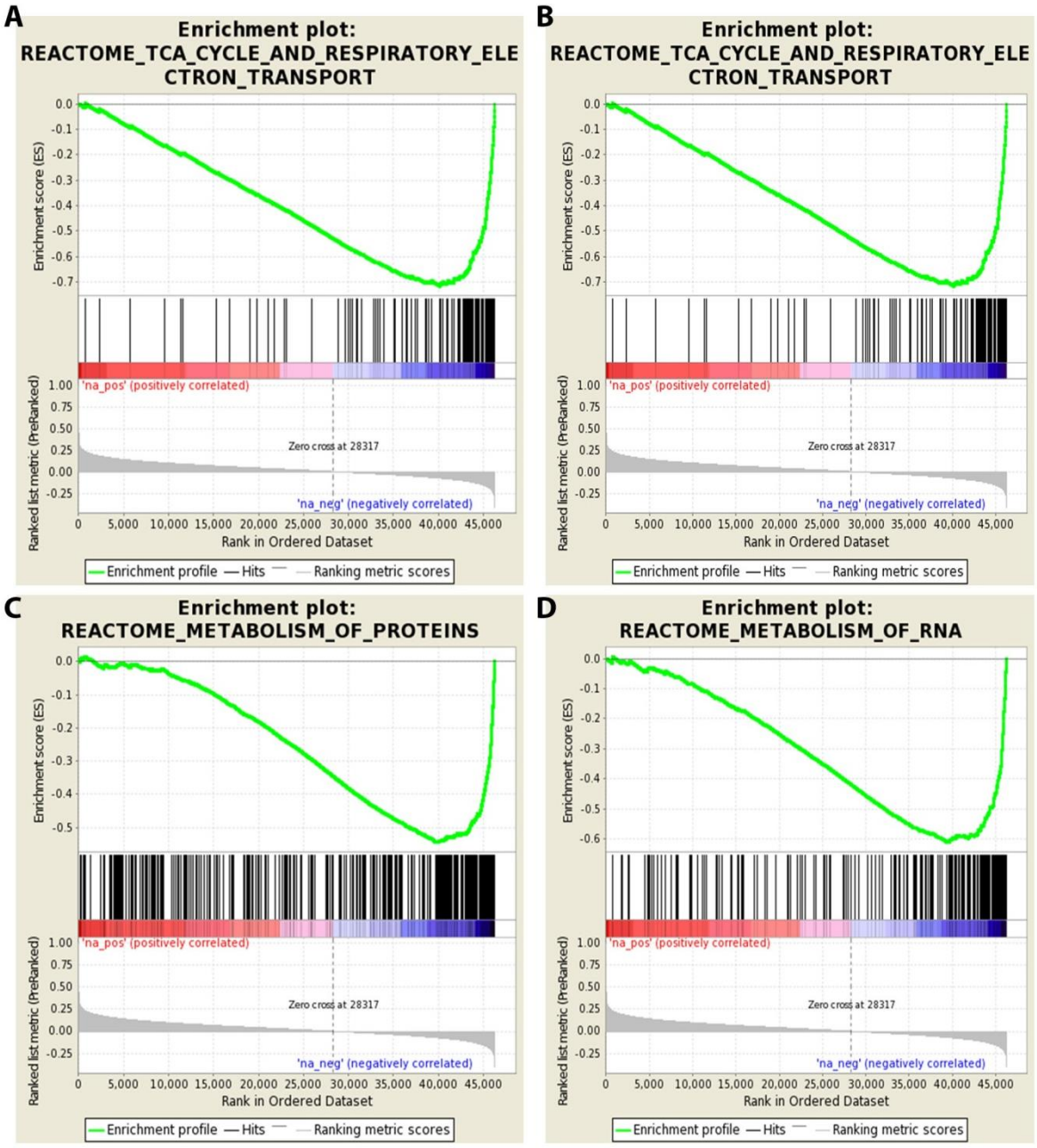


Figure 2.2: Metabolism-related gene set enrichment analysis of 261 ovarian cancer patients from the TCGA database showing correlations between NFAT3 expression and gene sets related to various metabolic pathways (A-C).

Constructs and Validation

NFAT proteins are primarily regulated through phosphorylation. Their regulatory domain includes several serine-rich and serine-proline-rich domains which are normally hyper-phosphorylated in the cytoplasm (Figure 2.4A). Serine dephosphorylation normally exposes a nuclear localization sequence, allowing NFAT to translocate to the nucleus [81, 82], where it can initiate or repress transcriptional activity when accompanied by various binding partners.

The A2780 and ID8 ovarian cancer cell lines were transduced with either a pGIPZ lentiviral expression construct with YFP (ctrl-YFP) or with a truncated NFAT3; the first 317 N-terminal amino acids of NFAT3 were deleted, and a YFP tag was fused to the C-terminal end, as described previously [179]. The 317-AA deletion includes the phosphorylation regions, thus permanently exposing the nuclear localization sequence, resulting in constitutively nuclear (and presumably transcriptionally active) NFAT, termed cNFAT3 (Figure 2.4B).

As the cNFAT3 model contains a significant deletion as well as a fluorescent tag comprising a significant fraction of the total protein's mass, we created a second model of constitutively active NFAT3 that better recapitulates the native protein structure. This model contains several point mutations that change the regulatory serines to alanines, leaving the remaining protein, including the transcriptional activation regions, intact [180]. Due to the lack of serine phosphorylation, the nuclear localization sequence is permanently exposed and the mutant NFAT3 is constitutively nuclear. This clone was transduced into the Hey1 ovarian cancer cell line with a commercially available doxycycline-inducible system (Tet-One inducible lentiviral transduction construct; Clontech) in order to achieve tighter control of expression of this

inducible and constitutive NFAT3 (IcNFAT3) (Fig. 2.4C). As a control, we also created a construct with the luciferase cDNA under the doxycycline-inducible promoter (iLuc).

We next validated constitutive NFAT3 expression and transcriptional activity in both cNFAT3 and IcNFAT3 lines. Transcriptional activity was measured through transcription of the RCAN gene, which is a validated NFAT target [182]. qRT-PCR analysis confirmed significant expression of cNFAT3 mRNAs when compared to Ctrl-YFP cells ($p<0.0001$) (Figure 2.5A). Indicating transcriptional activity of these cNFAT3 clones, we observed clear increased expression of the known NFAT target gene, RCAN ($p<0.001$) (Figure 2.5A). Similarly, we observed significant induction of IcNFAT3 with doxycycline treatment in two independent clones ($p<0.0001$) (Fig. 2.5B) with concurrent increased expression of RCAN ($p<0.0001$) (Fig. 2.5C). This induction was not observed in the ILuc cells for NFAT3 or RCAN.

Each model has some limitations. We observed in the cNFAT3 model that there was significant loss of expression within relatively short periods in culture. While the Ctrl-YFP cells maintained their expression of YFP, as measured by FACS (Fig. 2.6A, top), the cNFAT3 cells lost 80% of their transgene expression over 4 days in culture (Fig. 2.6A, bottom). The inducible luciferase model maintained tighter control of expression, but the IcNFAT3 clones showed three to six-fold induction of NFAT3 at baseline when compared to the ILuc cells, even in the absence of doxycycline (Fig. 2.6B).

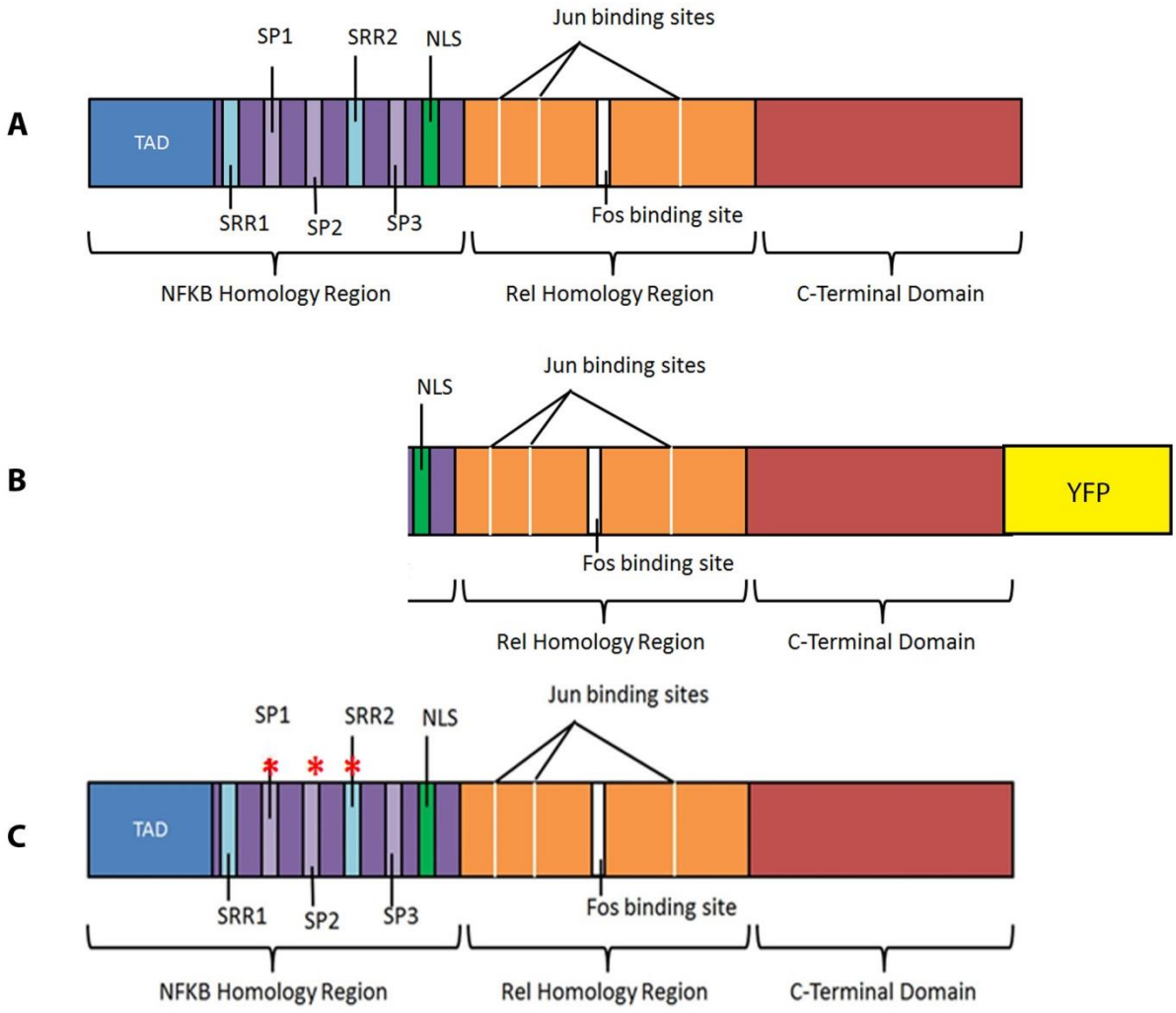


Figure 2.4: NFAT and construct structures. (A) Domain structure of NFAT proteins, including the transcriptional activation domain (TAD), serine-rich regions (SRR) and serine-proline-rich regions (SP), and interaction sites with transcriptional cofactors (Jun and Fos). (B) cNFAT3 structure, including deletion of the first 317 amino acids and addition of a C-terminal YFP. (C) I cNFAT3 structure, serine-alanine point mutations (*) in the regulatory region leading to constitutive nuclear localization with preserved domain architecture.

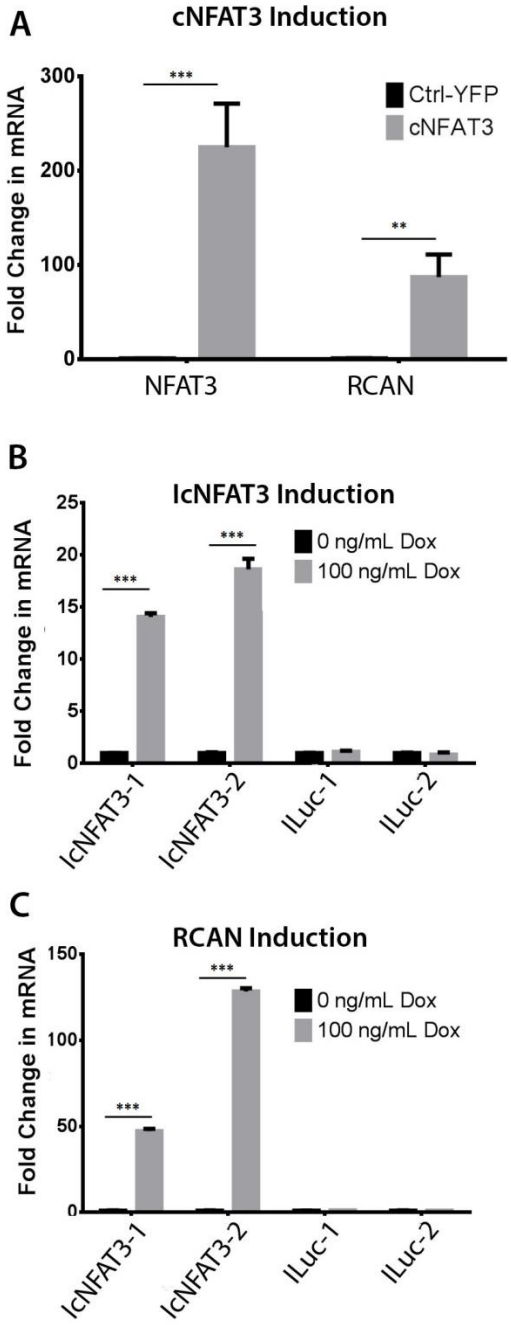


Figure 2.5. Construct validation. (A) Expression of cNFAT3 and its validated target gene RCAN in the cNFAT3 vs. Ctrl-YFP lines. (B) Doxycycline induction of NFAT3 mRNA in IcNFAT3 and ILuc lines. (C) Doxycycline induction of RCAN mRNA expression in IcNFAT3 and ILuc lines. **, $p<0.001$; ***, $p<0.0001$.

NFAT3 decreases cell proliferation without impacting viability or senescence

We tested the effects of NFAT3 activity on ovarian cancer cell growth by tracking cell numbers over time in ctrl-YFP cells vs. cNFAT3 cells in the A2780 and ID8 ovarian cancer lines. cNFAT3 expression was associated with a 2.76-fold decrease in ID8-cNFAT3 cell counts ($p<0.0001$) and a 1.99-fold decrease in A2780-cNFAT3 cell counts ($p<0.0001$) over four days compared to the ctrl-YFP lines (Fig 2.7A-B). A similar relationship was observed in the Hey1 IcNFAT3 cells; two IcNFAT3 clones showed Figure 3.1

1-fold ($p<0.0001$) and 1.65-fold ($p<0.0001$) reduced growth over four days of doxycycline induction (Fig 2.7 C-D), while doxycycline induction did not lead to a change in proliferation in the ILuc cells (Fig. 2.8A).

Given this significant change in cell number and links in the literature between NFAT proteins and apoptosis [104], we tested the effects of cNFAT3 on viability. Trypan blue staining indicated that total viability did not change in cNFAT3 or IcNFAT3 cells compared to their respective controls during four days of growth (Fig. 2.7E). We also analyzed apoptosis in the Hey1 IcNFAT3 model with Annexin-V FACS and in the A2780 cNFAT3 model with TUNEL staining to determine whether constitutive NFAT3 expression led to an increase in apoptosis. There was no difference in apoptotic rates in either model (Figure 2.7F and 2.8B). Similarly, although a small proportion of senescent cells exists in these cell lines, senescence-associated beta galactosidase staining did not change with constitutive NFAT3 expression (Fig. 2.8C). Therefore, it appears that NFAT3 expression decreases cell division without inducing death, apoptosis, or senescence.

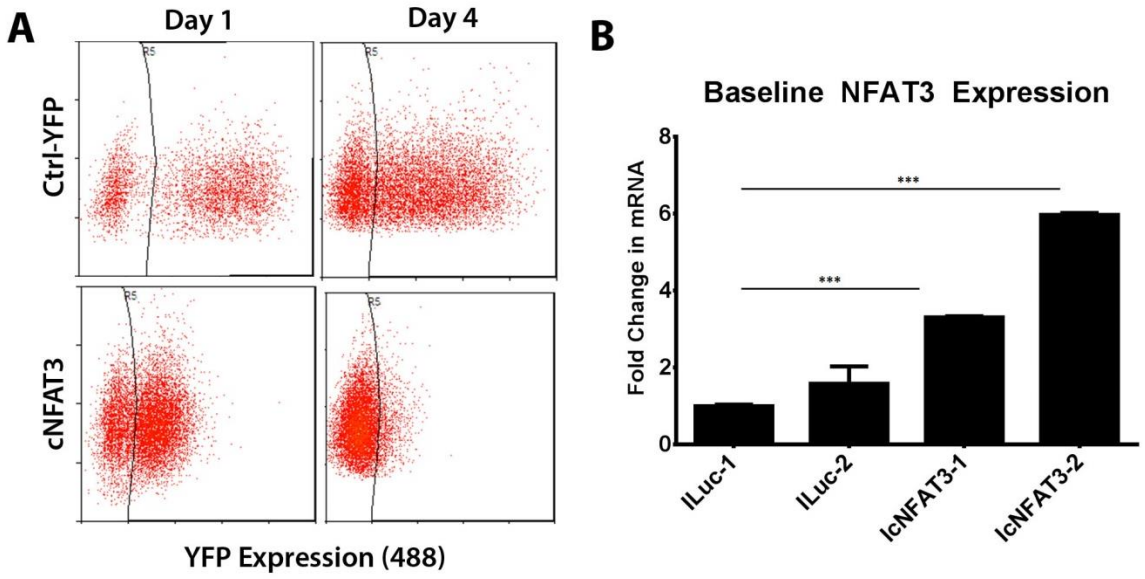


Figure 2.6. NFAT3 expression patterns in cNFAT3 and IcNFAT3 models. (A) FACS analysis of Ctrl-YFP cells (top) and cNFAT3 cells (bottom) showing YFP expression over 4 days in culture. (B) Baseline mRNA expression of NFAT3 in ILuc vs. IcNFAT3 cells without doxycycline induction. ***, $p<0.001$.

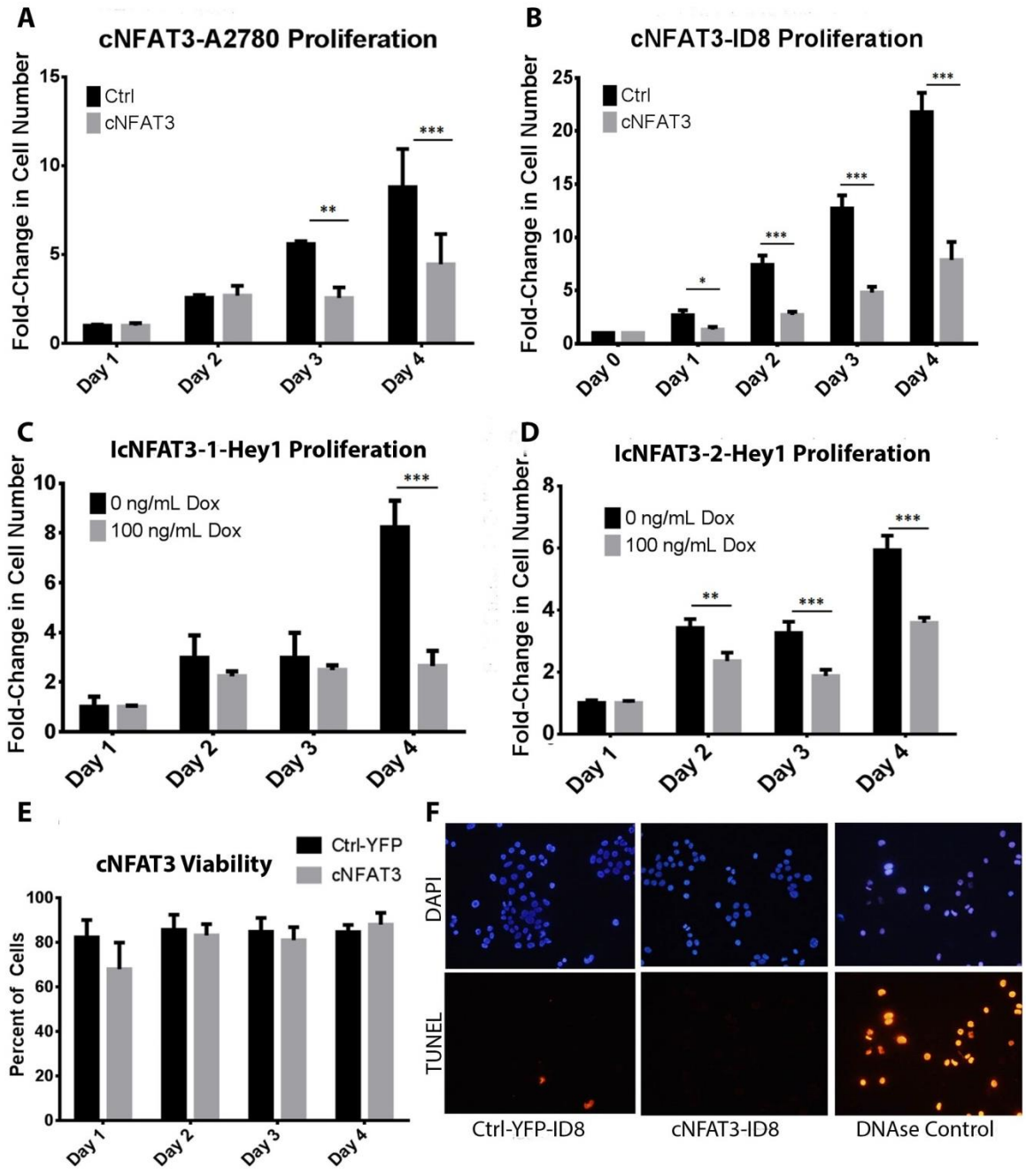


Figure 2.7. Effects of constitutive NFAT3 expression on proliferation and viability. (A-B) Proliferation of cNFAT3 vs. Ctrl-YFP cell lines in A2780 and ID8 ovarian cancer cells. (C-D) Proliferation of two IcNFAT3-Hey1 clones with or without doxycycline induction. (E) Viability over 4 days in cNFAT3-ID8 vs. Ctrl-YFP-ID8 cells. (F) TUNEL staining to quantify apoptosis in cNFAT3 vs. Ctrl-YFP-ID8 cells. *, $p<0.05$; **, $p<0.01$; ***$p<0.001$.

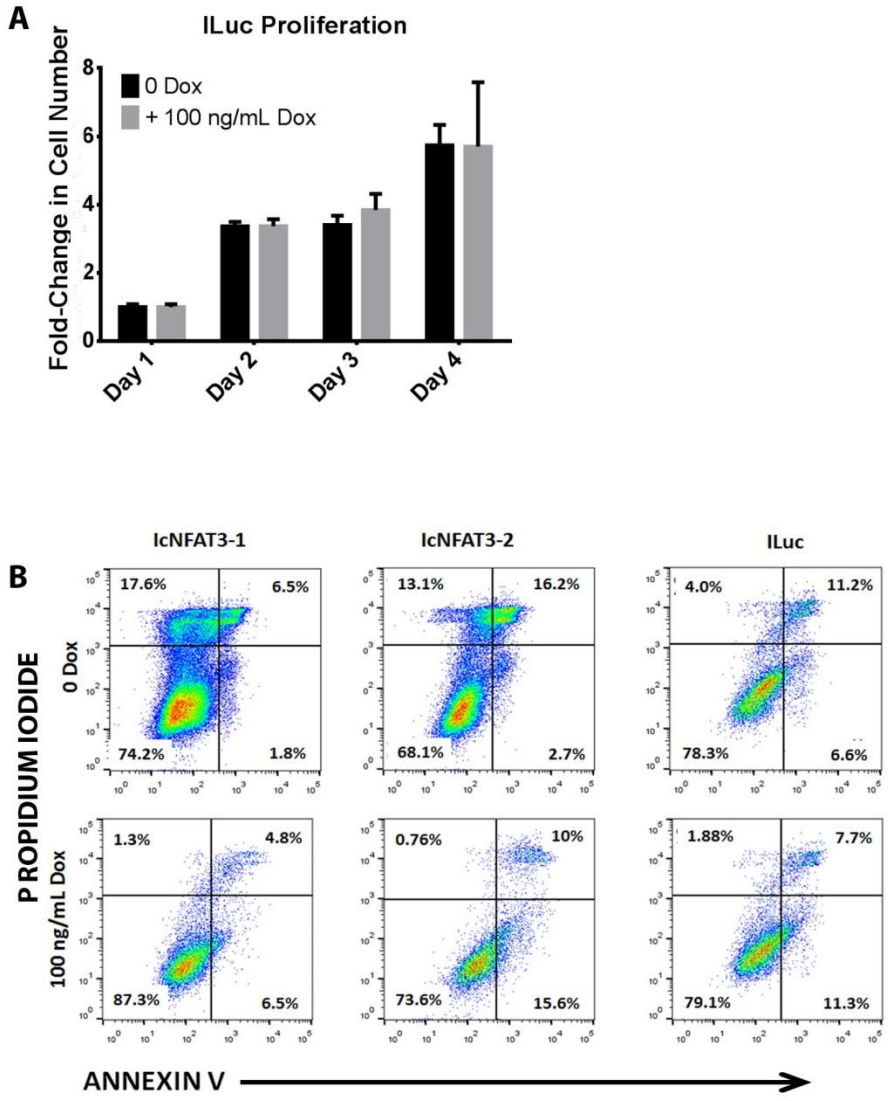
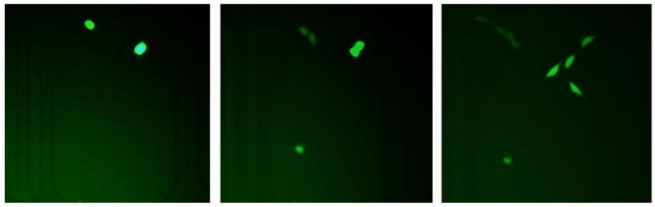


Figure 2.8. Viability effects of IcNFAT3 expression. (A) Effects of ILuc induction on cell proliferation. (B) Effects of doxycycline induction of IcNFAT3 or ILuc on apoptosis as measured by Annexin V staining.

To directly evaluate the impact of cNFAT3 expression on cellular proliferation rates, we analyzed cellular division at the single cell level. We loaded cNFAT3-A2780 or ctrl-YFP-A2780 cells into single cell microfluidic chips as previously described [21] and monitored cell divisions daily over the next four days (Fig. 2.9A). 39% of control cells and 20% of cNFAT3 cells underwent at least one cell division during this period. 41% of dividing control cells underwent a second cell division while only 4% of the cNFAT3 cells underwent a second division; this resulted in a final 3.4-fold decreased total cell number in the cNFAT3 cells vs. YFP cells (Fig. 2.9B). To further demonstrate reduced proliferation in cNFAT cells, we evaluated BrdU incorporation. Immunofluorescent analysis of BrdU incorporation confirmed a >2 fold reduction in studies confirm this decreased proliferation in cNFAT3 cells ($p<0.05$) (Fig. 2.9C-D). We also evaluated the cell cycle in IcNFAT3 and control cells. We observed an 8% increase in cells in the G0/G1 portion of the cell cycles with a reciprocal decrease in the numbers of cells in the G2/M phases. (Fig 2.9E-F).

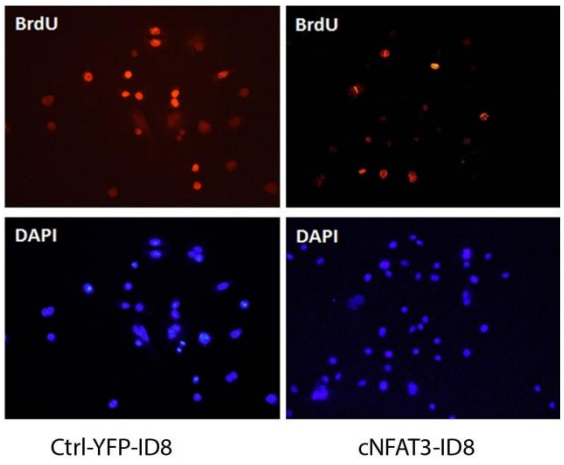
A



B

Number of First and Second Mitoses		
	Ctrl-YFP	**cNFAT3**
Initial Cell Number	180	120
First Mitosis	71 (39%)	25 (20%)
Second Mitosis	29(41%)	1 (4%)
Final Cell Number	605 (3.4-fold)	174 (1.4-fold)

C



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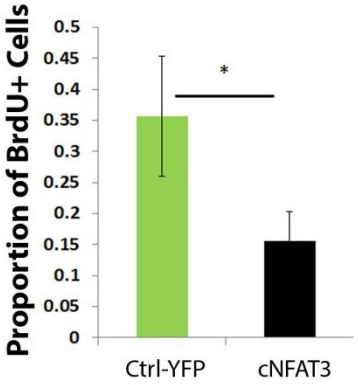

E

F

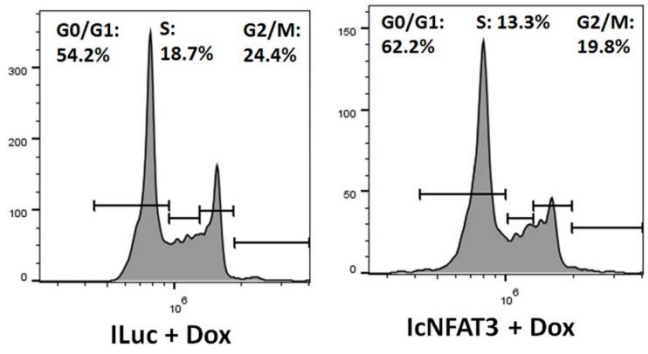

Figure 2.9. Detailed analysis of cNFAT3 and IcNFAT3 proliferation. (A) Ctrl-YFP cells growing over 3 days in single cell microfluidics chips that allow direct observation of divisions. (B) Quantification of cNFAT3 vs. Ctrl-YFP divisions in microfluidics chips. (C) Photomicrographs and (D) quantification of BrdU incorporation in cNFAT3 vs. Ctrl-YFP cells. (E-F) Cell cycle analysis of the percentage of cells in each phase of the cell cycle in cNFAT3 and IcNFAT3 cells.*, $p<0.05$.

cNFAT3 overexpression produces characteristics consistent with quiescence

Phenotypic characteristics of quiescent cells include a reduction in (i) cell size, (ii) total cellular RNA, and (iii) mRNA translation into protein, and (iv) metabolism. We therefore assessed the effect of NFAT3 on cell size. Doxycycline induction of cNFAT3 vs ILuc cells resulted in a clear decrease in size (Fig. 2.10A). Analysis of forward scatter in FACS plots (an indicator of size) revealed that while doxycycline treatment of iLuc cells resulted in a slight increase in cell size, doxycycline induction of IcNFAT3 cells resulted in an 11% decrease in cell size (Figure 2.10B,D). A2780-cNFAT3 cells were also significantly smaller than the ctrl-YFP cells; FACS analysis of forward scatter (Fig. 2.10C) demonstrated a ~50% reduction in cell size.

In quiescent cells, total cellular RNA decreases due to a reduction in rRNA, which normally comprises about 80% of cellular RNA [183]. Given the inverse correlation between NFAT3 expression and ribosome-related gene sets, we evaluated total cellular RNA levels upon cNFAT3 induction with doxycycline. While doxycycline treatment had no impact on total cellular RNA levels in iLuc control cells, doxycycline treatment of cNFAT3 cells resulted in a 20 to 50% reduction in total cellular RNA (Figure 2.10E). To confirm a quiescent phenotype, we performed Hoechst/Pyronin co-staining to identify quiescent cells based on RNA content and cell cycle phase. This assay relies on the fact that cells decrease ribosomal content as they enter a quiescent G0 state, presumably due to decreased metabolic demand. Therefore, we co-stained cells with Pyronin, an RNA-binding dye, and Hoechst 33442, a DNA-binding dye, as previously described [171]. Cells with low pyronin staining and 2N DNA were considered to be quiescent (Fig. 2.11).

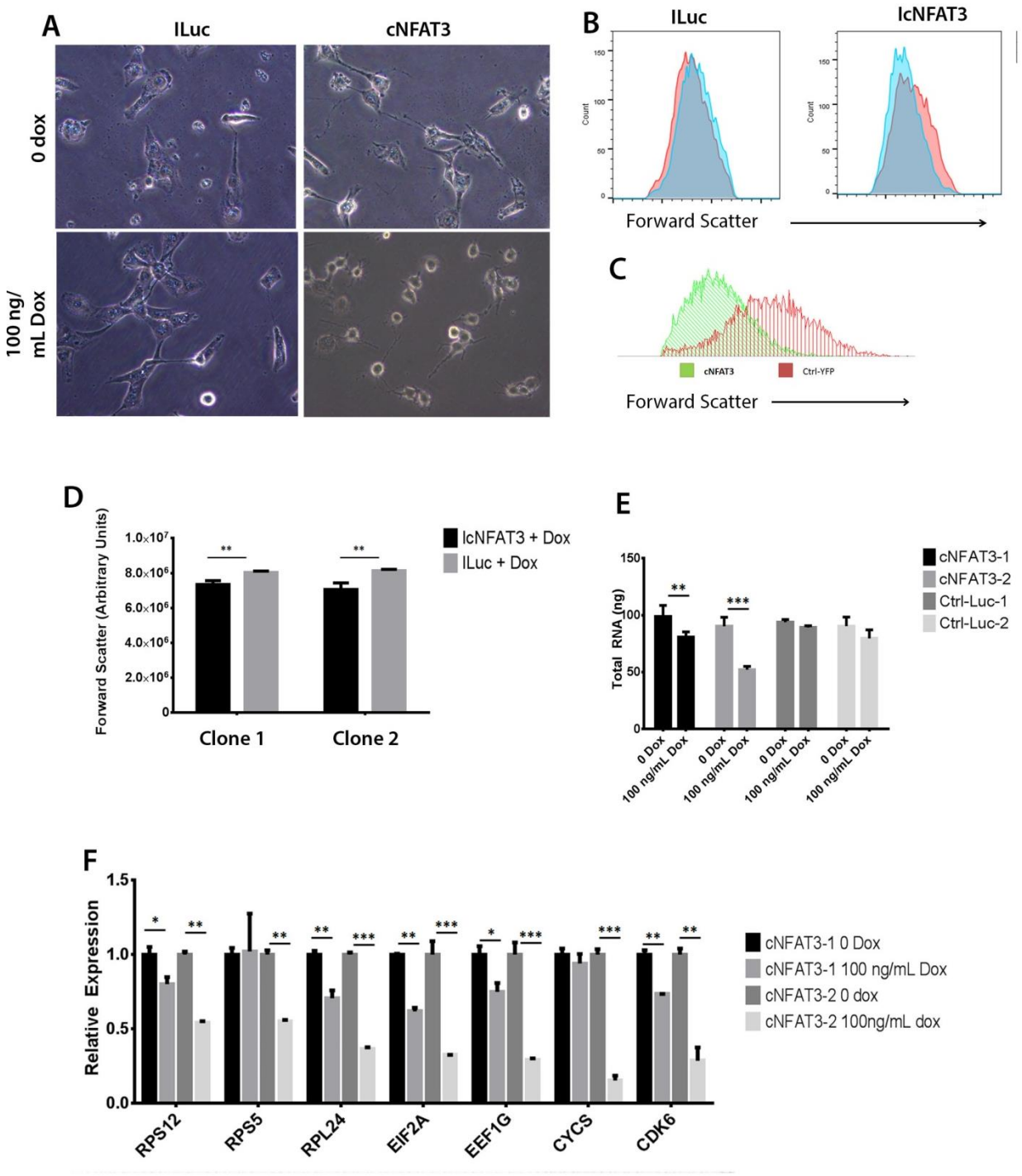

Figure 2.10. lcNFAT3 and cNFAT3 expression decrease cell size, as seen in phase contrast images (A) and using the flow-cytometry forward scatter parameter (B-D). This decrease in size was associated with a decrease in total RNA (E) as well as a decrease in transcription of ribosomal structural proteins and proteins required for translation and metabolism (F). *,p<0.05; **, p<0.01; ***, p<0.001.

We next evaluated specific ribosomal proteins and translation-related proteins whose mRNAs were strongly inversely correlated with NFAT3 in ovarian tumors based on GSEA analysis. qRT-PCR analysis found that cNFAT3 expression significantly decreased the levels of several critical ribosomal structural proteins such as RPL24, RPS5, and RRP40 (Fig. 2.10F). In addition, cNFAT3 expression also decreased the levels of multiple proteins required for translation, such as EIF2A and EEF1G (Fig. 2.10F). We also observed mRNA downregulation of factors required for oxidative metabolism, such as cytochrome C, as well as factors required for cell cycle progression, such as CDK6 (Fig. 2.10F). Together, these point to a general decrease in ribosomal translation and a general down-regulation of major cellular processes.

NFAT3 overexpression promotes chemotherapy resistance

As multiple reports have shown that quiescent/slower-cycling cells tend to be more chemotherapy resistant [44, 53, 54], we next tested the effects of constitutive NFAT3 expression on chemoresistance. NFAT3 is primarily regulated post-translationally and is only active when it has translocated to the nucleus. We treated multiple ovarian cell lines with cisplatin *in vitro* and evaluated NFAT3 subcellular localization. Suggesting that cisplatin activates NFAT3, treatment with cisplatin demonstrated nuclear translocation of native NFAT3 in all treated lines (Fig. 2.12A, 2.13A-B). Confirming increased NFAT3 activity, we observed cisplatin dose- dependent increases in the transcription of RCAN, a known NFAT target gene, in response to cisplatin in the Hey1, COV362, and A2780 ovarian cancer lines (Fig 2.12B, 2.13C-D).

We next directly evaluated the impact of cNFAT3 or native NFAT proteins on chemotherapy response. We treated A2780-cNFAT3 or ctrl-YFP cells with increasing doses of cisplatin.

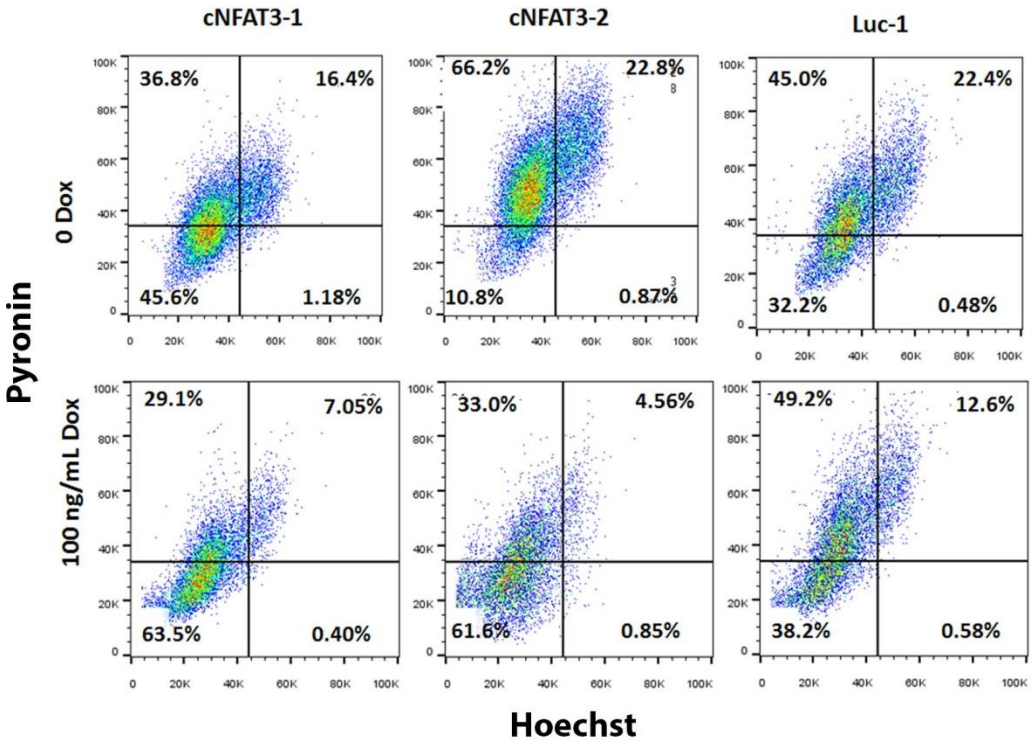

Figure 2.11. Hoechst/Pyronin analysis of quiescence induced by IcNFAT3 or ILuc expression.

When normalized to initial cell counts, cNFAT3 cells demonstrated significantly increased survival in response to cisplatin chemotherapy when treated with 0.25 and 0.5ug/mL cisplatin (Fig. 2.12C). In contrast, while the NFAT inhibitor VIVIT [92] alone had no impact on cell growth (data not shown), VIVIT sensitized cells to cisplatin chemotherapy cell survival over 3 days of cisplatin treatment (Fig. 2.12C).

NFAT3 overexpression significantly suppresses in vivo tumor growth

Due to the significant effects of constitutive NFAT3 activation *in vitro*, we examined its effects on xenograft tumor growth *in vivo*. A2780-cNFAT3 tumors demonstrated significant growth delay relative to controls ($p<0.0001$), with essentially no growth for three weeks and with 2/10 cNFAT tumors failing to initiate (Fig. 2.14A-B). After three weeks, tumors showed exponential growth. Analysis of the tumors that did develop from the cNFAT3 cells demonstrated loss of transgene expression (Fig. 2.15).

We performed a similar *in vivo* analysis using the IcNFAT3 model. In the presence of continuous doxycycline treatment, IcNFAT3 cells grow tumors that are 13.2-fold smaller than their ILuc controls ($p<0.0001$)(Fig. 2.14 C-E). In the absence of doxycycline treatment, these tumors also demonstrate slower growth than controls (Fig 2.14 F-G). This is consistent with 'leaky expression' from the inducible promoter (shown in Fig. 3). Despite the effects of the leaky doxycycline promoter, we observed a significant decrease in tumor growth in the cNFAT3 lines with dox induction, and doxycycline was able to arrest tumor growth when induced mid-experiment (Fig. 2.14 F-G).

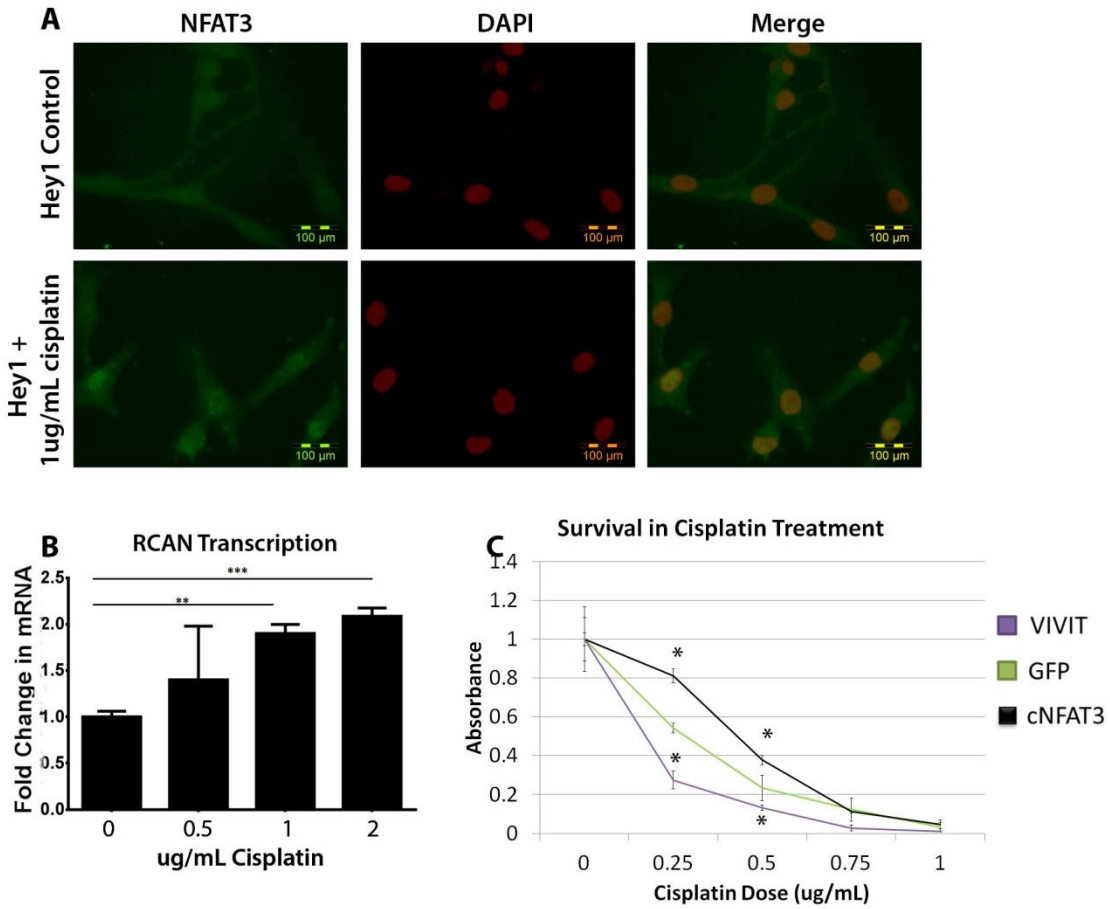

Figure 2.12: Response of NFAT3 to cisplatin treatment. (A) Native NFAT3 translocated to the nucleus in the Hey1 ovarian cancer line in response to cisplatin therapy. (B) Fold-changes in RCAN mRNA in response to cisplatin treatment. (C) Survival curves for cNFAT3 vs. Ctrl-YFP vs Ctrl-YFP cells treated with VIVIT, an NFAT inhibitor, in response to cisplatin. *, $p<0.05$; **, $p<0.01$, ***$p<0.001$.

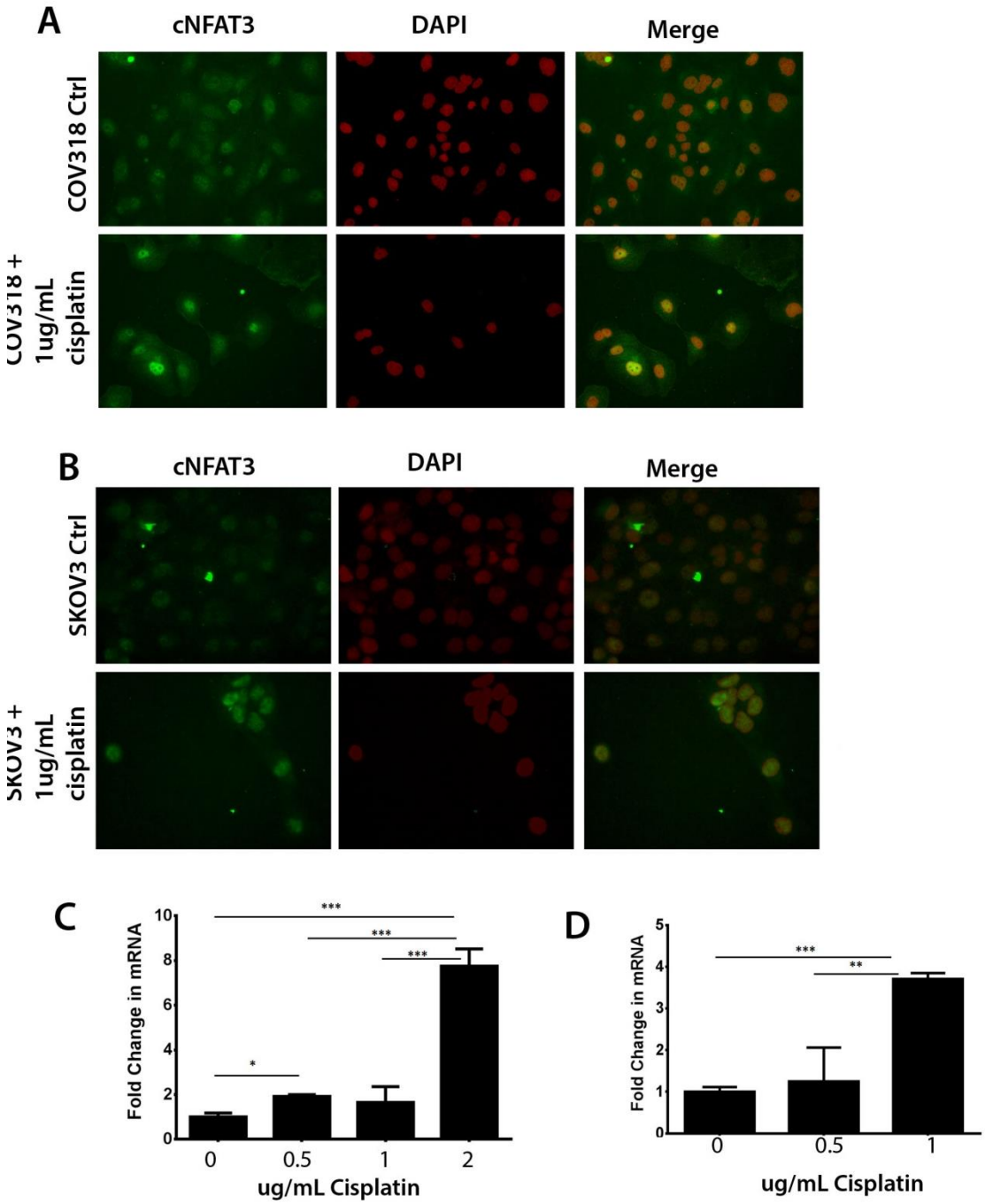

Figure 2.13. Effects of cisplatin on NFAT3. (A-B) NFAT3 nuclear translocation in response to cisplatin treatment. (C-D) qRT-PCR of RCAN mRNA levels in response to cisplatin treatment. *,p<0.05; **, p<0.01 ***, p<0.0001.

Discussion

The Nuclear Factor of Activated T-Cells (NFAT) family, comprised of NFAT1-4, is a group of transcription factors that are key regulators of cell proliferation [72-74], development [78, 79], cell cycle regulation [113], and regulation of stem cell proliferation [134] and quiescence [109]. More recently, NFAT family members have been implicated in multiple roles in cancer, including angiogenesis [176], cell transformation [177, 178], and response to chemotherapy [120, 121]. We report here a critical role for NFAT3 in the regulation of cellular quiescence in ovarian cancer. Expression of constitutively nuclear NFAT3 suppresses cellular proliferation and is associated with a reduction in cell size, decreased total cellular RNA levels, decreased transcription of ribosomal proteins, and decreased transcription of proteins associated with translation. As predicted for slow-cycling cells, cNFAT3 cells are resistant to chemotherapy. *In vivo* cNFAT3 expression is associated with a dormant tumor phenotype, which can be induced midway through tumor growth or at its outset.

NFAT3 and Quiescence: Based upon the role of NFAT1 in regulating quiescence in the hair follicle, we identified NFAT3 as a potential regulator of quiescence in ovarian cancer. NFAT3 is consistently expressed at higher levels in cancer stem-like cells (vs. the bulk population) in cell lines and in patients. Using two different constitutively nuclear NFAT3 constructs, one without and one with the N-terminal transcriptional activation domain, we observed a profound effect on cell proliferation both *in vitro* and *in vivo*. cNFAT3 expression leads to decreased proliferation and BrdU incorporation without increasing death, apoptosis, or senescence.

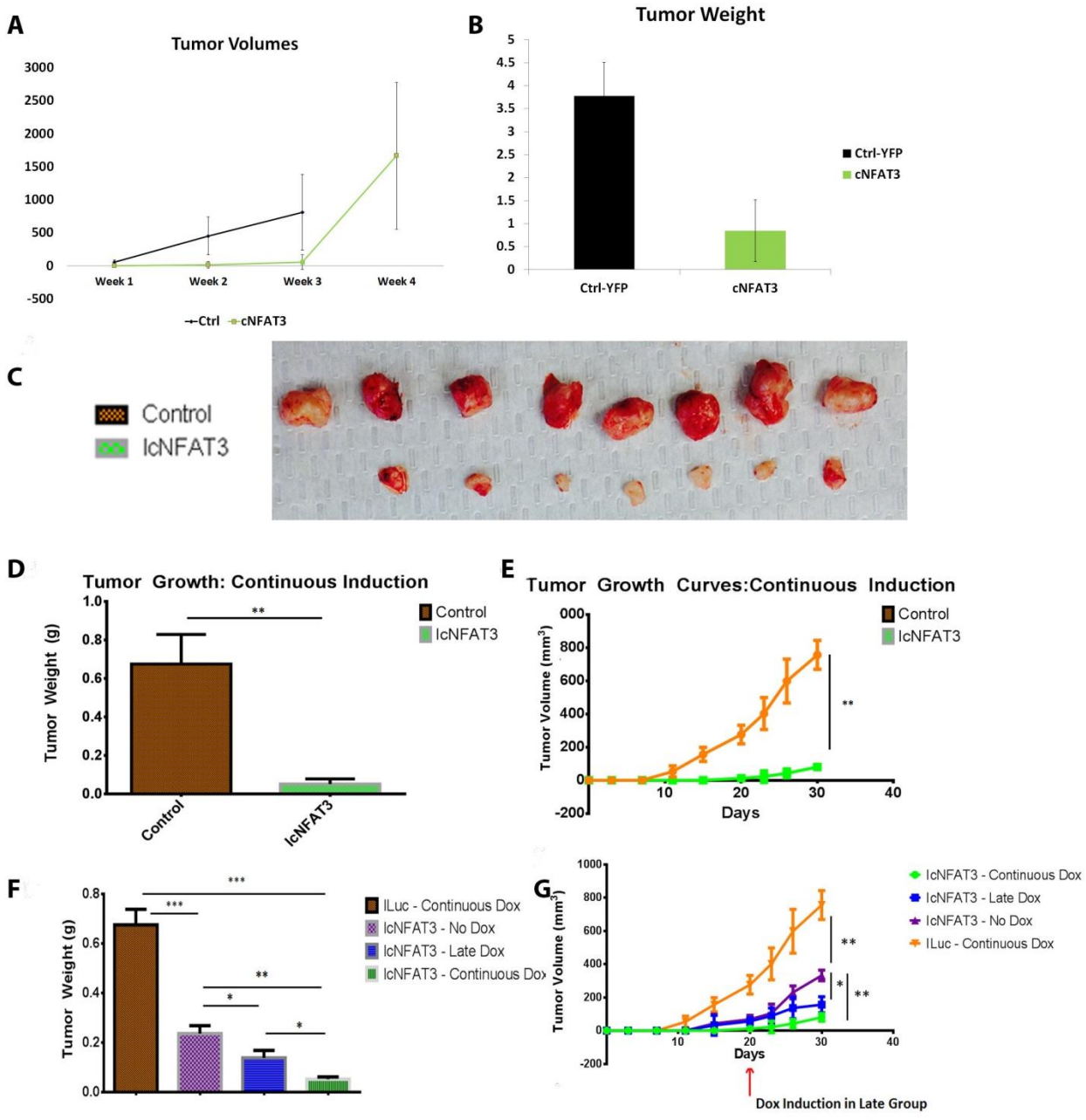

Figure 2.14. lcNFAT3 profoundly retards tumor growth *in vivo***.** Growth curves (A) and tumor weights (B) or A2780-cNFAT3 xenogragts. (C) Gross tumor appearance along with tumor weights (D) and growth curves (E) showing profound tumor growth retardation during lcNFAT3 induction. Doxycycline lcNFAT3 induction throughout tumor growth produced the smallest tumors, although induction during growth at 20 days led to smaller tumors and appeared to arrest growth (F-G). *p=0.05, ** p<0.05, ***p<0.01.

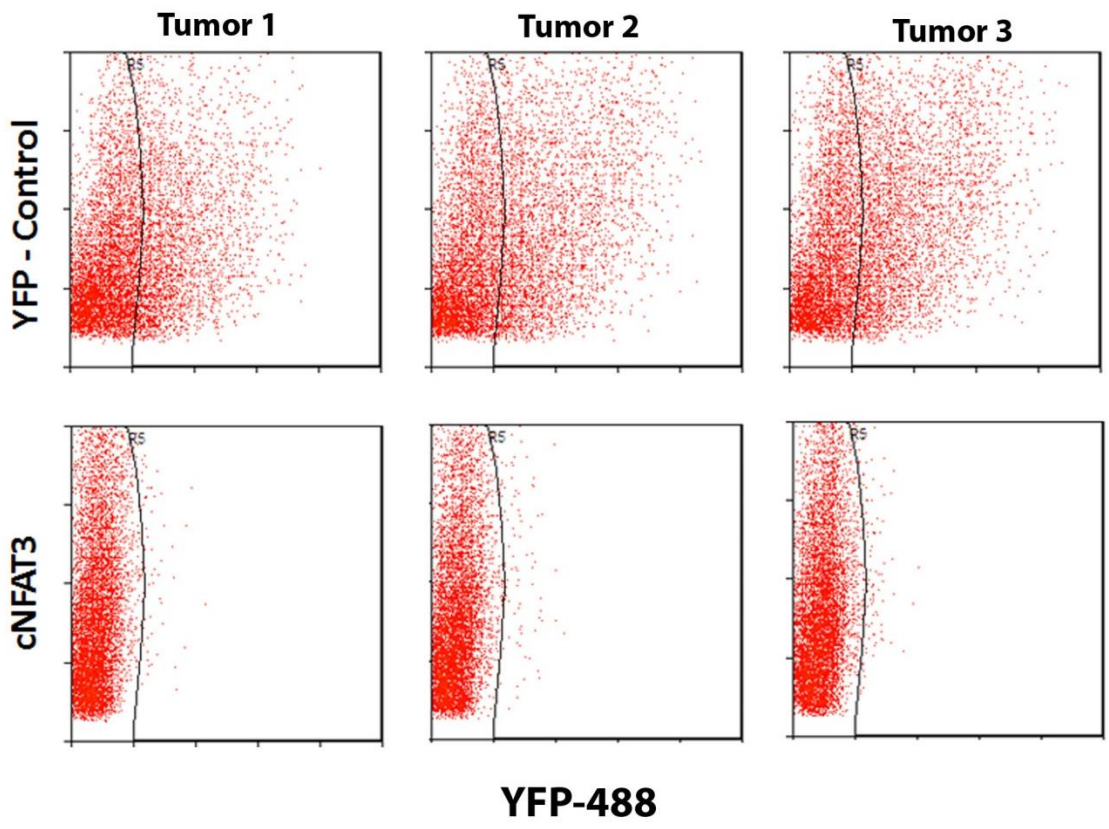

Figure 2.15. Expression of cNFAT3 and Ctrl-YFP in representative tumor xenografts by FACS.

The mechanism through which NFAT3 regulates cellular proliferation remains unclear. Consistent with NFAT1 transcriptional regulation of CDK4 to regulate cellular proliferation, we observed that cNFAT3 expression resulted in decreased CDK6 expression; both CDK4 and CDK6 are typically important cofactors in the G1-S-phase transition. We also observed a significant reduction in the amount of total cellular RNA. Furthermore, we observed downregulation of the mRNA for multiple ribosomal structural proteins and regulators of translation, including EIF2A and EEF1G. Although the absolute downregulation of each of these proteins is relatively low, they have significant collective impact. A 20% decrease in one protein based on qRT-PCR is insignificant; however, a 20% downregulation in most ribosomal proteins leading to a significantly decreased number of ribosomes and translational capacity is highly significant. This decrease in total cellular RNA was confirmed by Hoechst/Pyronin quiescence analysis, which showed a downward shift in total RNA with IcNFAT3 induction.

Given the significant and consistent reductions in proliferation observed through various assays, it was somewhat surprising that the cell cycle changes with cNFAT3 expression were relatively modest. However, these results are consistent with a study of NFAT4 in the brain [184] which observed significant changes in cell proliferation and vital dye retention with NFAT4 inhibition, yet observed minimal differences in the percentage of cells in each phase of the cell cycle. One potential explanation could be the induction of metabolic quiescence. While there is minimal research on metabolic quiescence in eukaryotic cells, one report described metabolic quiescence in yeast as a cell cycle-independent state induced by metabolic stress [185, 186]. More work is necessary to determine if NFAT3 is driving metabolic quiescence.

NFAT3 and chemotherapy resistance: NFAT3 has been poorly studied in cancer. In normal physiologic states, NFAT3 appears to function partly as a general stress response protein, as it serves a protective role in cardiomyocytes in response to radiation [115], is activated by mechanical stress in the heart [123] and bladder [124], and serves as a protective factor during hypoxia [128, 187]. We have shown that NFAT3 translocates to the nucleus and initiates transcription in response to cisplatin chemotherapy. Thus, NFAT3 may serve a similar stress response role in ovarian cancer cells to promote survival in response to chemotherapy. Supporting a role for NFAT proteins in chemotherapy resistance, we observed that inhibition of NFAT activity with VIVIT increased cell death during chemotherapy. It is important to note that VIVIT inhibits all NFAT family members and is not specific for NFAT3. However, this suggests that the NFAT family of proteins may be an important therapeutic target in ovarian cancer to overcome the chemotherapy resistance associated with slow-cycling cells. This hypothesis is supported by studies on cyclosporine, a commonly used immunosuppressant which inhibits the NFAT family. Cyclosporine has activity as a chemo-sensitizer and a phase II clinical trial demonstrated that cyclosporine could improve response to therapy in patients with chemotherapy-refractory disease [188, 189]. However, despite the encouraging Phase II trial, the drug has not been studied in Phase III trials. Furthermore, it has not been tested in patients with chemotherapy naïve disease, who may benefit the most from the elimination of slow cycling cells.

While cyclosporine is an FDA-approved drug that may hold promise as an adjunct therapeutic in ovarian cancer, it is a powerful immunosuppressant with numerous side effects and multiple targets in addition to NFAT proteins. Immunosuppression could have a

detrimental impact on antitumor immunity. Thus, development of NFAT3-specific inhibitors could be particularly useful, as NFAT3 is the only core NFAT family member that is not expressed in the immune system [71]. The development of specific NFAT3 inhibitors could allow chemosensitization of the NFAT3-expressing cancer stem-like cells without concomitant immunosuppression.

In summary, we have found that the master transcriptional regulator NFAT3 regulates a quiescent state in ovarian cancer and translocates to the nucleus in response to chemotherapy. Constitutively nuclear NFAT3 is associated with a reduction in cellular size and proliferation and the induction of chemotherapy resistance. In contrast, NFAT inhibition enhances chemotherapy resistance. Taken together, this data suggest NFAT3 is an important therapeutic target in ovarian cancer that warrants significant further study.

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Chapter 3: CDK4/6 Inhibition as Maintenance Therapy in High Grade Serous Ovarian Cancer

Abstract

Purpose: CDK4/6 inhibition, which blocks cell cycle progression from the G1 to the S phase, has shown promise as a maintenance therapy in multiple tumor types, but has never been tested in ovarian cancer. Due to the high relapse rate associated with high grade serous ovarian cancer, effective maintenance therapies that lengthen progression-free and overall survival are necessary. As multiple genes in the CDK4/6 pathway are commonly mutated or dysregulated in ovarian cancer patients, we hypothesized that CDK4/6 inhibition would be a rational therapy for this population. Therefore, we have tested the CDK4/6 inhibitor LEE-011 (Ribociclib; Novartis) in ovarian cancer models *in vitro* and *in vivo*.

Experimental Design: We determined dose ranges for LEE-011 in multiple ovarian cancer cell lines and evaluated its effects on cell division and cycling, viability, apoptosis, quiescence, senescence, and BrdU incorporation. In addition, we assessed the effects of various combinations of LEE-011 with cisplatin, a commonly used first-line ovarian cancer therapeutic, on cell proliferation, cell cycle, and response to chemotherapy *in vitro* and *in vivo*.

Results: CDK4/6 inhibition with LEE-011 significantly reduced the growth of Rb^{WT} high-grade serous ovarian cancer (HGSOC) cell lines at a mean IC50 of 360 nM. Rb^{null} cells were unaffected by CDK4/6 inhibition. LEE-011 treatment of Rb^{WT} ovarian cancer cells resulted in an increase in the percentage of cells in the G0/G1 phases of the cell cycle. Expression of senescence-associated β-galactosidase increased in a dose-dependent manner with LEE-011 treatment, but qRT-PCR of genes from the senescence-associated secretory profile showed mixed expression changes. Treated cells demonstrated an ability to proliferate after drug withdrawal, suggesting a potential pseudo-senescent state in which cells proliferate despite expression of senescence markers. Concurrent LEE-011 and cisplatin prevented ovarian cancer cells from resuming a normal cell cycle and delayed proliferation and recovery after cisplatin treatment. Treatment with LEE-011 as a maintenance or combination therapy significantly delayed cancer growth *in vivo* in platinum-sensitive and platinum-resistant murine ovarian cancer models.

Conclusions: CDK4/6 inhibition profoundly retarded growth in cellular models of high-grade serous ovarian cancer and acted to potentiate cisplatin, delaying recovery and cell cycle normalization after cisplatin treatment. LEE-011 delayed tumor growth in platinum-sensitive and platinum-resistant murine ovarian cancer models. This data supports the use of LEE-011 as a therapeutic agent in ovarian cancer.

Introduction

High-grade serous ovarian cancer (HGSOC) is the most lethal gynecological cancer in the United States, with a 5-year survival rate under 50%. The majority of patients present at Stages

III/IV and therefore have disseminated disease at diagnosis [190]. Despite a high rate of complete clinical remission with surgery and platinum/taxane-based chemotherapy, the majority of these patients relapse within two years and ultimately succumb to their disease [191] [3]. Non-cytotoxic or targeted maintenance therapies that delay disease recurrence could significantly improve patient quality of life and potentially improve overall survival. This is particularly relevant for patients with platinum-resistant ovarian carcinoma, who have a shorter progression free survival compared to patients with platinum-sensitive disease [8].

CDK4/6 inhibition is an emerging cytostatic maintenance therapy targeting cell cycle progression. A heterotrimeric complex of Cyclin D1, CDK4, and CDK6 is required to phosphorylate RB1, which eventually leads to transcription of genes required for the transition into S phase [192]. CDK4/6 inhibitors block the G1-S phase transition, inducing G1 arrest. CDK4/6 inhibitors have shown promise in many tumors *in vitro*, such as neuroblastoma [193], liposarcoma [194], and breast cancer [174], and multiple agents are currently under development. Clinical trials of CDK4/6 inhibitors have shown promise in mantle cell lymphoma [195], non-small cell lung cancer [196], and breast cancer [197]. In breast cancer, the CDK4/6 inhibitor Palbociclib combined with letrozole doubled progression-free survival from 10 to 20 months compared to letrozole alone [197]. Importantly, therapy was generally well tolerated with an acceptable side effect profile.

There are few agents which have demonstrated significant improvement in progression free survival for ovarian cancer. Bevacizumab, an anti-angiogenic agent, led to modest improvements in progression-free survival (on the order of months) without a clear impact on overall survival [10, 11]. Olaparib, a PARP inhibitor, improved progression free survival but had

no impact on overall survival [198] . Similarly, studies of chemotherapeutic agents as maintenance therapy have proven negative [199]. We report here a potential role for CDK4/6 inhibition as a maintenance therapy in HGSOC. We have shown that CDK4/6 inhibition significantly delayed growth *in vitro* and significantly improved outcomes in murine xenograft models of ovarian cancer. Importantly, CDK4/6 inhibition appears to augment chemotherapy response and is effective in both platinum-sensitive and platinum-resistant disease.

Materials and Methods

Cell Lines

The A2780 cell line was obtained from Dr. Susan Murphy at Duke. COV504 and OVSAHO lines were obtained from Dr. Deborah Marsh at the University of Sydney. The COV362 line was obtained from ATCC. Hey1, A2780, and COV504 lines were cultured in RPMI-1640 media with 10% FBS and 1% Penicillin/Streptomycin at 37°F and 5% CO2. OVSAHO, PEO1, and COV362 lines were cultured in DMEM with 10% FBS and 1% Penicillin/Streptomycin at 37°F and 5% CO_2.

Cell Cycle Analysis

Hey1 and COV362 cells were grown in 6-well plates in triplicate and treated for 72 hours with 0, 250nM, 1uM, or 3uM LEE-011 for three days. Cells were then harvested, fixed dropwise in 70% ethanol, and incubated with 0.1 ug/mL RNAse and 1 ug/mL Propodium Iodide (PI) for 1h at 37°F. 10,000 events were then analyzed on a BD Accuri flow cytometer.

Senescence Analysis

Cells were grown in in 6 well dishes in triplicate and treated for three days with 0, 250nM, 1uM, or 3uM LEE-011. Each well was stained overnight for senescence-associated β-galactosidase with the Senescence β-galactosidase Assay Kit (Cell Signaling) according to the manufacturer's instructions. Images were captured with an Olympus BX57 microscope.

Apoptosis Analysis

Cells were grown in 6 well dishes and treated for three days with 0, 250nM, 1uM, or 3uM LEE-011. Then, they were stained with an Annexin-V FITC apoptosis kit (BD Biosciences) according to the manufacturer's instructions and 10,000 events were analyzed on a Mo Flo Astrios FACS sorter (Beckman Coulter). The percentage of Annexin V^+, PI^+, Annexin V^+/PI^+, and Annexin V^-/PI^- cells was quantified.

Recovery Assays

To mimic residual disease after chemotherapy, 20,000 Hey1 cells were plated in each well of a 12-well dish and treated with cisplatin alone or with various combinations of LEE-011 and cisplatin. Thereafter, cells were counted every 2-3 days in duplicate samples with two technical replicates using trypan blue and the total cell number (relative to starting number) was plotted.

MTT Assays

2500 Hey1 cells were plated in each well of a 96-well plate. On the following day, cells were treated with LEE-011, cisplatin, or a combination. After 3-5 days, the media was aspirated and cells were stained with the Vybrant MTT Assay Kit (Thermo Fisher) as described in the manufacturer's instructions. We used MTT assays to quantify the effects of LEE-011 on absolute and relative cell numbers remaining after cisplatin chemotherapy. Absolute cell number was measured as raw absorbance from the MTT assay. Relative cell number was calculated as the absorbance of wells with LEE-011+cisplatin normalized to the wells treated with only the relevant LEE-011 concentration. This calculation was performed to correct for the decreased number of cells with LEE-011 treatment alone.

qRT-PCR

Hey1 cells were grown in 6-well dishes and treated for three days with 0, 250nM, 1uM, or 3uM LEE-011. Total RNA was extracted with an RNeasy Mini Kit (Qiagen) and quantified with a Nanodrop-1000 (Thermo Fisher). RNA was converted to cDNA with a SuperScript III Reverse Transcriptase cDNA Kit (Life Technologies), and 10 ng of cDNA was used for each reaction. Primers for senescence-associated qRT-PCR genes are as follows. CSF2: Forward, TCCTGAACCTGAGTAGAGACAC; Reverse, TGCTGCTTGTAGTGGCTGG. IL1A: Forward, TGGTAGTAGCAACCAACGGGA; Reverse, ACTTTGATTGAGGGCGTCATTC. IL6: Forward,

ACTCACCTCTTCAGAACGAATTG; Reverse, CCATCTTTGGAAGGTTCAGGTTG. SERPINB2: Forward, CAGCACCGAAGACCAGATGG; Reverse, CAAAATCGCATCAGGATAA. Angiopoietin: Forward, CTGGGCGTTTTGTTGTTGGTC; Reverse, GGTTTGGCATCATAGTGCTGG. Heregulin: Forward, CGGTGTCCATGCCTTCCA; Reverse, GCGAGTTTCTTAACAGGCTCT.

Tumor xenograft experiments

All experiments were conducted with the approval of the University of Michigan Institutional Animal Care and Use Committee. Nod/SCID/IL2R^{KO} or nude mice were injected bilaterally into the axillae with 100,000 Hey1 ovarian cancer cells or 2,000,000 PEO1 ovarian cancer cells. Three days later, mice were treated with vehicle (0.1% methylcellulose), cisplatin, LEE-011, or various combinations of cisplatin + LEE-011. Sample dosing schedules are shown in Fig. 3.8. Tumors were measured twice a week with calipers and tumor volumes were calculated by the formula: Volume = width*width*length. Tumor weights were collected when mice were sacrificed at the protocol endpoint.

Results

Mutations in and dysregulation of genes in the CDK4/6 pathway are common in ovarian cancer

We evaluated the expression/mutation pattern of genes linked with CDK4/6 regulation of the cell cycle in the HGSC TCGA database [200] . These included mutations, deletions or amplifications, and significant dysregulation of mRNA expression (z-scores <-2 or >2). Mutations

in the CDK4/6 pathway were relatively common in patients with ovarian HGSOC (Figure 3.1A). The tumor suppressor locus CDKN2A, which normally serves as a brake on cell cycle progression

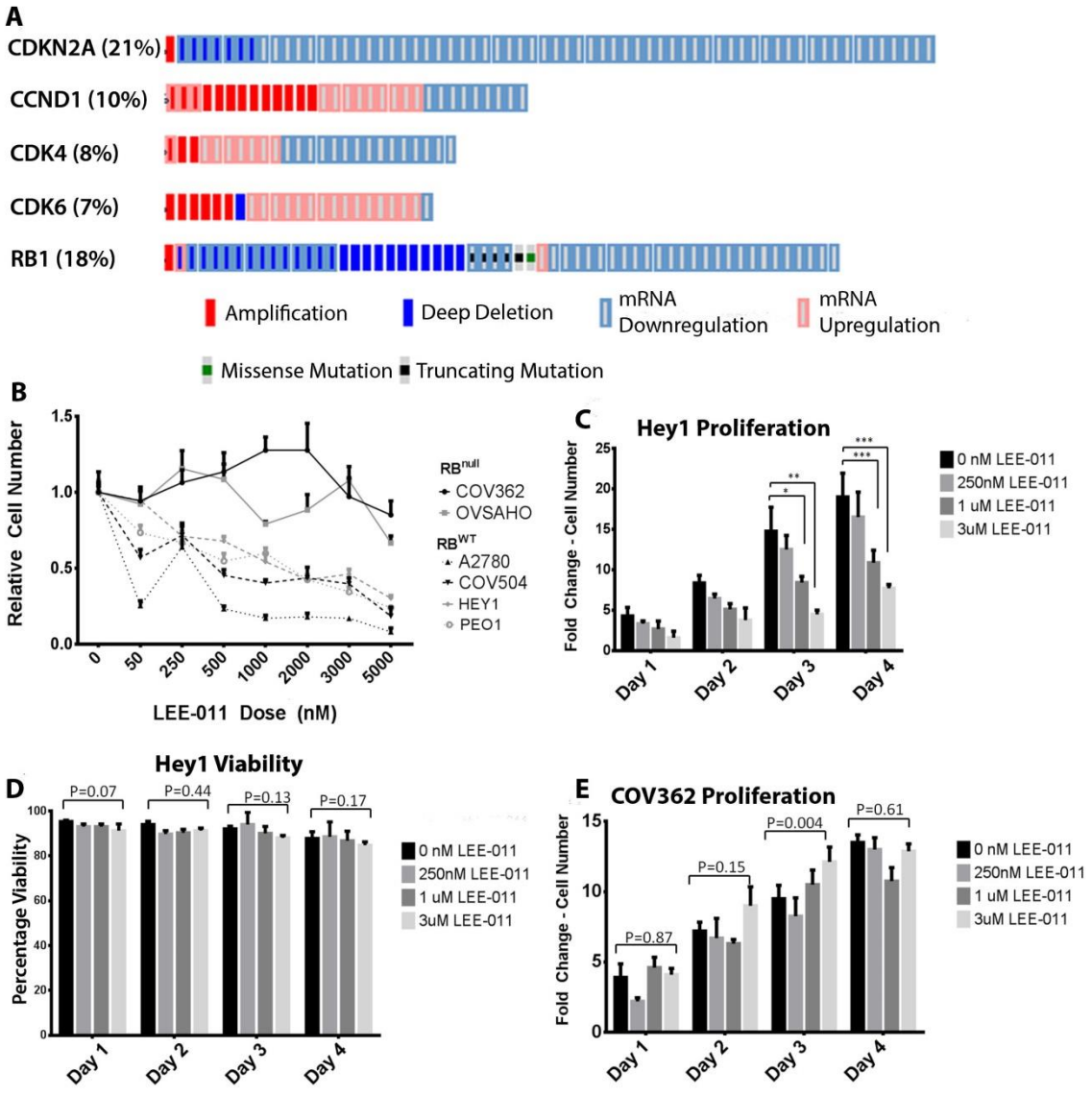


Figure 3.1. LEE-011 is a rational target in ovarian cancer and decreases proliferation in a dose-dependent manner in Rb^{WT} cell lines, but not Rb^{null} lines, without decreasing viability. (A) Analysis of 316 tumors from the TCGA database showing mutations and mRNA dysregulation of genes in the pathway controlling the G1-S phase transition. (B) Effects of LEE-011 on proliferation of six ovarian cancer lines over 3 days. (C) Effects of LEE-011 on Rb^{WT} Hey1 ovarian cancer cell proliferation and viability over 4 days, (E) Effects of LEE-011 on proliferation in the Rb^{null} COV362 cell line. *, $p<0.05$. **, $p<0.01$, ***, $p<0.001$.

by inhibiting CDK4 and CDK6 [201], was deleted or significantly down-regulated in 21% of HGSOCs. 16% of HGSOCs showed significant amplifications or increases in mRNA expression of CDK4, CDK6, and/or Cyclin D1 expression, which would speed the G1-S transition and promote tumor growth. 17% of patients demonstrated mutations in or significant downregulation of RB1, which would likely result in resistance to CDK4/6 inhibitors (Fig. 3.1A). Presumably, patients whose tumors do not have mutations in this pathway could also be benefit from CDK4/6 inhibition. Taken together, this data suggests that there is a large subset of ovarian HGSOC patients who could benefit from therapy with a CDK4/6 inhibitor.

LEE-011 affects cell proliferation in multiple ovarian cancer cell lines

To determine if CDK4/6 inhibition is active in HGSOC, Hey1, COV362, COV504, PEO1, A2780 and OVSAHO ovarian cancer cell lines were treated with increasing doses of the CDK4/6 inhibitor LEE-011 for 5 days and cell proliferation was quantified by cell counts with trypan blue. The $RB1^{WT}$ cell lines A2780, Hey1, COV504, and PEO1 all showed dose-dependent growth inhibition ($p<0.0001$ for all) (Figure 3.1B). As predicted, $RB1^{null}$ lines COV362 and OVSAHO were unresponsive (Fig. 3.1B). For more detailed analysis, we treated the Rb^{WT} cell line HEY1 and the Rb^{null} line COV362 with LEE-011 and assessed cell counts and viability daily. LEE-011 decreased the growth of HEY1 cells in a dose-dependent manner ($p<0.001$), but did not affect viability after 4 days of growth ($p=0.17$; Fig 3.1C-D). Once again, LEE-011 treatment did not affect proliferation ($p=0.61$ at Day 4; Figure 3.1E) or viability (Fig. 3.2A) in the COV362 line. Interestingly, Annexin-V/7-AAD staining showed that higher doses of LEE-011 treatment

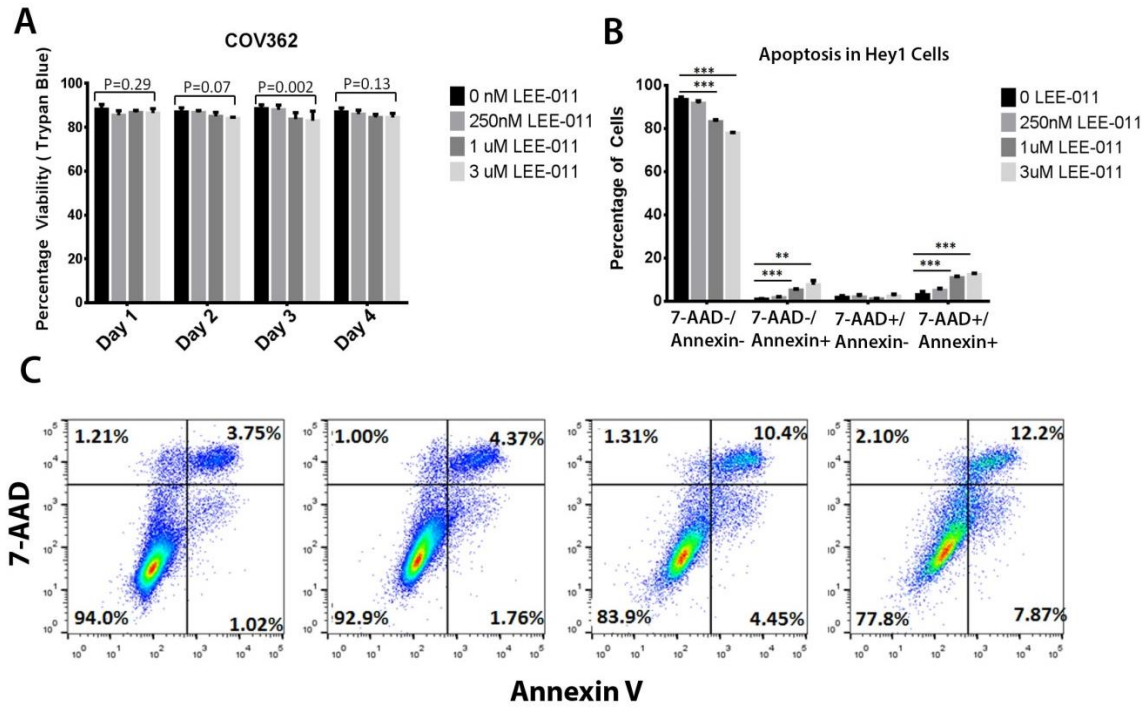


Figure 3.2: LEE-011 leads to mild apoptosis in Rb^{WT} Hey1 cells, but does not affect the Rb^{null} COV362 line. (A) Viability in COV362 cells measured with Trypan Blue. (B) The percentage of 7-AAD-/Annexin-, 7-AAD+/Annexin-, 7-AAD-/Annexin+, and 7-AAD+/Annexin+ Hey1 cells after 3 days of LEE-011 treatment. (C) Sample FACS plors for Annexin V/7-AAD staining for apoptosis. *, p<0.05 **,;p<0.01; ***, ***, p<0.001.

produced moderate increases in apoptosis in the Hey1 cell line, but not enough to explain the significant difference in live cell number (Figure 3.2B-C).

LEE-011 induces G1 arrest

We next performed cell cycle phase analysis following LEE-011 treatment to confirm its effects on the cell cycle. LEE-011 treatment led to a dose-dependent accumulation of Hey1 cells in the G1/G0 peak of the cell cycle ($p=0.009$), with a concomitant decrease in the percentage of S phase cells ($p=0.04$ and the G2/M peak ($p=0.0005$) (Fig. 3.3A-B); this pattern was confirmed in the Rb^{WT} cell lines A2780 and SKOV3 (Fig. 3.4A-B). We also observed corresponding decreases in BrdU incorporation from 28.9% to 8.9% in Hey1 ovarian cancer cells during this treatment (Fig. 3.3C). The Rb^{null} line COV362 showed no changes in the percentage of cells in G0/G1 phase ($p=0.99$), S phase ($p=0.85$), or G2/M phase ($p=0.55$) in response to LEE-011, regardless of dose (Figure 3.4C-D).

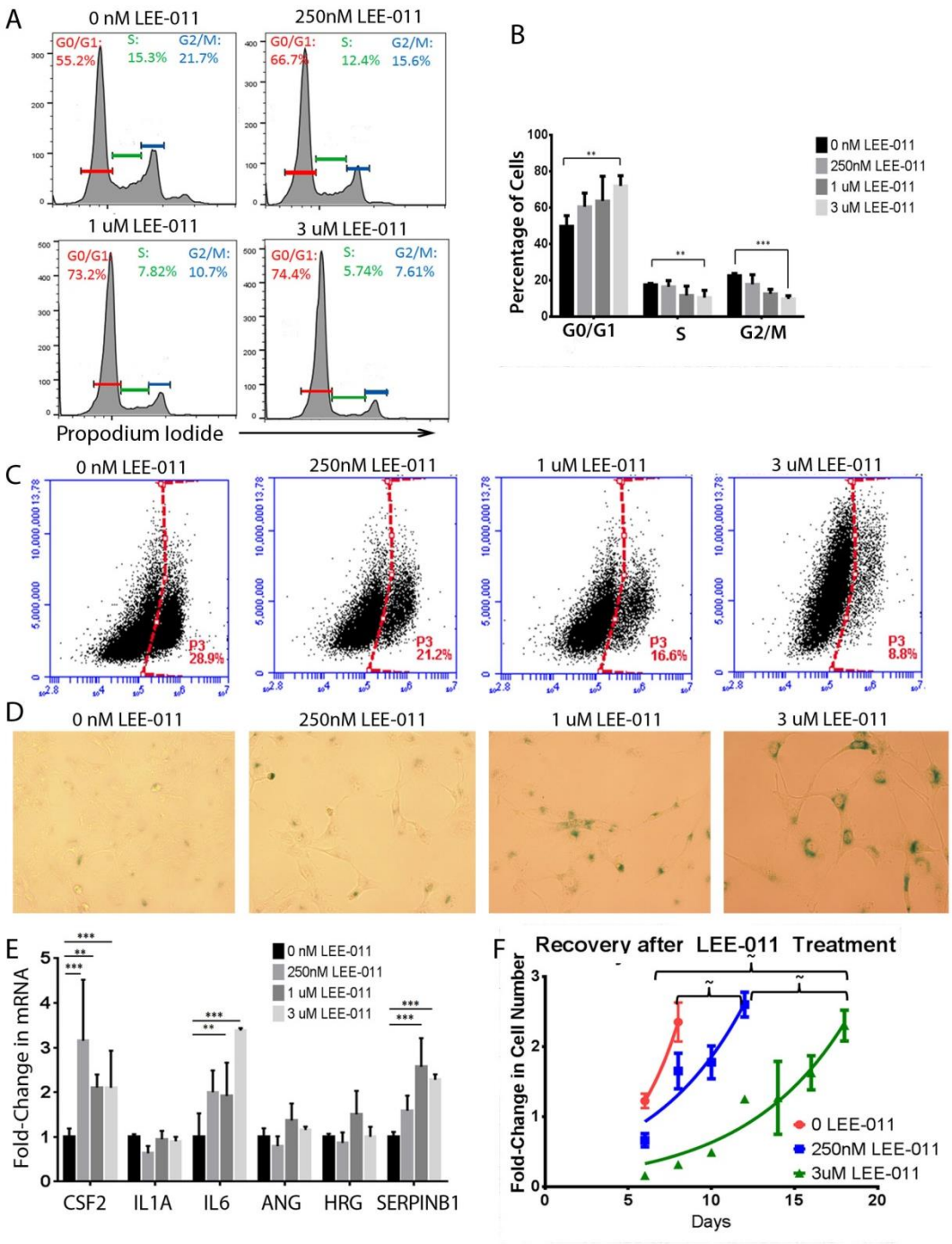


Figure 3.3. LEE-011 treatment leads to G1 arrest, decreased BrdU incorporation, and a potential pseudosenescent state. (A-B) Cell cycle profiles of LEE-011 treated cells along with summary. (C) BrdU and (D) senescence-associated beta-galactosidase staining during LEE-011 treatment. (E) qRT-PCR of genes from the senescence-associated secretory profile with LEE-011 treatment. (F) Proliferation of Hey1 cells after LEE-011 treatment. *,p<0.05; **, p<0.01; ***, p<0.001; ~, p<0.0001.

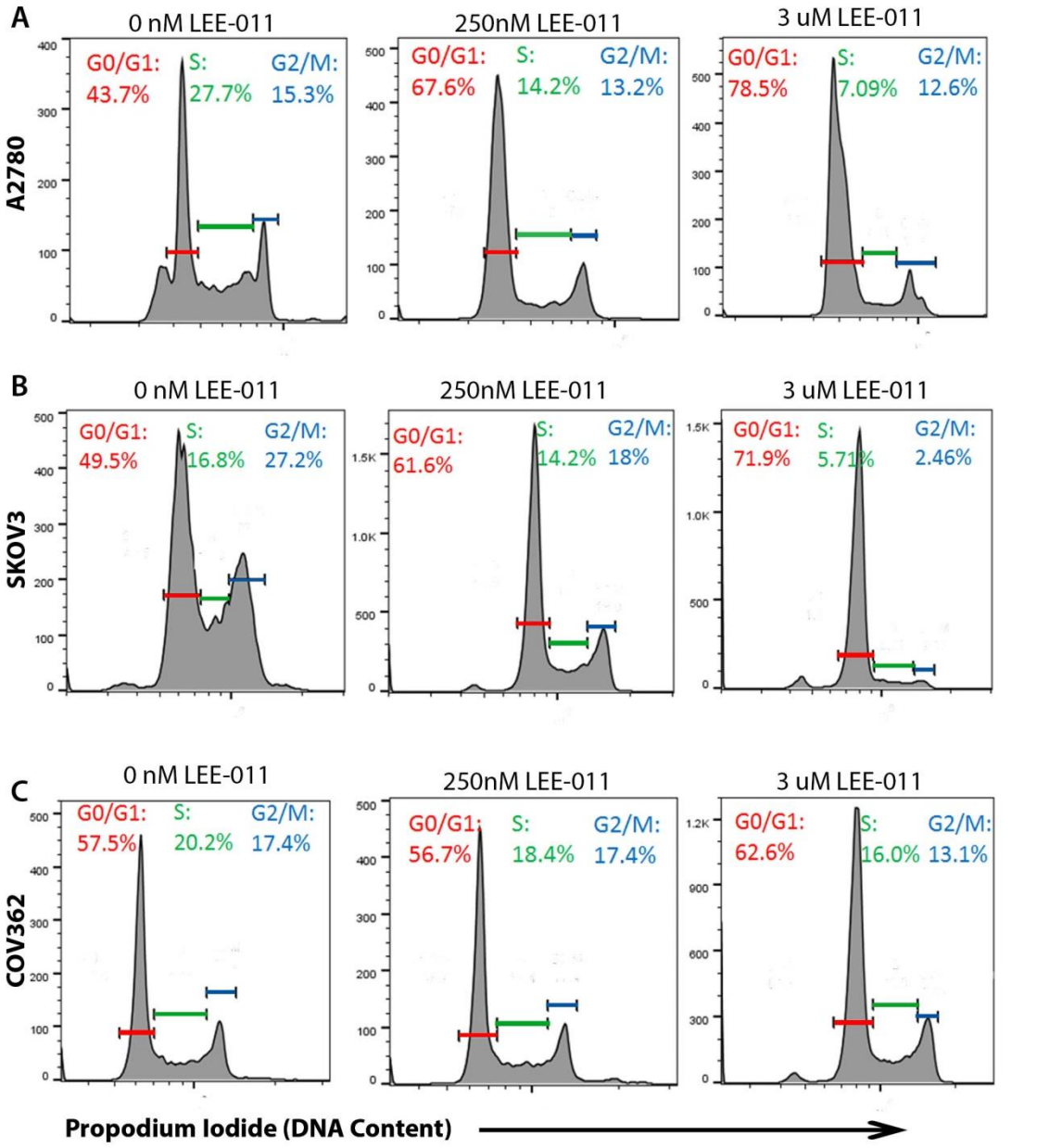


Figure 3.4. LEE-011 affects the cell cycle in Rb^{WT} but not Rb^{null} cell lines. (A-B) Cell cycle diagrams for the A2780 and SKOV3 Rb^{WT} ovarian cancer cell lines. (C) Cell cycle diagrams for the COV362 RB^{null} ovarian cancer line. (D) Quantification of cell cycle changes in COV362 ovarian cancer cells with LEE-011 treatment.

Effects of LEE-011 treatment on senescence markers

As CDK4/6 inhibition was reported to induce senescence in breast cancer cells [202], we have characterized this effect here in ovarian cancer. LEE-011 led to a significant, dose-dependent increase in the expression of senescence associated β-Galactosidase with LEE-011 treatment (Fig. 3.3D). We next evaluated the induction of expression of senescence associated secretary proteins, which serve as another marker of the senescent phenotype. qRT-PCR demonstrated an LEE-011 dependent increase in the expression of multiple genes associated with the senescence associated secretory profile, though other genes failed to show a response (Fig. 3.3E). Interestingly, at the highest does of LEE-011 treatment, ~95% of cells demonstrate SABG stain. However, when LEE-011 is washed out and cells are allowed to proliferate after 5 days of treatment, cell numbers continue to increase, indicating proliferation ($p<0.0001$, Fig. 3.3F). This may suggest the induction of a potential pseudo-senescent state, in which cells express senescence markers such as senescence associated β-Galactosidase but nonetheless remain capable of proliferating.

Effects of LEE-011 in Combination with Cisplatin

As platinum-based chemotherapy is the cornerstone of ovarian cancer therapy, we next evaluated the impact of LEE-011 treatment concurrent with cisplatin for 72 hours. Interestingly, concurrent therapy led to a decrease in the absolute number of surviving Hey1 cells compared to treatment with either drug alone ($p<0.001$, Figure 3.5A).

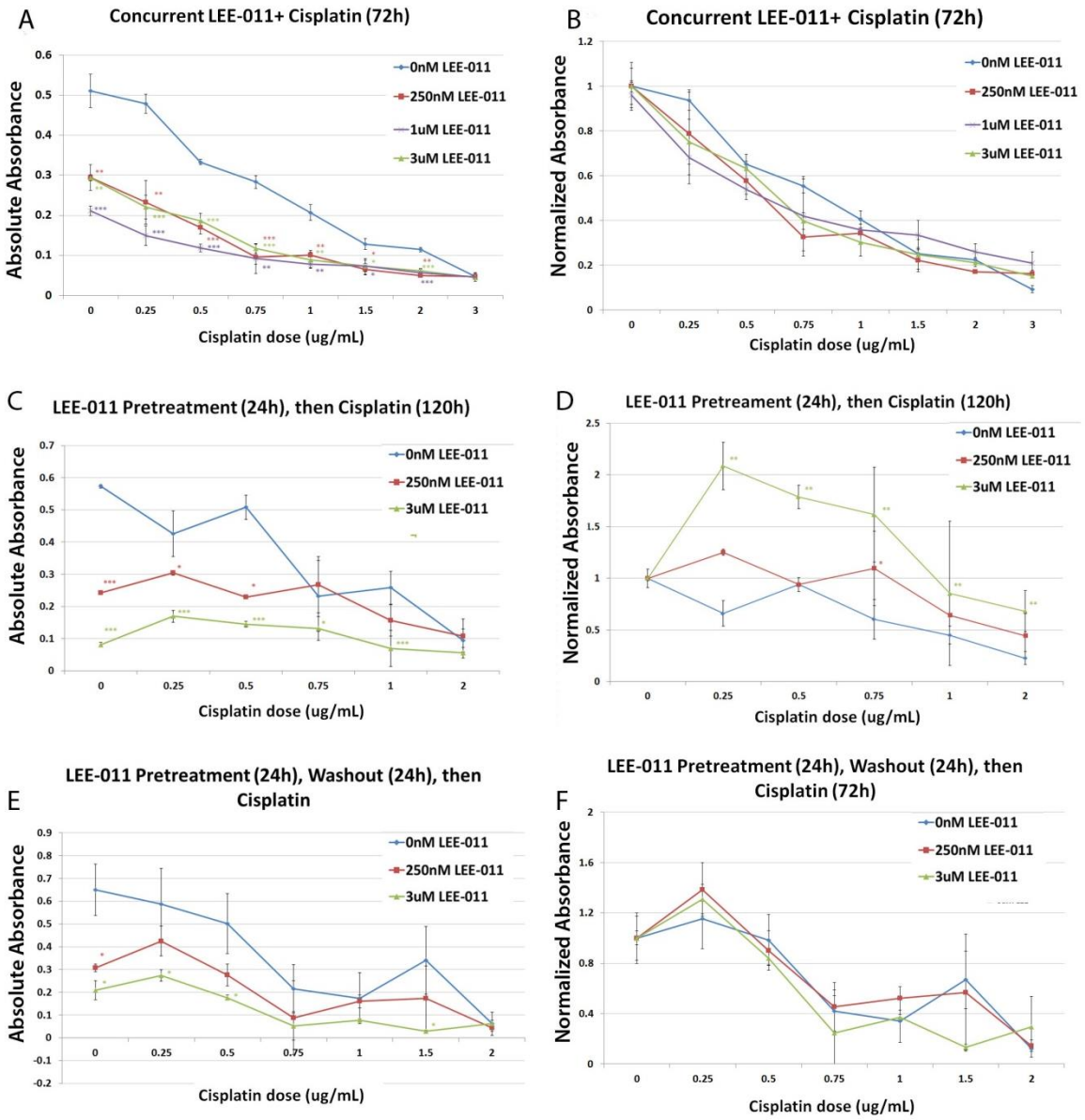


Figure 3.5. Effects of short-term combination treatment with LEE-011 and cisplatin. (A,B) MTT assay data showing absolute and normalized absorbance after 3 days of concurrent LEE-011 and cisplatin. Normalized absorbance represents each data point divided by the LEE-011-only control at each dose. (C-D) MTT assay data showing absolute and normalized absorbance after pre-treatment with LEE-011 and then cisplatin administration. (E-F) MTT assay data showing absolute and normalized absorbance after LEE-011 pretreatment with a washout period before cisplatin administration. *,$p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Normalizing cell numbers to adjust for the impact of LEE-011-associated decreases in cell numbers did not show any evidence of reduced chemotherapy efficacy (Fig. 3.5B). In theory cell-cycle arrest could protect cells from chemotherapies such as cisplatin, which predominantly target rapidly dividing cells. Therefore, we tested the effects of 24h LEE-011 pre-treatment before cisplatin and found that this regimen led to a decrease in the absolute number of cells ($p<0.001$, Fig. 3.5C). However, when normalized to the initial number of cells at each LEE-011 dose, we observed a reduction in chemotherapy efficacy, with a higher proportion of surviving cells ($p<0.01$, Fig. 3.5D), indicating that LEE-011 pre-treatment before cisplatin may serve as a protective factor. When the pre-treatment was followed by a 24-hr washout period before cisplatin treatment, this protective effect disappeared ($p<0.05$, Fig. 3.5 E-F). As Hey1 cells respond quite rapidly to LEE-011, we believe that this timing allowed normalization of the cycle before cisplatin administration, abrogating the protective effect of cell cycle arrest. However, it is clear that identifying the correct relative timing of drug administration is essential to harnessing the synergistic effect.

LEE-011 as Maintenance Therapy following Cisplatin or Concurrent Cisplatin and LEE-011

We next evaluated the impact of maintenance LEE-011 following cisplatin treatment *in vitro*. 20,000 Hey1 ovarian cancer cells were treated with 1ug/mL cisplatin for 72 hours and then we initiated daily treatment with vehicle alone (0 nM LEE) or with 250nM, 1uM, or 3uM LEE-011 and monitored cell recovery over time to mimic tumor recurrence from minimal residual

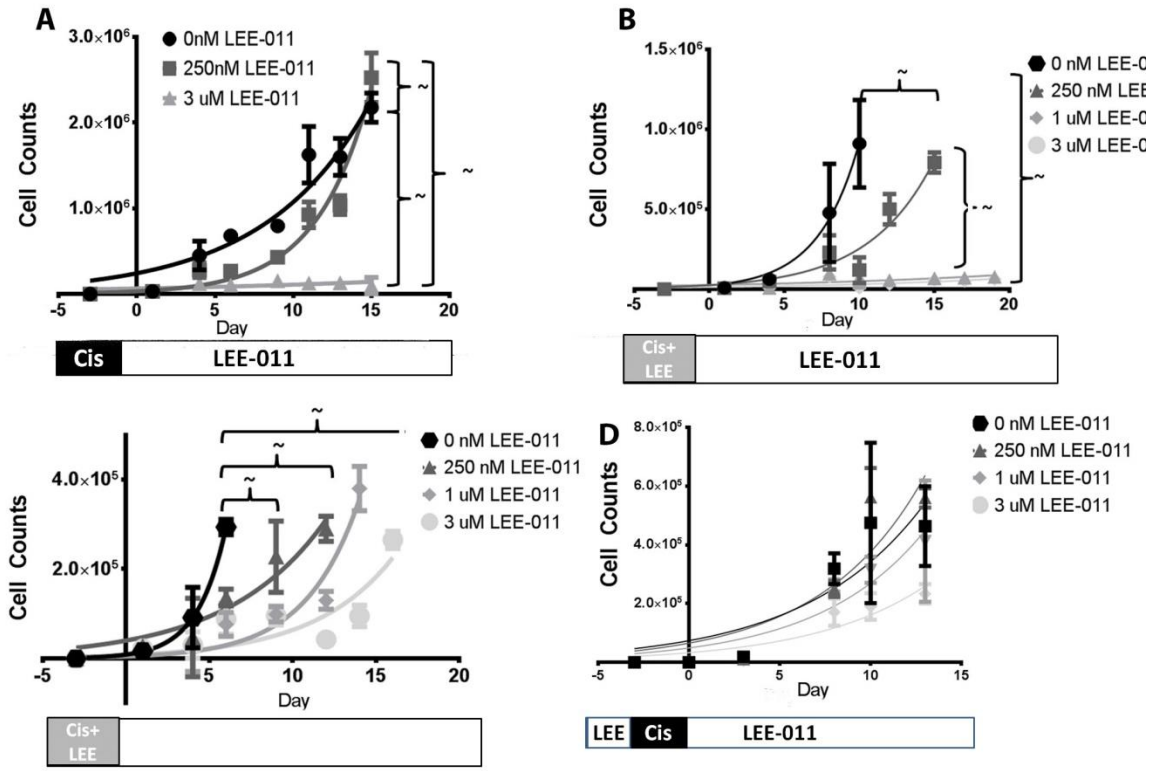


Figure 3.6: Maintenance therapy with LEE-011 delays regrowth after cisplatin. (A) Maintenance therapy with LEE-011 after 3 days of cisplatin treatment. (B) Concurrent cisplatin+LEE-011 with LEE-011 maintenance therapy afterwards. (C) Concurrent cisplatin+LEE-011, with no subsequent treatment. (D) Prereatment with LEE-011 followed by cisplatin treatment and LEE-011 maintenance therapy. ~, $p<0.0001$.

disease. Cells treated with LEE-011 demonstrated slow but consistent proliferation over time, consistent with a cytostatic but not cytotoxic mechanism of action (Fig. 3.1C). In contrast, we observed complete growth arrest at higher doses of LEE-011 when used as a maintenance therapy after cisplatin treatment ($p<0.0001$, Fig. 3.6A). Concurrent treatment with cisplatin and LEE–001 followed by maintenance with LEE-011 was similarly associated with complete growth arrest, consistent with synergy between the two drugs ($p<0.0001$, Fig. 3.6B). Interestingly, concurrent treatment for 72h with LEE-011 and cisplatin, followed by no maintenance therapy, still significantly delayed recovery after chemotherapy ($p<0.001$, Fig. 3.6C). However, as predicted from the short-term cell kill data described above, pretreatment of cells with LEE-011 prior to cisplatin exposure was less effective (Fig. 3.6D). This study of recovery after cisplatin chemotherapy confirms our short-term viability data indicating that the timing of relative administration of LEE-011 and cisplatin is crucial to maximizing their combined effect. Due to the impact of a short pulse of combined cisplatin and LEE-011 on the ability of cells to recover after chemotherapy, we investigated the effects of this combination therapy on the cell cycle.

LEE-011 impairs normal cell cycling after cisplatin treatment

We investigated the cell cycle over time in control, cisplatin, LEE-011, or LEE-011+Cisplatin treated cells. 24 hours after cisplatin only treatment, the majority of cells were in the S-G2/M phases of the cell cycle, (Fig. 3.7A). This is consistent with previous reports on cisplatin's effects on the cell cycle, and is presumably because cells sustain DNA damage and fail to pass the G2M mitotic checkpoint [203]. In contrast, 24 hours after concurrent treatment with LEE-011 and

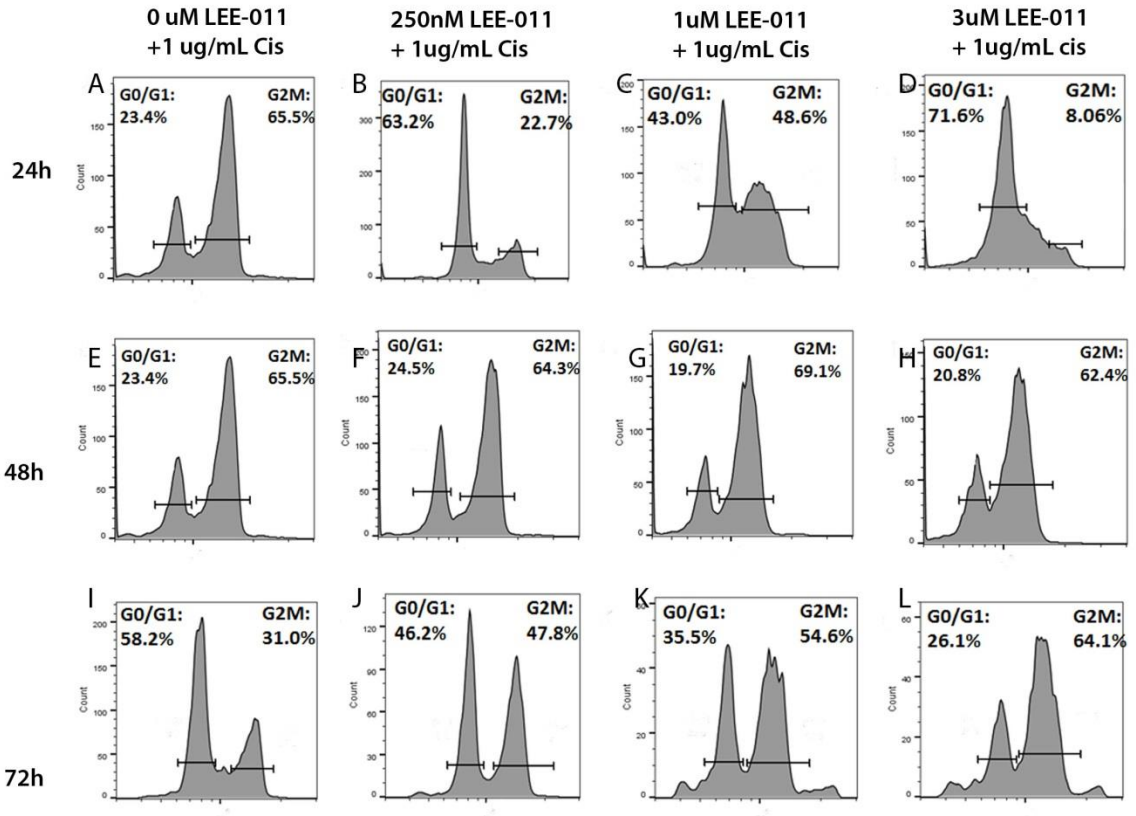
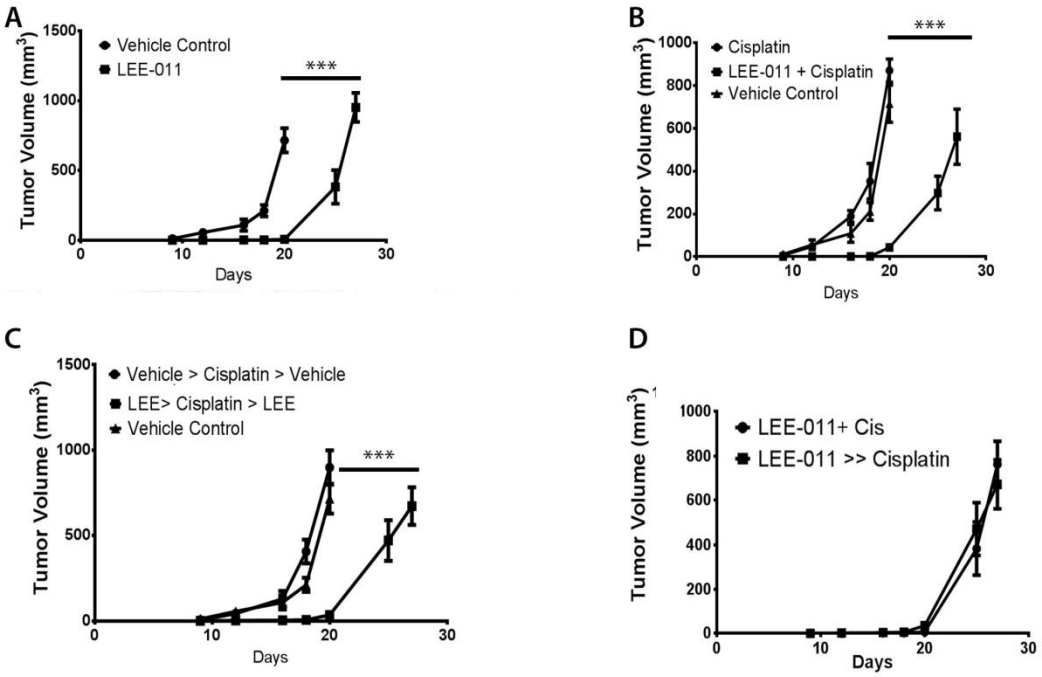


Figure 3.7: Cell cycle phase diagrams of concurrent LEE-011 and cisplatin administration. (A-D) Cell cycle after 24h of 1 ug/mL cisplatin along with 0 (A), 250nM (B), 1 uM (C), or 3 uM (D) LEE-011. (E-H) Cell cycle after 48h of concurrent 1 ug/mL cisplatin along with 0 (E), 250nM (F), 1 uM (G), or 3 uM (H) LEE-011. (I-L) Cell cycle after 72h of concurrent 1 ug/mL cisplatin along with 0 (I), 250nM (J), 1uM (K). or 3 uM (L) LEE-011.

cisplatin, the majority of cells were in the G0/G1 portion of the cell cycle (Fig. 3.7 B-D). At 48 hours, both cisplatin only and concurrent LEE-011+Cisplatin treated cells demonstrated a majority of cells in the S-G2/M phase of the cell cycle (Fig. 3.7E-H). Finally, the cell cycle profile began to normalize 72 hours after treatment with cisplatin only (Fig. 3.7I). However, 72 hours after concurrent LEE-011+cisplatin therapy, the majority of cells were still arrested in the S-G2/M portion of the cell cycle, with increased doses of LEE-011 showing a more profound arrest (Fig. 3.7I-L). Compared to cisplatin alone treatment, in which 32% of cells were in the S-G2/M phase, over 80% of cells treated with 1 ug/mL cisplatin and 3uM LEE-011 for three days remained arrested in the S-G2/M phase (Fig. 3.7L).

LEE-011 is effective alone and in combination with cisplatin in in vivo *xenograft models*

We next evaluated LEE-011 activity *in vivo*. We injected NSG mice with 100,000 Hey1 cells and began treatment with LEE-011 three days after tumor initiation. Treatment with LEE-011 alone increased the time to tumor endpoint from 18 to 26 days in comparison with the vehicle ($p<0.0001$) (Fig. 3.8A). We next evaluated the impact of LEE-011 as a maintenance therapy following cisplatin therapy in cisplatin resistant Hey1 cells. The addition of LEE-011 maintenance therapy after cisplatin, once again resulted in a ~40% increase in time to tumor endpoint (Fig. 3.8B).



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	Day																		
Group	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
No treatment																			
LEE-011 only																			
Cis only																			
Cis> LEE maintenance																			
Cis+LEE-011> LEE maintenance																			

Cisplatin
Cisplatin+LEE-011
LEE-011
No Dose

Fig 3.8: LEE-011 delays tumor growth in *in vivo* xenografts. (A) Hey1 xenografts treated with vehicle or LEE-011. (B) Hey1 xenografts treated with a vehicle control, cisplatin, or cisplatin and LEE-011. (C) Hey1 xenografts treated with vehicle control alone or pretreated with and then maintained with vehicle or LEE-011. (D) Hey1 xenografts treated either with concurrent cisplatin and LEE-011 or with cisplatin and then LEE-011. **(E) Sample dosing schedules.**

Discussion

The majority of patients with HGSOC present with advanced stage disease. Despite a complete clinical remission in response to first-line therapy, 70% of these patients eventually relapse. While recurrent ovarian cancer can be treated, there is currently no cure, particularly once patients become resistant to platinum. Therefore, in the absence of curative chemotherapy, identifying rational cytostatic therapies that can prolong progression free and overall survival is essential for improving patient survival and quality of life, particularly in patients with recurrent or platinum-resistant disease.

We report here a potential role for CDK4/6 inhibitors as a maintenance therapy in HGSOC. Mutational analysis of ovarian cancer has shown that 38% of patients have mutations and dysregulated expression of CDKN2A, CDK4, CDK6, and CCND1 that would likely make them good candidates for CDK4/6 inhibitor therapy. 20% of patients have deletions or downregulations of CDKN2A, which normally serves as a brake on the G1-S transition. The loss of this tumor suppressor locus has significant clinical consequences, as meta-analyses have implicated aberrant methylation of this gene in the development of head and neck squamous cell carcinoma [204] and prostate cancer [205]. CDKN2A downregulation also correlates with poor prognosis in glioma [206]. This subset of patients, who have lost the natural brake on the Cyclin D1/CDK4/CDK6 complex, are likely to benefit significantly from CDK4/6 inhibitor therapy. Along with CDKN2A downregulation, amplification of CDK4 or CDK6 were correlated with sensitivity to CDK4/6 inhibition in breast cancer [174], and patients with overactive Cyclin D, CDK4, or CDK6 are likely to benefit from LEE-011. In contrast, 17% of ovarian cancer patients have homozygous deletions or significant downregulations of RB1. Because RB1 is the final

effector for this pathway and is necessary for cell cycle progression from G1 to S phase, patients with significant Rb downregulation or deletion are predicted to receive significantly less benefit from CDK4/6 inhibitor therapy. Therefore, careful study of tumor responses and biomarkers is needed to optimize patient selection for CDK4/6 inhibitor therapy.

Our data show that LEE-011 significantly retards proliferation, causes G1 arrest, and induces a pseudo-senescent phenotype in ovarian cancer cells. Despite the fact that senescence is defined as irreversible cell cycle arrest, and that cells show a strong increase in senescence-associated B-galactosidase, they have a clear ability to proliferate after drug withdrawal. This expression of senescence markers in combination with the ability to divide could potentially be a pseudo-senescent state.

LEE-011 demonstrated clear efficacy in slowing ovarian cancer growth *in vitro* and *in vivo*. Interestingly, we have observed significant synergy between LEE-011 and cisplatin. While LEE-011 alone retards cell proliferation but allows it to move forward steadily, the combination of concurrent and maintenance LEE-011 with and after cisplatin completely arrested growth in vitro and delayed growth in vivo. Ovarian cancer cells are sensitive to differences in the timing and dose schedule when LEE-011 and cisplatin are combined. Concurrent administration along with maintenance, if tolerated, appears to be more effective than post-cisplatin maintenance alone, potentially due to DNA damage incurred during the cisplatin treatment. This is consistent with a previous report showing that CDK6 silencing increased cisplatin-induced cell death by repressing transcription of ATR, a protein involved in the DNA damage response [207]. Dysregulation of the DNA damage response may explain our observation that LEE-011 prevents cells from recovering and cycling normally when given in combination with cisplatin. Further

research into the mechanism by which LEE-011 potentiates cisplatin's effects and prevents normalization of cell cycling may yield significant insights into this combination therapy.

LEE-011 also significantly delayed tumor growth in *in vivo* xenograft experiments when used as a single agent and after cisplatin. LEE-011 is effective as a maintenance therapy even in platinum-resistant disease. Particularly given that these patients typically have shorter survival times and fewer treatment options, a novel targeted maintenance therapy that is effective for this population would constitute a significant improvement in quality of life, progression free survival and potentially overall survival. Further study of LEE-011 maintenance therapy as a single agent and in combination with cisplatin in validated platinum-resistant models is warranted.

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Chapter 4: Discussion

Summary of Thesis Work

This thesis has taken two approaches to investigating recurrent ovarian cancer, by investigating mechanisms of quiescence and relapse and also studying therapies that may prolong survival in patients with recurrent disease. We have identified NFAT3 as a transcription factor that is correlated with poor prognosis in ovarian cancer patients and is differentially overexpressed in ovarian cancer stem-like cells. NFAT3 appears to induce a quiescent phenotype in ovarian cancer, significantly decreasing proliferation without increasing cell death. Interestingly, this decrease in proliferation is not accompanied by a significant shift into the G0 phase or by any large changes in the percentage of cells in each phase of the cycle. However, NFAT3 appears to induce a quiescent phenotype, as its overexpression leads to smaller cells with decreased total RNA and decreased transcription of mRNA for many ribosomal structural proteins, translation-related proteins, and proteins involved in oxidative metabolism. Constitutive NFAT3 overexpression also induces chemotherapy resistance, and native NFAT3 translocates to the nucleus and initiates a transcriptional program in response to

cisplatin in multiple ovarian cancer cell lines. Constitutive NFAT3 expression also causes profound xenograft growth retardation *in vivo*, and loss of NFAT3 expression in these tumors is correlated with tumor growth; conversely, induction of NFAT3 in xenograft tumors is associated with slowing of tumor growth.

Our work on NFAT3 shows that its effects on cellular proliferation are likely multifactorial. In addition to global effects on the transcription of translation- and metabolism-related genes, we have identified moderate transcriptional repression of CDK6 in response to constitutive NFAT3 expression. This is consistent with other reports of NFAT1-mediated transcriptional repression of CDK4, which regulates the cell cycle in hair follicle stem cells. Both CDK4 and CDK6 are active in promoting the G1-S-phase cell cycle transition, and these proteins have been recently targeted by dual CDK4/6 inhibitors. This is a newer cytostatic drug class that blocks the G1-S phase transition. Phase 1 clinical trials of CDK4/6 inhibitors are ongoing in many cancers, but CDK4/6 inhibition has never been tested in ovarian cancer. Due to the promising effects of CDK4/6 inhibition in other cancers [174], the differential expression of NFAT proteins in ovarian cancer stem-like cells, and the cytostatic and quiescence-promoting effects of NFAT family members through regulation of CDK4 and CDK6, we have investigated CDK4/6 inhibition as a potential therapeutic pathway in high grade serous ovarian cancer.

We performed *in silico* analysis of TCGA data from 316 ovarian cancer patients and found that a significant proportion of patients have mutations in or dysregulation of genes involved in the G1-S phase transition; therefore, CDK4/6 inhibition may be a promising rational therapeutic for patients with ovarian cancer, particularly given the lack of currently available maintenance agents. We therefore characterized CDK4/6 inhibition by LEE-011 (Ribociclib,

Novartis) in ovarian cancer and have shown that LEE-011 significantly decreases cell proliferation and induces G1 arrest in sensitive Rb^{WT} cell lines. These arrested cells display a pseudo-senescent phenotype, delaying cancer growth both *in vitro* and *in vivo*. We also have observed a synergistic effect of LEE-011 in combination with cisplatin, where concurrent treatment with both drugs impairs subsequent cell cycling compared to treatment with cisplatin alone. Further research into the mechanism of synergy between LEE-011 and cisplatin is required, but this data hints that LEE-011 may be effective for the majority of ovarian cancer patients.

NFAT3 and Quiescence

As discussed in Chapter 1, quiescence and senescence tend to be poorly defined, though they are both generally considered to fall into the G0 phase of the cell cycle. Quiescent cells are typically thought to have exited the cell cycle, but are capable of dividing again; in some cases, they may be less metabolically active. Senescent cells, on the other hand, are defined by permanent cell cycle exit and by definition can never divide again. In practice, this distinction can be difficult to draw because it is technically challenging to differentiate very slow cycling cells from cells that have ceased to cycle. In addition, all the currently used markers of senescence lack sensitivity, specificity, or both, as described in Chapter 1. The absence of definitive markers of senescence further complicates technical attempts to distinguish deeply quiescent cells from senescent cells. A third technical challenge in the study of quiescence lies within the nature of cancer itself: rare, quiescent cells are rapidly outnumbered by the

aggressively growing cells which make up most of a cell line or tumor. Despite these technical challenges, our work with NFAT3 has uncovered an interesting and relatively novel quiescence phenotype that appears to be independent of cell cycle phase.

We have investigated the proliferation-restricted phenotype described in Chapter 2 with two models of constitutively active NFAT3. Though constitutive NFAT3 expression leads to tumor growth retardation and a significant decrease in proliferation, it does not lead to cell death or the expression of senescence markers. In addition, when NFAT3 is induced and then withdrawn in a doxycycline-driven system, the cells begin to proliferate normally again. This ability to resume proliferation excludes a senescent phenotype and suggests that NFAT3 induction produces quiescent/slow-cycling cells. Quiescent cells are almost universally defined as those that have exited the cell cycle into G0 phase [54, 208]. Despite extensive cell cycle characterization of two different constitutive NFAT3 models, both of which show significant decreases in proliferation, we have failed to observe large differences in cell cycle profiles, or significant shifts into the G0 phase, that may explain the proliferation differences between cNFAT3 or IcNFAT3 cells and their respective controls.

Interestingly, this lack of evidence is not unprecedented. A group studying NFAT4 in the brain observed significant and reversible decreases in cell proliferation and neurosphere size with NFAT4 inhibition. However, there was a negligible difference in the percentage of cells that were in the cell cycle, as measured by ki-67 expression. In addition, this group observed minimal differences in the percentage of cells in each phase of the cell cycle, despite significant differences in dye retention assays [184]. This is precisely what we have observed in both of our constitutively active NFAT3 models. When combined with our proliferation and cell cycle data,

this report suggests that multiple NFAT family members may regulate cell cycle-independent entrance into a quiescent or pseudo-quiescent state. Based on the definition of quiescence as requiring a G0 phase, the referenced paper concluded that NFAT4 slowed progression through the cycle without inducing quiescence and did not further investigate other quiescence-related parameters. However, our characterization of cNFAT3 cells shows that they display many properties inherent to a quiescent state. cNFAT3 cells are significantly smaller than their control counterparts and display a 20-40% decrease in total cellular RNA. In addition, we have observed significant decreases in the mRNA transcription of multiple ribosomal structural components, which is consistent with the known downregulation of ribosomes in the quiescent state. These observations are supported by gene set enrichment analysis showing that NFAT3 expression is correlated with downregulation of transcriptional sets for ribosomal structural proteins, translation, and oxidative metabolism. These functional parameters of quiescence correlate with the reduced proliferation that we have observed in bulk culture and in single cell microfluidics chips *in vitro* and in xenograft tumors *in vivo*. Despite the clear differences in proliferation, cell cycle phase analysis indicated few differences in the proportion of cells in each phase of the cell cycle between cNFAT3 or IcNFAT3 and control cells. Therefore, this reduced proliferation is not due to a G0 or G1 arrest and may be based at least partly on NFAT3-dependent regulation of multiple basic survival functions, such as translation and oxidative metabolism.

To the best of our knowledge, there are no vertebrate studies or studies in cancer that characterize cells as quiescent without a G0 arrest. However, the concept of a cell cycle-independent quiescent phenotype is not completely unknown. Studies in *Saccharomyces* have

shown that yeast cells can enter a reversible quiescent state from any phase of the cell cycle in response to carbon exhaustion, a metabolic stressor [185, 186]. Particularly given the widespread roles of NFAT family members as stress-response proteins, as discussed in Chapter 1, it is plausible that NFAT proteins may drive a metabolically-based quiescence. We have described a relatively novel quiescence phenotype in which proliferation is significantly decreased, despite the lack of a G0 or G1 arrest. This quiescent phenotype is consistent with reports on stemness and metabolic differences in slow cycling stem cells and sometimes in cancer stem-like cells [54]. The presence of a single master regulator that impacts many disparate aspects of cellular physiology is unusual and should be further investigated for its clinical implications. Most cancer therapeutics target various phases of the cell cycle, and treatment regimens tend to assume that quiescent/senescent cells are in the G0 phase and will exit into the G1 phase [55]. If this is not the case, novel therapeutic strategies may be required to treat cells entering the cycle into a phase other than G1.

Despite the lack of large cell cycle changes, we have observed that cNFAT3 modestly decreases CDK6, which is usually a factor promoting cell cycle progression. While NFAT1, NFAT2, and NFAT4 all have well-described roles in the cell cycle (reviewed in [104]), this is the first study to define a role for NFAT3 in the transcriptional regulation of cell cycle factors. As we observe few differences in the percentage of cells in each phase of the cycle, NFAT3 does not appear to cause arrest at any phase; instead, the total time per cycle appears to be increased instead. It is possible that NFAT3 mediates its effects on cell cycling largely by affecting fundamental cellular processes such as translation and metabolism instead of cell cycle regulation. However, the proportional lengthening of each phase could also potentially be due

to a modest effect on a large number of cell cycle-related proteins, similar to its apparent effect on ribosomal structural proteins, and this phenotype would be consistent with the aforementioned studies of NFAT4 in the brain. Detailed expression analysis of cyclins and CDKs from various cell cycle phases, possibly along with analysis of cell cycle progression with compounds that block the cycle at specific phases, are likely to elucidate the influence of NFAT3 on the cell cycle regulatory apparatus.

Future work will help to elucidate the role of NFAT3 in metabolism, translation and ribosomal downregulation, and cell cycling. Though we have excluded senescence based on the ability to reversibly decrease proliferation, label retention studies will help to further characterize division parameters of cNFAT3 cells and identify changes in the length of the cell cycle. RNA-seq analysis of cNFAT3 and IcNFAT3 vs. control cells will provide definitive evidence for gene sets that are up- and down-regulated and is likely to provide targets for further exploration. One major challenge to mechanistic analysis of NFAT3's effects is that this protein appears to modulate many cellular functions to a relatively small degree. For example, in Chapter 2, we showed a modest (20%) decrease in the transcription of ribosomal structural proteins and proteins required for translation. In general, a 20% decrease is a relatively insignificant change in qRT-PCR; however, a 20% downregulation in the number of ribosomes in the cell or in overall translation is likely to have profound effects. In fact, the decreased total cellular RNA and decreased total size are likely to be consequences of this reduction. Further work characterizing detailed transcriptional activity of NFAT3 will likely help to clarify its mechanisms of action.

NFAT3 and Chemotherapy Resistance

As described in Chapter 2, we have shown that NFAT3 translocates to the nucleus and activates transcription in response to cisplatin treatment. In addition, cNFAT3 expression increases viability during cisplatin treatment; conversely, concurrent treatment with cisplatin and VIVIT, an NFAT inhibitor, decreases viability. The mechanisms by which NFAT3 mediates chemotherapy resistance are unknown, but there are two broad categories of plausible mechanisms. Given the cNFAT3-induced growth retardation described in Chapter 2 and the known association of quiescence with a chemotherapy-resistant phenotype, it is possible that NFAT3's effects on the cell cycle may also be responsible for chemotherapy resistance. However, NFAT proteins also function as stress-response proteins, as described in Chapter 1. Therefore, it is also possible that NFAT3 initiates a transcriptional program in response to cisplatin that is independent of its effects on the cell cycle; this may include the transcription of detoxification enzymes or efflux pumps. This would be consistent with the known inhibitory effects of cyclosporine on P-glycoprotein, a well-characterized efflux pump responsible for multi-drug resistance [209]. A combination of quiescence and more direct chemotherapy resistance mechanisms may also be at work. However, we have not yet characterized the gene signature that is responsible for chemotherapy resistance. RNA-seq of control vs. cNFAT3 cells and ovarian cancer cells before and after cisplatin treatment might be helpful in identifying the mechanism, particularly if certain gene signatures overlap between cNFAT3 cells and cells treated with cisplatin. Due to the significance of these findings suggesting a quiescent and chemotherapy resistant stem-like phenotype in this subset of ovarian cancer cells, further research on specific mechanisms of chemotherapy resistance is warranted.

Clinical Implications of Thesis Work

The bulk of this thesis work has focused on quiescence, which can carry significant clinical implications. NFAT proteins are known to balance quiescence and proliferation of stem cells [109], and NFAT3 may play a similarly dual role in regulating survival and proliferation of stem-like cells in ovarian cancer. NFAT3 expression leads to RNA downregulation and a quiescent phenotype, including reduced cell cycling. During chemotherapy or radiotherapy performed with an intent to cure, quiescence is a significant negative factor; as these therapies tend to target rapidly dividing cells, slower-cycling cells may have a survival advantage, which can lead to relapse. However, during maintenance chemotherapy performed to increase progression-free or overall survival without curative intent, the induction of a quiescent state can be a positive factor; under these conditions, small deposits of very slowly-cycling cells may survive for months or years without causing clinical symptoms. Although tumor dormancy and subsequent reactivation is a concern [210], ovarian cancer is relatively aggressive and overall survival is under five years [1]; patients with platinum-resistant disease often have a prognosis of about a year. Therefore, an NFAT-promoting maintenance agent may significantly improve survival by inducing quiescence, as late relapses are less of a concern for most ovarian cancer patients due to the aggressive nature of this tumor. Although such NFAT3-upregulating agents do not exist, we have noted the suppressive effects of NFAT family proteins on CDK4 and CDK6 and characterized the effects of CDK4/6 inhibition in ovarian cancer; this combination may serve as a proxy for quiescence induction by continuous NFAT activation. The consistent inhibition of CDK4 and CDK6 leading to cytostasis and a decrease in cell cycling could potentially serve as an effective maintenance therapeutic in ovarian cancer.

As described in Chapter 1, most patients with high grade serous ovarian cancer experience relapse after the completion of first-line chemotherapy; the primary goals of treatment for these patients with recurrent disease consist of lengthening progression-free and overall survival [7]. There are currently no approved maintenance therapies that actually increase overall survival in patients with relapsed disease. The only two targeted maintenance therapies approved in ovarian cancer are Bevacizumab, an anti-angiogenesis agent, and Olaparib, a PARP inhibitor. Bevacizumab lengthens progression-free survival by 3-4 months without increasing overall survival at all [10, 11], at a cost of $50,000-$100,00 per year [211], while Olaparib similarly increases progression-free survival without appreciably changing overall survival [198] at a similarly elevated cost [212]. Therefore, the efficacy of current maintenance therapies is severely lacking. We have shown that CDK4/6 inhibition is a rational therapy for the majority of ovarian cancer patients in Chapter 3, and it has been shown to have significant benefit in breast cancer patients. Though CDK4/6 inhibition may be of particular benefit to the 40% of patients who already have mutations in or dysregulation of the relevant pathway genes, as described in Chapter 3, this does not preclude its use in patients who do not carry these genotypes. The cytostatic effects of CDK4/6 inhibitors in ovarian cancer therapy is a promising characteristic that deserves further clinical study.

The promotion of chemotherapy resistance by NFAT3 is another characteristic that can be exploited therapeutically. In this case, NFAT inhibition may promote chemosensitivity, increasing the kill rate and decreasing recurrence; we have shown evidence for NFAT3 as a chemoprotective agent and NFAT inhibition as a chemosensitizing factor in Chapter 2. This is a therapeutic avenue that holds significantly more promise for current use, as multiple pan-NFAT

inhibitors exist and are quite well-characterized. Cyclosporine A (CsA) and tacrolimus (FK506) are two pan-NFAT inhibitors that have been used for close to half a century as immunosuppressant drugs [72, 82]. NFAT inhibition with cyclosporine (or with the NFAT-specific agent VIVIT [92]) presents an alternative therapeutic strategy to the maintenance approach described above. NFAT inhibition is likely to lead to an increased cycling rate. If quiescence is in fact protecting these cells from DNA damage during cytotoxic chemotherapy, then NFAT inhibition followed immediately by chemotherapy may be an effective way to eradicate these formerly slow-cycling cells. This could be a potentially curative approach instead of a maintenance therapy. This strategy has been proposed before [55], and there is some evidence supporting this approach [56]. Our data have shown convincingly that NFAT inhibition (even pan-NFAT inhibition with VIVIT) tends to make cells more sensitive to chemotherapy. This is consistent with two clinical trials showing that cyclosporine treatment reversed platinum resistance in a significant percentage of ovarian cancer patients [188, 189]. This is precisely what we would expect from chemosensitization induced by NFAT; as cells lose NFAT expression and move into the cycle, they are more likely to be sensitive to platinum chemotherapy. Though these studies showed some promise, clinical trials of cyclosporine have never seen significant follow-up. Our data suggests that this might be a highly promising avenue for exploration.

While cyclosporine has the significant advantages of known dosing schedules, side effects, pharmacokinetics, and toxicity, the development of a new and more specific NFAT3 inhibitor is likely to have several advantages over pan-NFAT inhibition with cyclosporine. The adverse effects and side effects of cyclosporine are nontrivial; it inhibits all of calcineurin's

targets as well as all the NFAT family members. This leads to profound immunosuppression, which is why calcineurin was widely used for organ transplantation. However, a more specific NFAT3 inhibitor would be invaluable for precisely this reason – NFAT1, NFAT2 and NFAT4 all have known roles in the adult immune system, but NFAT3 does not [71]. Therefore, an NFAT3-specific inhibitor may have significant effects on ovarian cancer without significantly disrupting the immune system. This is particularly significant in light of data showing that tumor infiltrating lymphocytes play a key role in the body's natural defenses against ovarian cancer [213] and the fact that dysregulation of NFAT3, but not any of the other NFATs, is correlated with poor overall survival in ovarian cancer patients. Specific inhibition of NFAT3 that leaves the adult immune system intact may prove to be a powerful tool to treat chemotherapy resistant ovarian cancer. In the absence of specific NFAT inhibitors, further trials with cyclosporine and exploration of gene expression profiles, cell cycling, and chemosensitization in this system may be significant for ovarian cancer therapy.

We have also explored chemosensitization by CDK4/6 inhibitors in Chapter 3 and noted the potential for synergy between CDK4/6 inhibition and platinum agents, which are standard first line therapy in ovarian cancer. We have presented data showing that the concurrent combination of LEE-011 and cisplatin is more potent than cisplatin alone or cisplatin followed by maintenance with LEE-011. Based on our analysis of cell cycle dynamics, it appears that concurrent treatment sensitizes cells to the DNA-damaging effects of cisplatin, rendering them unable to recover and progress through the cell cycle. This is consistent with reports suggesting that cells are maximally sensitive to cisplatin in the G1 phase [214], which LEE-011 appears to lengthen. LEE-011 given in combination with and then as a maintenance therapy after cisplatin

could lead to better initial response rates and longer remissions, based on our data regarding chemosensitization. As this drug can be given orally, a long maintenance interval with a CDK4/6 inhibitor would represent a significant increase in quality of life as well as overall survival. For this reason, Dr Buckanovich has initiated a Phase I clinical trial of LEE-011 in high grade serous ovarian cancer patients to test the effects of concurrent LEE-011 and carboplatin administration followed by LEE-011 maintenance therapy.

Conclusions

This body of work has identified multiple novel functions for NFAT3 in ovarian cancer stem-like cells, including a role in proliferation and regulation of cell size and ribosomal content. We have also identified a quiescent state that is characterized by significantly decreased cellular division and metabolism, but not by arrest in the G0 phase, which has not been previously described in vertebrate systems. NFAT3 expression also leads to a chemotherapy-resistant phenotype and profound growth inhibition of xenograft tumors *in vivo*. Based on NFAT3 modulation of CDK6 and the significant impact of NFAT1-mediated modulation of CDK4 on quiescence in the hair follicle stem cell, we have also investigated CDK4/6 inhibition by LEE-011 (Ribociclib) in ovarian cancer. LEE-011 retards growth by impairing the G1-S phase transition and is a rational maintenance therapeutic for most patients with high grade serous ovarian cancer. We have identified significant synergy between LEE-011 and cisplatin which suggests that these may serve as a promising combination therapy, and Dr Buckanovich initiated a clinical trial to test this. Taken together, these data show that LEE-011 is a promising

ovarian cancer therapeutic that may be useful as a combination therapy or a maintenance therapy in a wide range of ovarian cancer patients.

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