

**Implications of PI3K/AKT inhibition on REST protein stability and neuroendocrine  
prostate cancer**

by

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## **Abstract**

Treatment-induced neuroendocrine (NE) prostate cancer (t-NEPC) is an aggressive subtype of prostate cancer (PCa) that can arise as a consequence of rigorous androgen receptor pathway inhibition (ARPI) therapies now used to treat castration resistant disease (CRPC). While the PI3K/AKT pathway has been investigated as a co-therapeutic target with ARPI for advanced prostate adenocarcinoma, whether this strategy has implications on t-NEPC progression remains unknown. Findings from this work indicate that PI3K/AKT inhibition alone reduces protein expression of the RE-1 silencing transcription factor (REST) and induces multiple NE markers in PCa cells. The loss of REST by PI3K/AKT inhibition is through protein degradation mediated by the E3-ubiquitin ligase  $\beta$ -TRCP and REST phosphorylations at the S1024, S1027, and S1030 sites. Since AR inhibition was previously reported to deplete REST, results from this project reveal that the combined inhibition of PI3K/AKT and AR further aggravates REST protein reduction. Upon profiling the transcriptomes of AKT inhibition, AR inhibition, and AKT/AR co-inhibition in the LNCaP cell model, Gene Set Enrichment Analysis (GSEA) shows that these transcriptomes are highly correlated with the REST-regulated gene signature. Co-targeting AKT and AR resulted in an even higher correlation comparing to those of single treatment. Comparing these transcriptomes to the RNA-seq gene signature of t-NEPC patients by GSEA, it was observed that adding AKT inhibition to AR blockade enhanced the expression of neurogenesis-related genes and resulted in a stronger and broader upregulation of REST-regulated genes specific to t-NEPC. Collectively, these results indicate that AKT pathway inhibition can induce NE transdifferentiation in PCa cells via REST protein degradation. It delineates a potential risk for the AR and PI3K/AKT co-targeting strategy as it may further facilitate t-NEPC development.

## **Lay abstract**

One in every eight men is diagnosed with prostate cancer (PCa), rendering this disease the second most common cancer affecting Canadian men. The primary treatment for advanced PCa is androgen receptor (AR) pathway inhibition (ARPI). However, PCa will almost certainly come back with this treatment. Consequently, new therapies targeting the AKT signaling pathway are being actively investigated. In this project, we have found that AKT inhibitors can potentially contribute to the development of one of the most lethal subtypes of therapy-resistant PCa called neuroendocrine prostate cancer (NEPC). In fact, we observed that treating cancer cells with AKT inhibitors and AR inhibitors have an additive effect on reducing a protein called REST, a master suppressor for NEPC development. Results from this study provide a critical implication on the potential adverse effect of this new therapeutic strategy targeting the AKT signaling pathway in PCa.

## **Preface**

Chapter 2&3 of this thesis is currently being peer-reviewed for publication: “Chen R, Li Y, Buttyan R, Dong X. (2017) Implications of PI3K/AKT inhibition on REST protein stability and neuroendocrine prostate cancer.” The experimental work reported was accomplished by a research team within Vancouver Prostate Centre (VPC). Dr. Xuesen Dong was the senior author of this manuscript and directly led the project. Dr. Ralph Buttyan provided critical comments and reviewed the work. Estelle Li and Saki Konomura from Pathology Core of VPC prepared and conducted immunohistochemistry analyses. Mrs. Ning Xie and Mr. Yinan Li from Dong lab trained me with molecular and cellular techniques. I wrote the manuscript, which was revised by my supervisor Dr. Xuesen Dong.

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## List of abbreviations

|      |                                       |
|------|---------------------------------------|
| AdPC | adenocarcinoma                        |
| ADT  | androgen-deprivation therapy          |
| AKT  | protein kinase B                      |
| AR   | androgen receptor                     |
| ARE  | androgen response element             |
| ARPI | androgen receptor pathway inhibition  |
| ATP  | adenosine triphosphate                |
| AUA  | American Urological Association       |
| BPH  | benign prostatic hyperplasia          |
| CIHR | Canadian Institute of Cancer Research |
| CRPC | castration-resistant prostate cancer  |
| CSS  | charcoal stripped serum               |
| CZ   | central zone                          |
| DBD  | DNA-binding domain                    |
| DHT  | dihydrotestosterone                   |
| DRE  | digital rectal examination            |
| EAU  | European Association of Urology       |
| ENZ  | enzalutamide                          |
| EGF  | epidermal growth factor               |
| EPX  | epoxomicin                            |
| FC   | fold change                           |
| FBS  | fetal bovine serum                    |

|      |                                     |
|------|-------------------------------------|
| IHC  | immunohistochemistry                |
| MS   | manuscript                          |
| NE   | neuroendocrine                      |
| NEPC | neuroendocrine prostate cancer      |
| TZ   | transitional zone                   |
| Padj | adjusted p-value                    |
| PBS  | phosphate-buffered saline           |
| PCa  | prostate cancer                     |
| PDX  | patient-derived xenograft           |
| PI3K | phosphatidylinositol 3-kinase       |
| PSA  | prostate-specific antigen           |
| PTEN | phosphatase and tensin homolog      |
| PZ   | peripheral zone                     |
| RD   | repressor domain                    |
| RE-1 | repressor element 1                 |
| REST | RE-1 silencing transcription factor |
| RTK  | Receptor tyrosine kinase            |
| TMA  | tissue microarray                   |
| UGE  | urogenital sinus epithelium         |
| UGM  | urogenital sinus mesenchyme         |

## **Acknowledgements**

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# **Chapter 1: Background, hypothesis, and objective**

## **1.1 Prostate cancer**

### **1.1.1 Overview**

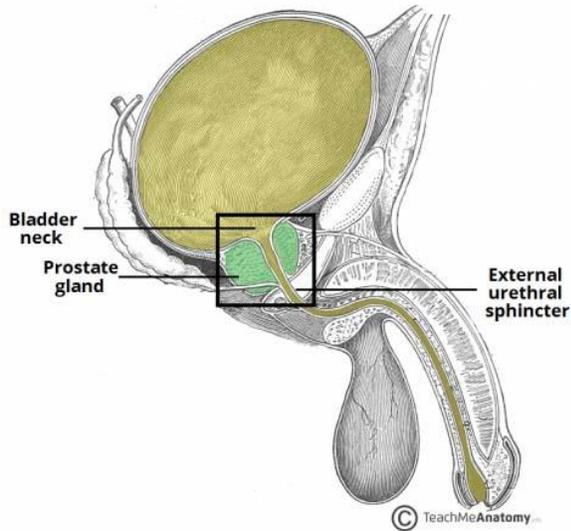
More than 1.1 million patients were diagnosed with prostate cancer (PCa) and more than 300,000 deaths were PCa related worldwide in the year of 2012 according to a report from the Globocan Project (1). In Canada, PCa affects one in every eight Canadian men and is the third leading cause of cancer-related mortality in men (2). Due to the immense health and financial burdens this disease confers to patients, family, and the society, there is a great interest to improve PCa detection, treatment, and management. This thesis aims to provide a contribution to our current pool of knowledge in understanding PCa.

### **1.1.2 The prostate**

The human prostate is one of the male-specific accessory glands. It is a walnut-sized gland that surrounds the urethra underneath the bladder and anterior to the rectum (Figure 1.1). Prostate development begins during week 10-12 of gestation (3). During this time, testicular testosterone acting through androgen receptors (AR) to promote urogenital sinus epithelium (UGE) budding, epithelial morphogenesis, and differentiation (4–6). With the maturation of the prostate stroma later on (7), the epithelial-mesenchymal interactions further induce the development of several distinct cellular subtypes within the prostatic epithelium including luminal epithelial, basal epithelial, and neuroendocrine cells (8) (Figure 1.2). The luminal cells are columnar and express cytokeratin 8 and 18. These cells are the main source for the production and secretion of prostatic fluid. The basal epithelial cells are localized along the basement membrane of the ductal lumen and express cytokeratin 5 and 14. In addition, interspersed within the basal

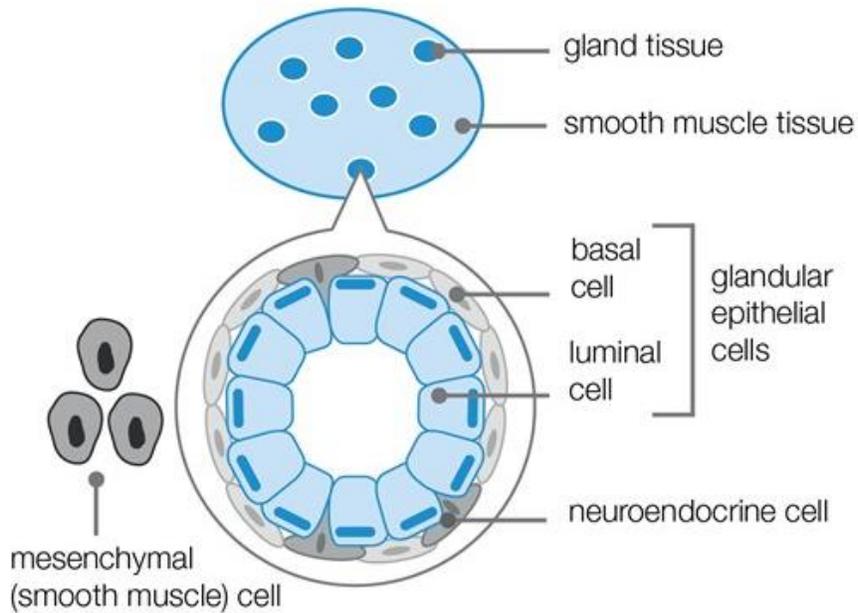
epithelial cells are the rare neuroendocrine (NE) cells that are mostly non-proliferating and secrete NE products. While both luminal and basal epithelial cells are derived from the UGE, the origin of the benign NE cells is still controversial as reviewed previously (9). Benign NE cells may share common origins with other prostatic cells, while may also be derived from migratory neural crest cells during early prostate development. The human prostate continues to grow in size throughout one's lifespan. It weighs approximately 1-2 grams at birth and 20 grams at the onset of puberty. Anatomically, the adult prostate has three histologically classified lobes including the peripheral zone, the central zone, and the transition zone (10,11) (Figure 1.3). The peripheral zone, almost equivalent to the lateral and posterior parts of the prostate, comprises 70% of the prostatic tissue and is a region where most PCa arise (12). The central zone is found between the peripheral and transition zones, comprising of 20-25% of the prostate tissue. Finally, the transition zone enfolds the urethra and is prone to the development of benign prostatic hyperplasia (BPH) (13).

Functionally, the human prostate, as one of the two glands that produce most of the ejaculate, secretes fluid related to semen gelation, coagulation, and liquefaction (14). Some prostatic proteins were also implicated in the coating/uncoating of spermatozoa and the interaction with cervical mucus. The most notable secretion of the prostate is prostate-specific antigen (PSA) (15). Its secretion from the prostate, along with the prostate gland's growth and differentiation, are tightly regulated by androgens (6,16). Furthermore, the prostate also helps expel semen during ejaculations. Interestingly, it has been reported that the prostate, but not the prostatic secretion, is required for fertility, suggesting possible unknown functions of this gland (3).



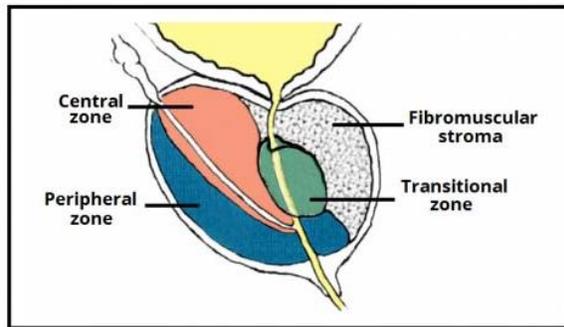
**Figure 1.1 The prostate gland**

Copied from <http://teachmeanatomy.info/pelvis/the-male-reproductive-system/prostate-gland/> with permission



**Figure 1.2 Prostate tissue structure**

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**Figure 1.3 The basic prostate anatomy**

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### **1.1.3 Prostate cancer**

The prostate naturally grows in size over time as one ages and can frequently lead to a benign disease called BPH (10). It is estimated to affect 1 in 2 males aged between 51-60 and the incidence rate further increases with aging (17,18). The enlarged prostate can squeeze the urethra to cause urinary symptoms such as frequency, urgency, dysuria, and urinary retention etc.

However, BPH is not correlated with PCa risk. In fact, the strongest PCa risk factors are age, race, and family history (19). Age is the strongest factor for PCa as patients are mostly diagnosed after the age of 55. Stochastic mutations and replication errors, along with environmental factors such as chronic inflammation, could lead to oncogenic cellular processes such as hyper-proliferation, prostate cell de-differentiation, and disruption of microenvironment homeostasis (20). Moreover, men with a family history (the definition is confined to first-degree relatives) of PCa had a 30%-60% probability of having the disease by the age of 75 compared to 4.8% in those who do not have the family history (21). From a racial perspective, black men across the

globe have a 60% higher risk of developing PCa with poorer prognosis compared to their counterparts (22). In addition, other factors such as germline genetic mutations and diet are beginning to emerge as new indicators for PCa (23,24).

PCa differs from BPH because the former can grow uncontrollably, invade surrounding tissues, and spread to other distant organs. Therefore, early detection of locally confined PCa has emerged as a major theme in this disease. According to the newest European Association of Urology (EAU) guideline, early stage PCa is usually asymptomatic due to its relatively small size and slow growth, in contrast to late stage PCa that usually leads to hematuria, dysuria, fatigue, weight loss, as well as pelvic/lower back pain (25). Both EAU and American Urological Association (AUA) suggest that early signs of PCa can be detected by abnormalities from the digital rectal examination and serum PSA levels (25,26). It has been hypothesized that elevated PSA levels in serum may be due to the disruption of the prostate cellular architecture, resulting in diffusion of PSA into the circulation (27). Because PSA can also rise due to other conditions such as BPH and prostatitis, additional factors such as PSA velocity, free/total PSA ratio, -2proPSA, PCA3 levels are also frequently taken into consideration by clinicians when diagnosing PCa. According to the EAU and AUA guidelines, once PCa is suspected, prostate biopsies with a minimum of 12 cores, with or without the aid of multiparametric MRI, will be performed to confirm PCa diagnosis (25,26). Tissue samples from biopsies are further evaluated via histopathological assessment by pathologists. CT and bones scans are necessary if metastasis is suspected. Finally, a urological pathology report is provided that includes information regarding histopathological type, Gleason grading, Tumor, Node, Metastasis (TNM) staging, tumor focality, size/location of the dominant tumor, and surgical margin. Conventionally, the

Gleason Grading system includes 5 grades (from 1-5) based on tissue histology, followed by combining the most common and second most common patterns to get Gleason scores ranging from 2-10 (28). Currently, Gleason scores of 2-5 are not assigned with Gleason 6 prostate cancer being low risk, Gleason 7 being intermediate risk, and Gleason 8-10 being high risk (29). However, there have been attempts recently to create a new prostate cancer grading system (30). The new system suggested in 2015 includes grade groups instead of Gleason score and they range from group 1-5 with 5 being the most aggressive. In addition, in the TMN system, T pertains to the tumor within and immediately adjacent to the prostate tissue, N describes lymph node metastasis, and M denotes to bone metastasis.

Per EAU and AUA recommendations, treatment options for low and intermediate risk PCa include active surveillance (particularly in low risk Gleason 6 PCa), watchful waiting (particularly in patients with other significant comorbidities), surgery, and radiation therapy. In high-risk PCa, defined by T stage  $\geq$ cT2c, Gleason score  $\geq$ 8, and/or PSA $>$ 20 ng/ml according to the D'Amico classification (31), radiation therapy (brachytherapy or external beam radiation therapy) with neoadjuvant androgen-deprivation therapy and radical prostatectomy with salvage radiation therapy are recommended (32), suggesting that combination therapies in high risk PCa are more effective. Nonetheless, high risk PCa has increased chance of recurrence, metastatic progression, and death from PCa. Interestingly, radiation therapy and surgery have similar outcome in terms of cancer treatment but can lead to different side effects. In general, surgery does have more limitations in treating PCa due to comorbidities and tumor invasion status (e.g. surgical margin).

According to the EAU and AUA recommendations, in the case of metastatic PCa, androgen deprivation therapy (ADT) that includes suppressing testicular androgen secretion and inhibiting the actions of androgen on AR has become the standard of care (33,34). To lower testicular androgen productions, luteinizing-hormone-releasing hormone (LHRH) agonists and antagonists are the two main therapeutic options (35). The first-line anti-androgens include steroidal (e.g. abiraterone acetate) and non-steroid compounds (e.g. enzalutamide). Unfortunately, these first-line standard of care treatment for metastatic PCa can only delay its progression in most cases (36). Combination treatment of more than one ADT treatments, co-treatment of ADT and chemotherapy, as well as intermittent ADT were all studied as part of randomized clinical trials previously but minimum improvements in clinical outcome were observed, particularly with regard to the quality-adjusted life-year (33,34).

#### **1.1.4 Castration-resistance prostate cancer**

Although ADT is effective in prolonging the survival of metastatic PCa patients, relapse occurs in most cases and castration-resistant prostate cancer (CRPC) emerges. Upon diagnosis, CRPC patients have a 16-18 months of median overall survival (37). These CRPC tumors can emerge both as AR-dependent and AR-independent tumors via adaptation. It has been suggested that there are three main mechanisms by which the CRPC tumors evade first/second-line therapies: 1) AR-dependent CRPC with secondary alterations in AR via AR mutation, amplification, and alternative splicing that circumvent the current androgen-AR blockade, 2) AR-dependent CRPC with bypass/crosstalk mechanisms through GR overexpression and PI3K/AKT overactivation, and 3) AR-independent CRPC is usually accompanied by the loss of the luminal-epithelial phenotype (38). The first two mechanisms comprise the majority of CRPC. Treatment options

for the AR-dependent CRPC patients (excluding CRPC without metastasis) include additional androgen receptor pathway inhibition (ARPI) and systematic chemotherapy. Abiraterone acetate is one of the next-generation ARPI drugs that prolongs survival by primarily inhibiting the cytochrome P450 isoform 17A1 (CYP17), an enzyme critical to androgen steroidogenesis (39,40). Another one is enzalutamide, which is a potent oral nonsteroidal AR antagonist and has been shown to have a higher potency in blocking AR than bicalutamide (41,42). On the other hand, doxorubicin is also frequently used as the first-line chemotherapeutic agent, particularly in vulnerable older men (43). This taxane-based microtubule-depolymerizing drug has been shown to improve both progression-free and overall survival in metastatic CRPC patients comparing to a previously used chemotherapy agent mitoxantrone (44). Cabazitaxel, as a second-line chemotherapy agent, also has been reported to have disease-delaying effects (45). Radium-223, approved by the FDA in 2013, is recommended for CRPC patients with symptomatic bone metastasis (46). Unfortunately, these treatments often only prolong patients' survival for months, and disease progression often recurs after tumors have adapted to these treatments. In fact, with the applications of more potent ARPI inhibitors, there is an increased incidence of AR-independent neuroendocrine (NE)/anaplastic PCa. These tumors are resistant to ARPI treatments and usually have rapid progression. No effective therapies are currently available for this type of CRPC besides platinum-based systematic chemotherapy (47).

## **1.2 Neuroendocrine prostate cancer**

### **1.2.1 Epidemiology**

Neuroendocrine prostate cancer (NEPC) is a main subtype of AR-independent CRPC. NEPC, in particular small-cell NEPC, is one of the most lethal CRPC tumors with a median survival of

only 7 months upon the diagnosis owing to the lack of early detection methods and treatment options (48). Although NEPC is rare as the primary form of PCa (<1%), it is estimated to consist of up to 25% in CRPC patients status post first/second lines of ARPI treatments (49). The origin of NEPC is yet conclusive, but findings have strongly argued that this disease is treatment induced and evolved from CRPC-adenocarcinoma (AdPC) under the selection pressure of ARPI. For example, treatment-induced NEPC (t-NEPC) and AdPC often share similar genomics but distinct transcriptomes, suggesting shared clonal origins (50). Consistently, the development of AdPC tumors into t-NEPC over months in patient-derived xenograft (PDX) models and the transdifferentiation of luminal epithelial PCa cells to NEPC cells in genetically modified mice (GEM) models further supported this theory (51). Because t-NEPC is most likely emerged from ARPI treatments from AdPC as a resistance mechanism, the prevalence of t-NEPC is expected to rise with the applications of more potent anti-androgens, indicating the need to find more efficacious ways to prevent and manage t-NEPC.

### **1.2.2 Clinical challenges**

There are several major challenges that impede effective management of t-NEPC. First, because PSA is used as the “gold-standard” of detecting PCa recurrence/progression and t-NEPC tumors do not express this biomarker due to their AR-independent nature, early detections of t-NEPC is difficult. To solve this issue, investigations have been focusing using other noninvasive biomarkers such as serum chromogranin levels, t-NEPC-specific circulating tumor cells, and circulating tumor DNA (52,53). Moreover, heterogeneity among t-NEPC is also an area that is only beginning to be understood due to the lack to biopsies from CRPC patients in the past. Yet, studies to date have already shown a spectrum of t-NEPC that has varied pathological and

clinical features. For example, Beltran and Epstein et al. have proposed the pathological classification of NE differentiation in PCa into six groups: 1) usual prostate adenocarcinoma with NE differentiation, 2) adenocarcinoma with Paneth cell NE differentiation, 3) carcinoid tumor, 4) small cell carcinoma, 5) large cell NE carcinoma, and 6) mixed small and large cell NE carcinoma (47,54). Soon after, Beltran et al. have found varied genomic, epigenetic, and transcriptomic characteristics even among t-NEPC patients, further supporting the heterogeneity of this disease (50). These indications of numerous subtypes of t-NEPC imply varied treatment response and prognosis even within the t-NEPC tumors, thus possibly hindering the development of t-NEPC treatment with high efficacy. Nevertheless, targeted therapies for t-NEPC have been ongoing as led by an aurora-A kinase (AURKA) inhibitor MLN8237 (NCT01799278) in phase II clinical trial. EZH2 inhibitors are also being evaluated in pre-clinical models for t-NEPC.

### **1.2.3 Review of molecular events involved in t-NEPC development**

There has been a rapid progress of molecular understanding with regard to t-NEPC development and progression in the recent years. Although a plethora of future investigations are still needed, findings have strongly suggested that these molecular events involved in t-NEPC can be broadly summarized in a model consisting of lineage plasticity, NE differentiation, and proliferation.

#### ***i) Gain of lineage plasticity***

Lineage plasticity describes a state of PCa cells of being able to transform from luminal epithelial phenotype to other phenotypes. Previous studies have shown remarkable lineage plasticity among PCa cells both as a result of genomic alterations and microenvironment changes. From a genomic perspective, the loss-of-function of retinoblastoma 1 (Rb1) and tumor

protein 53 (TP53) has been shown to enhance PCa tumor lineage plasticity. It was first observed that upon the expression of the transforming region of SV40 large T antigen (SV40 Tag) in the TRAMP mice model, metastatic prostate cancers including tumors with the NEPC phenotype were developed as Rb1 and TP53 were sequestered and rendered inactive (55). Later studies have confirmed that Rb1 and TP53 knockout mice can develop NEPC from metastatic tumors initiated from PTEN loss (56). Concurrently, Mu et al. demonstrated that Rb1 and TP53 knockdown in PCa cells *in vitro* could induce rapid dedifferentiation within 48 hours (57). These findings indicate that Rb1 and TP53 loss not only promote cell survival and proliferation through their canonical pathways, but also induce lineage plasticity in PCa cells. Another important factor reported is N-Myc, a known oncogene for neuronal-related tumors such as neuroblastoma, medulloblastoma, glioblastoma multiforme, and retinoblastoma (58). The overexpression of this oncogenic driver led to the emergence of NEPC tumors in mice models along with other types of tumors (59,60).

Clinically, deletion/mutation of Rb1 (90% vs 30%) (61) and TP53 (67% vs 30-50%) (62,63), as well as amplification of N-Myc (40% vs 5%) (64) were more prevalent in t-NEPC patients compared to those from AdPC patients, suggesting that these factors may be t-NEPC specific. Interestingly, upregulations of SOX2 (a transcription factor involved in maintaining stem-cells in the central nervous system) and EZH2 (an epigenetic regulator as a part of the Polycomb Repressive Complex 2 System) were observed in both Rb1/TP53 knockout and N-Myc overexpressing cell and animal models (56,57,59), implying that SOX2 and EZH2 may in part represent the nodes of signaling pathways involved in NEPC development. However, Rb1/TP53 loss or N-Myc overexpression likely confers PCa tumors the lineage plasticity but do not

specifically drive them toward NEPC because these genetic alterations in animal models led to the emergence of various kinds of tumors with NEPC being part of the spectrum. Supporting this hypothesis are the DU145 cells, which are AR-negative and have non-functional Rb1 and TP53, yet do not show the NEPC phenotype (65,66).

In addition to genomic aberrations, microenvironment changes can also dictate PCa lineage plasticity. This was exemplified by the findings of Nouri et al., where the group has shown that LNCaP cells cultured with a neural crest Stem Transition Medium were capable of transforming into a neural crest-like phenotype, which upon further stimulus could differentiate into neuronal-like, oligodendrocyte-like, and osteoblast-like lineages (67). Although exact factors causing these changes are yet to be determined, these findings supported that microenvironment factors can induce PCa cell lineage plasticity for differentiation. The arguably most important pathway in regulating PCa lineage plasticity is the androgen-AR axis because t-NEPC can only be ARPI-induced. Kregel et al. (2013) elucidated that ARPI can promote PCa lineage plasticity by upregulating SOX2 through reduced AR-mediated transcriptional repression of the stem cell regulator (68). Moreover, ARPI was also reported to indirectly induce SOX2 via the upregulation of BRN2 (69). These findings, combined with the implications of ARPI on other lineage changes including epithelial-mesenchymal transition and epithelial-myeloid transition, support the regulatory role of the androgen-AR axis on PCa lineage plasticity (70). Other microenvironment factors can also induce phenotypical changes in PCa cells, particularly in the Rb1/TP53/NMyc wildtype LNCaP cells, including cAMP, interleukin-6, melatonin, and hypoxia (71–76).

*ii) NE-specific differentiation:*

Although genomic and environmental factors can affect AdPC lineage plasticity, there are multiple lineage directions for AdPC to adapt ARPI resistance. For example, on rare occasions, AdPC cells undergoing the treatment of ARPI can emerge as highly metastable AR-negative and non-neuronal cells exemplified by PC3 and DU145 cells (66). On other occasions, ARPI could induce cells to undergo phenotypical changes similar to the gaining of stem-like properties, epithelial-mesenchymal transition, and epithelial-myeloid transition (70). Therefore, this phenomenon raises the question of why neuroendocrine is an often preferred phenotype for AdPC to turn to? A possible explanation may be that neuroendocrine properties play an important role in PCa survival and proliferation second to the androgen-AR axis. In mice, autonomic nerve signals infiltrated in the PCa stroma and tumors could promote PCa survival, invasion, migration, and distant metastasis (77). Patients with PCa and preneoplastic lesions have increased global nerve density in tumor foci compared to benign regions, and axonogenesis in PCa tumors was correlated with ARPI resistance as well as poor outcome (78). Interestingly, patients with spinal cord injury were reported to have smaller prostate and lower PCa incidence (79,80). These findings, together, suggest a potentially important role of neuronal signaling for prostate and PCa development alike. Neuroendocrine tumors, on the other hand, not only have neuronal-like morphologies and interact with the autonomic nerve system, but also secrete factors such as bombesin, chromogranin family peptides, thyroid-stimulating hormone-like peptides, calcitonin-gene family peptides to promote cell survival, proliferation, and chemoresistance (81–86). Therefore, NE differentiation likely confers selection advantage to AdPC cells under stress. This differentiation process is likely controlled by factors such as the master neural-specific alternative splicing factor SRRM4 aka nSR100 (87), the transcription

suppressor of neurogenesis REST (will be discussed in section 1.4), the lineage regulator FOXA1/2 (55,88–90), as well as the neuro-specific transcription factor BRN2 (69,91).

***iii) Proliferation:***

Benign neuroendocrine cells are mostly post-mitotic and have minimal proliferative abilities, similar to those of most neuronal cells (81). In PCa, PCa cells with NE marker expressions were observed in 30-100% of the PCa population (82,92), yet only roughly 25% could be transformed into NEPC under the selection of ARPI (49). In fact, NE phenotype acquisition often occurs during a stress period as an adaptive survival mechanism. *In vitro* studies have found that the emergence of the NE phenotype in PCa cells was often accompanied by reduced proliferation rate under various treatment (e.g. ARPI, IL-6, cAMP, melatonin, hypoxia (71,74,76,93,94)). Therefore, these observations suggest that NEPC cells may have to gain additional clonal expansion and tumor formation abilities in order to populate. In fact, findings to date indicate that there are several genes that are specific to NEPC because they are upregulated only in NEPC tumor cells but not in AdPC cells, yet only facilitate cell proliferation and invasion but not NE differentiation when investigated in cell and animal models. These genes include aurora kinase A (64,95), PEG 10 (96), MEAF6-1 (97), and cyclin D1 (98,99).

In short, genes involved in lineage plasticity permit AdPC to evade ARPI in an AR-independent fashion. These genes tend to give rise to various AR-independent phenotypes in PCa tumors. In addition to having lineage plasticity, AdPC tumors may require the input of NE-specific differentiation genes to eventually transform into NEPC. SRRM4, REST, BRN2, FOXA2 could play critical roles in determining the final lineage of NEPC that may have selective advantages

among other AR-independent and -dependent tumors under the current treatment regimen. Furthermore, NEPC cells require the ability to outgrow other PCa cells for clonal expansion, tumor establishment, and treatment resistance. This process may be aided by the expression of NEPC-specific growth facilitators such as AURKA, PEG10, MEAF6, and cyclin D1. However, this proposed process is likely non-linear because it is possible that fast growing tumors that have already acquired phenotypical plasticity may differentiate into NEPC en masse upon ARPI treatment. In addition, these genes discussed may involve in more than one process such as TP53 and Rb1 loss that also confers treatment resistance and cell proliferation. Nonetheless, the lineage plasticity-NE differentiation-proliferation model provides a parsimonious summarization of current molecular events involved in t-NEPC development and progression.

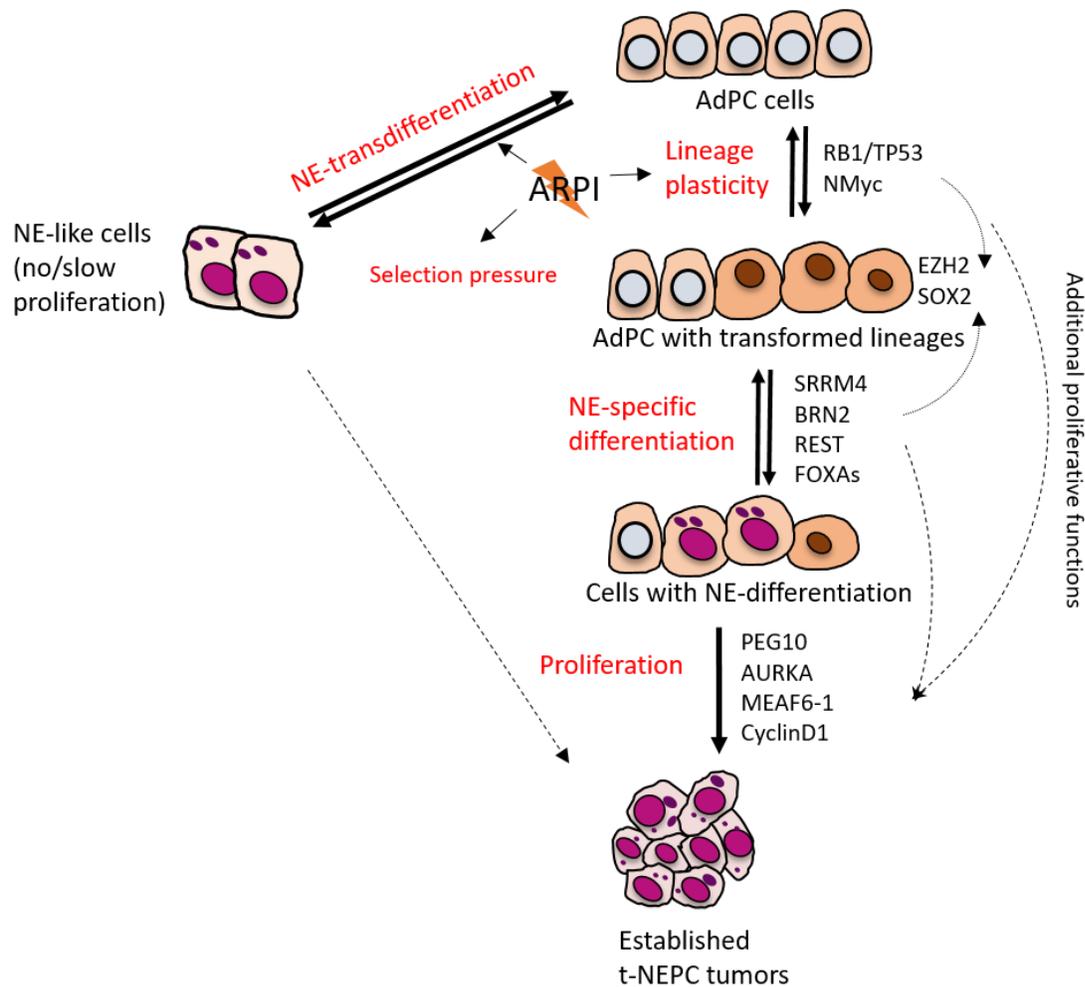


Figure 1.4: A schematic summary of putative NEPC development process

### 1.3 PI3K/AKT signaling

#### 1.3.1 The PI3K/AKT signaling pathway

The PI3K/AKT signaling pathway is one of the main oncogenic drivers of PCa (100). The family of the phosphoinositide 3-kinase (PI3K) lipid kinases consists of many activators, inhibitors, effectors, and secondary messengers that entail a broad spectrum of biological functions such as cell survival, cell cycle, and metabolism, motility, and genomic stability (101). There are primarily 3 classes of PI3Ks based on structures and substrate specificities as reviewed by

Thorpe et al. (102). Briefly, the class I has subclasses IA and IB. Class IA PI3Ks are heterodimers consisting of a p110 catalytic subunit (p110 $\alpha$ , p110 $\beta$ , p110 $\delta$  encoded by PIK3CA, PIK3CB, and PIK3CD, respectively) and a p85 regulatory subunit (p85 $\alpha$  and p85 $\beta$ , and their respective splicing variants). Class IB PI3Ks consist of p110 $\gamma$  and the p101 or p87 regulatory subunit. P110 $\alpha$  and p110 $\beta$  are ubiquitously expressed in human cells whereas p110 $\delta$  and p110 $\gamma$  were restricted to leukocytes only. In addition, there are also class II and class III PI3Ks, which are less well-understood and have different structures as well as divergent roles. Since this thesis work entails the PI3K/AKT signaling pathway that only involved PI3K IA, we will focus on the mechanistic actions of PI3K IA isoforms.

As reviewed by Vivanco and Sawyers (103), activation of the PI3K signaling pathway begins when growth factors bind to tyrosine kinase receptors (RTK), leading to dimerization and the heterologous autophosphorylation at the tyrosine residuals. The inactive PI3K is recruited via the binding of its Src-homology 2 (SH2) domain on the PI3K p85 regulatory subunit to the phosphotyrosine residues on the RTK receptor or the SH2 docking sites on the adaptor proteins IRS1 and IRS2. These SH2-phosphotyrosine interactions relieve the p85 inhibition effect on PI3K and activate the p110 catalytic subunit. The active PI3K then migrates to the inner side of the plasma membrane and binds to phosphatidylinositol-4,5-bisphosphate (PIP2) and converts this lipid into phosphatidylinositol (3,4,5)-triphosphate (PIP3). PIP3, in turn, can interact with protein kinase B (PKB) aka AKT (named after its homologous protein in retrovirus AKT8) via the pleckstrin-homology (PH) domain. However, AKT is yet fully active as phosphorylation and dissociation of the carboxy-terminal modulator protein (CTMP) by an unknown kinase is required. Once CTMP is detached, AKT can be fully activated upon phosphorylation at Thr308

and Ser473 by PDK1 and PDK2, respectively. In addition to activating the serine threonine kinase AKT, PIP3 can also be converted back to the inactive PIP2 form by the action of phosphatase and tensin homolog (PTEN).

There are three isoforms of AKT (AKT1, AKT2, and AKT3), which are encoded by three separate genes and have more than 80% similarities in amino acid sequences (100). Specific functions of each of these isoforms are poorly understood despite extensive research. In general, the active AKT interacts with numerous downstream effectors in the cytosol (Fig 1.5) (100). For example, AKT can hinder apoptosis by binding to BAD to prevent it from creating holes in the mitochondria and the subsequent caspase activation cascade (104). Similarly, AKT can phosphorylate the E3 ligase MDM2, which in turn facilitates the degradation of p53 in order to circumvent apoptosis, DNA repair, and cell-cycle arrest (105). Another classical function of AKT is the regulation of protein translation. Specifically, AKT can lead to the activation of the mammalian target of rapamycin (mTOR) (106). mTOR phosphorylates S6K, leading to its recruitment to the large subunit of ribosome and subsequent protein synthesis initiation. Furthermore, AKT regulates cellular metabolism in part by directly phosphorylating and inhibiting the glycogen synthase kinase 3 (GSK3), a normally constitutively active kinase acting as a negative regulator in the hormonal control of glucose homeostasis by inactivating glycogen synthase (GYS1 or GYS2), in the Wnt signaling by inhibiting CTNNB1/beta-catenin, and in the regulation of microtubules through phosphorylation of MAPT/TAU and MACF1 (107). In summary, the PI3K/AKT signaling pathway is an intricate system that is involved in numerous cellular functions via interactions with other factors. Because of the pro-survival and pro-proliferation functions of the PI3K/AKT pathway, its aberrant activations are often associated

with oncogenesis in almost all forms of cancers such as lung, colon, pancreas, ovarian, breast, and prostate.

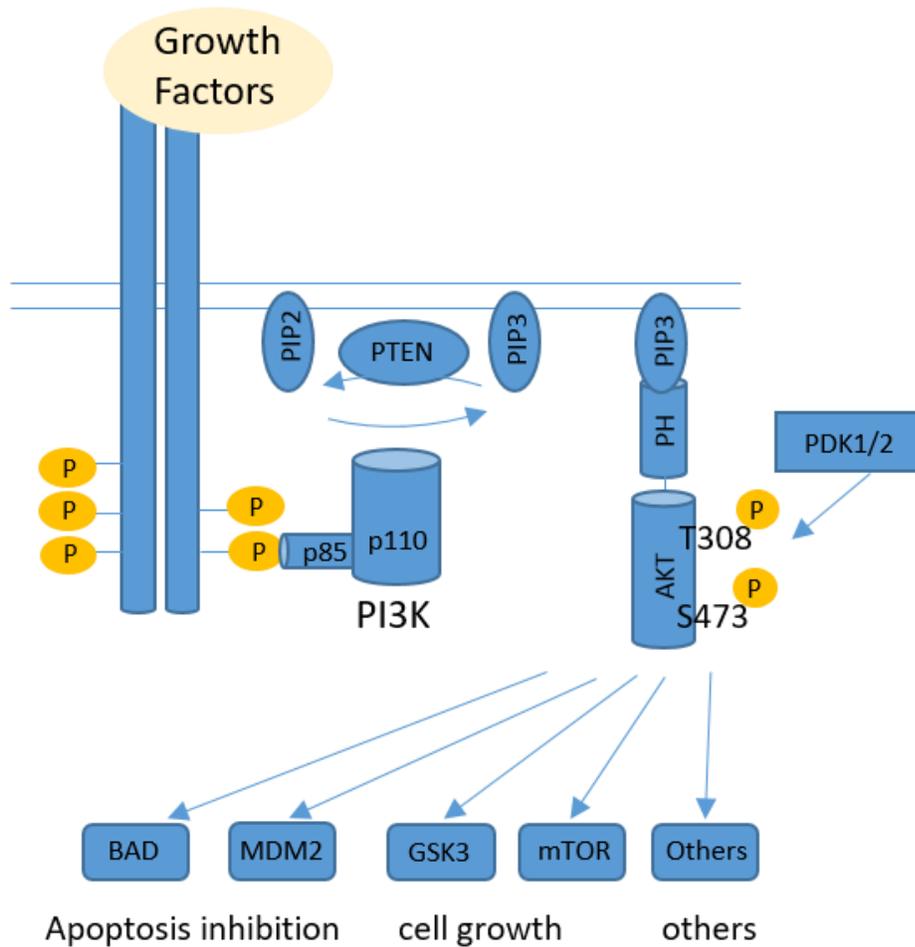


Figure 1.5 A simplified schematic summary of the PI3K/AKT signaling pathway

### 1.3.2 The PI3K/AKT signaling pathway in prostate cancer

Aberrant activation of the PI3K/AKT signaling pathway can be found in 40-70% of CRPC tumors (62,108). Interestingly, PI3K and AKT amplifications and mutations are rare in PCa while PTEN loss-of-function mutation and deletion were frequently observed, suggesting that

PTEN loss is the main mechanism of PI3K/AKT overactivation in PCa (100). Amplifications of the tyrosine kinase activities were also reported in PCa but not as dominant as PTEN loss (109). In addition, the PI3K/AKT signaling pathway has been reported to have reciprocal activation mechanisms with the AR signaling in PCa. Two groups have reported in 2011 that down-regulation of AR promotes PI3K/AKT activation in driving cancer cell survival and proliferation, meanwhile blocking the PI3K/AKT pathway upregulates the AR signaling pathway (110,111). As revealed from these studies, AR checks AKT activity at least in part via upregulating FKBP5 that suppresses the AKT phosphatase PHLPP. On the other hand, PI3K/AKT activation reduces AR transcriptional output via HER kinases. Because of these important roles of PI3K/AKT signaling pathway in promoting PCa progression, a significant effort has been made in designing small-molecules targeting this signaling pathway.

### **1.3.3 Small molecule therapeutics**

One of the first identified small molecule inhibitor for the PI3K/AKT pathway is Wortmannin (112–114). As a mold metabolite, it is a competitive pan-PI3K inhibitor (all forms of PI3K) by covalently binding to the ATP-binding site of p110. Therefore, this drug can be considered irreversibly blocking PI3K activities. Soon after, the first synthetic compound, LY294002, was discovered (115). LY294002 competitively inhibits PI3K by making a critical hydrogen bond with the p110 subunit in a reversible fashion. Since then, numerous drugs have been developed and can be categorized into 5 categories: 1) Pan PI3K inhibitors, 2) dual pan-PI3K and mTOR inhibitors, 3) isoform-selective PI3K inhibitors, 4) AKT-specific inhibitors, and 5) mTOR-specific inhibitors (116–118).

Because of the importance of PI3K/AKT signaling pathway in PCa, many clinical trials have been conducted in testing new small molecule drugs targeting this pathway despite the fact that no drugs have successfully entered the phase III clinical trials yet. The following sections will evaluate all PI3K/AKT inhibitor clinical trials either ongoing or completed after 2015 as listed on [clinicaltrials.gov](http://clinicaltrials.gov).

***Pan PI3K inhibitors:***

PX-866 failed a phase II clinical trial in recurrent or metastatic CRPC in 2015 (NCT01331083). BKM120 failed a phase II clinical trial in mCRPC patients in 2015 (NCT01385293) and now is being studied as a neoadjuvant therapy in high risk prostate cancer (NCT01695473).

***Dual pan-PI3K and mTOR inhibitors:***

GDC-0980 is current on phase Ib clinical trial in a three arm study that compares the efficacy of abiraterone plus GDC-0980, ipatasertib, of placebo in CRPC patients after docetaxel treatment (NCT01485861).

BEZ235 has completed a three-arm phase Ib trial along with BKM120 that compared the effects of abiraterone plus BEZ235, BKM120, and placebo in CRPC patients in 2015. No results have been released yet (NCT01634061).

***Isoform-selective PI3K inhibitors:***

GSK2636771, as a p110b selective inhibitor, has had a completed phase I/IIa trial in CRPC patients with PTEN loss as single treatment in 2015 and now being studied in a phase I clinical trial for combination treatment with enzalutamide (NCT01458067).

***AKT inhibitor:***

MK2206 (AKT allosteric inhibitor (119)), is on a phase I clinical trial in combination with hydroxychloroquine in CRPC patients (NCT01480154).

AZD5363 (AKT competitive inhibitor (120)), is on a phase II clinical trial in combination with enzalutamide in PTEN-loss CRPC patients (NCT02525068).

***mTOR inhibitor:***

AP23573 failed a phase II trial in taxane-resistant CRPC (NCT00110188).

Temsirolimus failed as monotherapy in chemorefractory CRPC men (121) and in combination with bicalutamide in CRPC patients (122) (NCT00887640).

RAD001 showed limited efficacy in combination with bicalutamide in CRPC patients phase II (123) (NCT00630344).

Since the above drugs are rapamycin analogues that only block the effects of mTOR on S6 kinase activity via FKBP12, but do not block the mTOR-EIF4E axis, mTOR ATP-binding site competitive inhibitors have been investigated due to their dual inhibitory effects:

MLN0128 is being studied in a phase II clinical trial in chemorefractory CRPC patients (NCT02091531).

CC-115 is being studied in a phase I clinical trial in combination with enzalutamide in CRPC patients (NCT02833883).

## **1.4 The transcription repressor REST**

### **1.4.1 The REST gene**

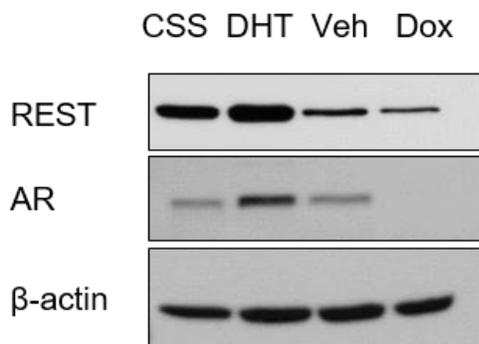
Repressor element-1 silencing transcription factor (REST), also known as neuron-restricted silencing factor (NRSF), is an important protein involved in embryonic development and neuronal-differentiation (124,125). Aberrant expression or function of REST has been associated with a range of disease such as Down's syndrome (126) and Huntington's disease (127). Located on chromosome 4q12, REST is highly expressed in almost all human cells except neuronal cells. It is a 1097 amino acid protein containing eight zinc fingers and two repressor domains (RD1 and RD2) that are located in the N- and C- terminals respectively (128). Within the cluster of zinc fingers, there is a DNA binding domain (DBD) that can bind to the 21-bp repressor element 1 (RE-1) normally located within the regulatory region of target genes (including numerous neurogenesis-related genes). The REST DBD can recognize two types of RE-1 motifs: canonical and non-canonical. The canonical RE-1 motif consists of two 10bp consensus sequences separated by one random nucleotide, whereas the non-canonical RE-1 motif has the two 10bp consensus sequences separated by variable length of nucleotides (129,130). REST normally has higher binding affinity to the canonical RE-1 motif (131). As a transcription repressor, REST binds the target DNA to recruit various cofactors such as mSIN3A and CoREST to its RD regions (132). Loss of REST allows de-repression of genes required for neural cell differentiation.

In addition to the predominant isoform of REST (isoform 1), there are three more truncated isoforms of this protein. Notably, REST4 (isoform 3) has a truncated DNA binding domain and no RD2; its expressions have been implicated in neuroblastoma and small-cell lung cancer (133).

The biological function of this isoform remains controversial but it may involve in repressing REST activities through its interactions with REST.

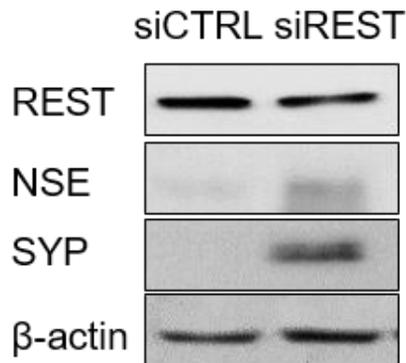
#### 1.4.2 REST in NEPC

With regard to cancer, REST plays paradoxical roles depending on the type of cells. It plays a role of tumor suppressor in human epithelial cells while acts as an oncogene in neuronal-related tumors as well as other non-epithelial cancers (134,135). In PCa, REST has not been implicated in PCa carcinogenesis or the development of castration resistance (136). However, loss of full-length REST protein expression was associated with NEPC progression both in clinical samples and animal models (136,137) (Figure 1.6). Knockdown of REST by siRNA also induced neuronal signatures in PCa cells that include NE markers such as SYP and NSE (Figure 1.7) (138). Mechanistically, ARPI has been reported to down-regulate REST, putatively through  $\beta$ -TRCP-mediated protein degradation (138).



**Figure 1.6 AR blockade and REST expression levels**

Parental LNCaP cells were cultured under CSS for 2 days followed by the addition of vehicle or 10 nM dihydrotestosterone (DHT) for 2 days. Doxycycline-inducible LNCaP(shAR) cells (139) were cultured in normal FBS medium followed by the treatment of either vehicle (Veh) or 2 nM doxycycline (Dox).



**Figure 1.7 REST knockdown and NE marker expressions**

LNCaP cells were transfected with siRNA against control or siRNA against REST. Cell lysates were collected and REST, NSE, SYP, and  $\beta$ -actin protein levels were measured by immunoblotting.

### 1.4.3 REST regulation

REST is regulated both at the mRNA and protein levels. At the mRNA level, REST is regulated by the serine/arginine repetitive matrix4 (SRRM4) via alternative splicing (87,140). SRRM4 can splice REST into REST4 by recognizing the UGC motif in REST intron 3 and incorporating a neural-specific exon (exon N) between exons 3 and 4 of REST (87). At the protein level, REST is tightly regulated by ubiquitination and deubiquitinating processes (141). SCF<sup>TRCP1</sup> and SCF<sup>TRCP2</sup> (SKP1-CUL1-F-box protein) are two out of sixty-nine well characterized F-box E3 ligases and has been shown to recognize REST for ubiquitination (142).  $\beta$ -TRCP1 and  $\beta$ -TRCP2 can both recognize the degron regions of REST upon phosphorylation (phosphodegron) (142). The phosphodegron regions of REST were described at S1024, S1027, S1030 (142) and S1013 (143). Although REST phosphorylation at its degron regions has been described as a major mechanism for REST protein stability regulation, there are only a few signaling pathways

reported involving REST phosphorylation. Kaneko and colleagues reported that Casein kinase 1 can directly phosphorylate REST degrons in HeLA cells (143). In addition, ERK1/2 kinases were reported to phosphorylate a proline-directed phosphodegron motif at S861/864, which in turn recruits Pin1 and  $\beta$ -TRCP (144). In the same study, the authors also described that the C-terminal domain small phosphatase 1 (CTDSP1) dephosphorylates S861/864 to stabilize REST. Similarly, HAUSP (the herpesvirus-associated ubiquitin-specific protease, also known as USP7) has been shown to dephosphorylate the phosphodegron regions of REST(145).

## **1.5 Hypotheses and aims**

Loss of REST is a key factor for prostate adenocarcinoma cells to gain NE phenotypes under various conditions, including AR inhibition. Because of the reciprocal activation mechanism between PI3K/AKT and AR signaling pathways, we originally hypothesized that PI3K/AKT inhibition would increase AR function in PTEN-deficient cells, thereby stabilizing REST expression to prevent neuroendocrine differentiation. However, preliminary results revealed that treatment of the pan-PI3K inhibitor LY294002 reduced REST protein expression. These observations raised interesting questions on the potential PI3K/AKT-REST-NE differentiation axis in PCa. Therefore, two main hypotheses for further investigations were proposed: (1) PI3K/AKT inhibition can reduce REST expression and NE differentiation in AdPC cells and (2) the combination treatment of PI3K/AKT inhibition and ARPI can accelerate the development of NEPC from AdPC cells.

Main Objective: To study the potentially unexpected implications of PI3K/AKT inhibition on the NE differentiation in AdPC cells either as single treatment or combination treatment, with a special focus on the interaction between PI3K/AKT inhibition and the NE-suppressor REST.

*Aim I:* To confirm whether PI3K/AKT inhibition confers PCa cells with NEPC phenotype through REST-mediated pathway using in vitro cell models

*Aim II:* To determine the mechanism by which PI3K/AKT inhibition reduces REST expression

*Aim III:* To investigate the impacts of combination treatment of ARPI and PI3K/AKT inhibitor on NEPC development

## **Chapter 2: Materials and methods**

### **2.1 Materials**

R1881, DHT, LY294002, BKM120, AZD5363, MK2206, Rapamycin, MG132, and cyclohexamide were purchased from Cedarlane (Burlington, ON, Canada). Other chemicals, solvents, and solutions were obtained from Sigma-Aldrich (Oakville, ON, Canada).

### **2.2 Cell lines materials**

Human PCa cell lines LNCaP and PC3 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The LNCaP95 cell line is androgen-independent as it was derived from LNCaP cells cultured under long-term androgen depletion conditions in 1995. LNCaP95 cells were generously gifted from Dr. Alan Meeker of Johns Hopkins University. The LNCaP(shAR) cell line is a stable cell line derived from LNCaP cells with the exogenous introduction of doxycycline-inducible shRNA targeting AR expression (139). It was provided by Dr. Paul Rennie (Vancouver Prostate Centre). LNCaP and LNCaP(shAR) cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS). PC3 cells were cultured in DMEM medium with 10% FBS. LNCaP95 cells were cultured in phenol-free RPMI-1640 medium with 10% charcoal-stripped serum (CSS) (Hyclone). All cell lines were authenticated by short tandem repeat assays or RNA sequencing assays.

### **2.3 Transfections**

Cells were transfected with control siRNA (Dharmacon) and siRNA targeting AKT1/2 (cat#.sc-43609, Santa Cruz) using Lipofectamine 3000 according to the manufacturer's protocol.

Transient DNA plasmid transfections also used Lipofectamine 3000. Detailed information on

plasmid DNA and siRNA is listed in table 2.1. All plasmids and mutations were verified by DNA sequencing at the CMMT/BCCHR DNA Sequencing Core Facility.

|                               |                             |                |
|-------------------------------|-----------------------------|----------------|
| Control siRNA                 | Dharmacon D-001210-01-20    | D-001210-01-20 |
| AKT1/2 siRNA                  | Santa Cruz Biotech sc-43609 | sc-43609       |
| Myc-tagged REST-FL            | Gerald Thiel Group          |                |
| Myc-tagged REST4              | Gerald Thiel Group          |                |
| HA-tagged ubiquitin           |                             |                |
| Flag-tagged REST-FL           | Stephen Elledge Group       |                |
| Flag-tagged REST-FL (S1024A)  | Stephen Elledge Group       |                |
| Flag-tagged REST-FL (S1027A)  | Stephen Elledge Group       |                |
| Flag-tagged REST-FL (S1030A)  | Stephen Elledge Group       |                |
| Flag-tagged REST-FL (Tri-S/A) | Stephen Elledge Group       |                |

**Table 2.1 SiRNA and plasmid information**

## **2.4 Real-Time PCR**

RNA extraction: Total RNA of cells was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. Specifically, cell medium were vacuumed and the cells were washed twice with phosphate-buffer saline (PBS) before 1 ml trizol reagent was added to cells in 60 mm or 100 mm plates. After lysing cells by pipetting the cells and trizol mixture, the cell lysates were transferred to a 1.5 ml Eppendorf tube and allowed to homogenize for 5 minutes at room

temperature. Chloroform (200  $\mu$ l) was then added to the tube and shaken vigorously by hand for 15 seconds. After incubation at room temperature for 3 minutes, the cell lysates were centrifuged at 12,000g for 15 minutes at 4°C. The upper aqueous phase was then transferred to a new tube without disturbing the lower layers. This chloroform extraction step was repeated one more time. Afterwards, 500  $\mu$ l of 100% isopropanol was added to the aqueous phase in the new Eppendorf tube, incubated for 10 minutes at room temperature, and centrifuged at 12,000g for 10 minutes. After centrifuge, the supernatant was removed, the RNA pellet was washed by 1 ml of 70% ethanol. After the ethanol was removed, RNA pellet was allowed to air dry for 5-10 minutes until becoming transparent. 20-50  $\mu$ l RNase-free water was added to each Eppendorf tube to dissolve RNA pellets, followed by incubation for 10 minutes at 65°C. The RNA concentration and purity ( $260/280 > 1.9$  and  $260/230 > 2.0$ ) was measured by NanoDrop2000.

Reverse-transcription: 2  $\mu$ g of total RNA was pre-treated with at the 10  $\mu$ l volume a DNase solution containing DNase I (Invitrogen) at room temperature for 15 minutes before DNase inactivation by treatment of 1  $\mu$ l EDTA. Then, 1  $\mu$ l of 10 mM dNTP mix and 1  $\mu$ l of 10 uM Random Hexamer were added to the mixture solution and incubated at 65°C for 5 minutes. The 7  $\mu$ l of master mix containing 4  $\mu$ L of 5X First Strand Buffer, 2  $\mu$ L 0.1 M dithiothreitol (DTT), 0.5  $\mu$ L SuperScript II Reverse Transcriptase, and 0.5  $\mu$ L double-distilled water (ddH<sub>2</sub>O) was then added to the mixture for a randomprimed reverse transcription to create a cDNA pool.

Real-time PCR: real-time PCR was performed using the ABI PRISM 7900 HT system (Applied Biosystems, Burlington, Canada) with 5 ng of cDNA, 1  $\mu$ M of each primer pair and SYBR Green PCR master mix (Roche) in 96- or 384-well plates. Default conditions of the 7900 HT

Software was used including 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15-sec 95°C and 1-min 60°C. Relative quantification of gene transcription was measured by comparing the Ct values of gene transcriptions of interest to the housekeeping gene GAPDH. All real-time PCR experiments were carried out with at least three technical replicates and independent cDNA syntheses. Information of the primers is listed in Table 2.3.

| Primer Name | Sequence                              |
|-------------|---------------------------------------|
| GAPDH F     | 5'- GGA CCT GAC CTG CCG TCT AGA A -3' |
| GAPDH R     | 5'- GGT GTC GCT GTT GAA GTC AGA G -3' |
| AKT1F       | 5'- TGG ACT ACC TGC ACT CGG AGA A -3' |
| AKT1R       | 5'- GTG CCG CAA AAG GTC TTC ATG G -3' |
| REST F      | 5'- GCC GCA CCT CAG CTT ATT ATG-3'    |
| REST R      | 5'- CCG GCA TCA GTT CTG CCA T-3'      |
| CHGA F      | 5'- TAA AGG GGA TAC CGA GGT GAT G -3' |
| CHGA R      | 5'- TCG GAG TGT CTC AAA ACA TTC C -3' |
| CHGB F      | 5'- CGA GGG GAA GAT AGC AGT GAA -3'   |
| CHGB R      | 5'- CAG CAT GTG TTT CCG ATC TGG -3'   |

|          |  |
|----------|--|
| ENO2 F   | 5'- CCG GGA ACT CAG ACC TCA TC -3'     |
| ENO2 R   | 5'- CTC TGC ACC TAG TCG CAT GG -3'     |
| SYP F    | 5'- TTA GTT GGG GAC TAC TCC TCG -3'    |
| SYP R    | 5'- GGC CCT TTG TTA TTC TCT CGG TA -3' |
| SCG3 F   | 5'- GTC TTC ATC AAC TAG ACG GGA CT -3' |
| SCG3 R   | 5'- ACA ATC TTG TCA AAC ACG GCT C -3'  |
| KCNH6 F  | 5'- GTC GCT CCC CAA AAC ACT TAC -3'    |
| KCNH6 R  | 5'- CGA AGA GTT CGC AGA AGC C -3'      |
| SYT4 F   | 5'- TCCCGAGAGAGGAATTAGAACTT -3'        |
| SYT4 R   | 5'- TCCCGAGAGAGGAATTAGAACTT -3'        |
| NKX3.1 F | 5'- CCC ACA CTC AGG TGA TCG AG -3'     |
| NKX3.1 R | 5'- GAG CTG CTT TCG CTT AGT CTT -3'    |
| REST4 F  | 5'- GCG TAC TCA TTC AGT GGG GTA T -3'  |
| REST4 R  | 5'- GAT TAG AGG CCA CAT AAC TGC AC -3' |

**Table 2.2 Primer Information**

## 2.5 Western blot

After washing and centrifugation, pelleted cells dispersed into the lysis buffer (50 mM Tris pH8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS) with proteinase and phosphatase inhibitors (Roche) in 1.5 ml Eppendorf tubes. The cell lysates were briefly sonicated and the protein lysates were extracted from the supernatant layer after centrifuging at 14,000g for 10 min at 4°C. After collecting the protein lysates, protein concentrations were measured with the Pierce BCA Protein Assay Kit (Thermo Fisher, Mississauga, Canada) according to the manufacturer's protocol. Approximately equal weight of total protein (30-70 µg) were then mixed with and denatured by sodium dodecyl sulfate (SDS) and boiled at 95°C for 5 min. Prepared protein samples were separated by electrophoresis on an 6%-10% SDS polyacrylamide gel, then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, USA) in transfer buffer (25 mM Tris-HCl, 250 mM glycine, 0.1% SDS, pH 8.3) either with the semi-dry transfer method (40-60 min at 25 V at room temperature with the The Trans-Blot® SD Semi-Dry Transfer Cell) or the wet-transfer method (240 min at 280 mA at 4°C). Upon the completion of protein transfer to the PVDF membranes, they were blocked with the Odyssey Blocking Buffer, followed by immunoblotting with specific primary antibodies (1:500 to 1:2000 dilution) listed in table 2.4 at 4°C overnight. Then, after washing the membranes with the tris-buffered saline plus TWEEN-20 (TBST) buffer 3 times with 10 min each, secondary antibodies (anti-rabbit/mouse/goat IgG-HRP antibodies (Santa Cruz, Dallas, USA) (dilution 1:10,000) were added to the membranes for incubation at room temperature for 1 hour. After three additional 10 min TBST wash, the membranes were probed by Pierce ECL Western Blotting Substrate (Thermo Fisher), exposed to autoradiography films (Genesee Scientific, San Diego, US), and developed/fixed by a film processor (EL-RAD, Vancouver, Canada). Experiments were repeated

in three independent experiments and one representative result was shown. Image J software was used to perform densitometry analyses of protein bands.

| Antibody   | Description | Cat No.   | Supplier           |
|------------|-------------|-----------|--------------------|
| Flag       | M5          | F4042     | Sigma Aldrich      |
| HA         |             | Ab137838  | Abcam              |
| Myc-tag    |             | Ab9106    | Abcam              |
| Beta-actin | C11         | Sc-1615   | Santa Cruz Biotech |
| AR         | N-20        | Sc-816    | Santa Cruz Biotech |
| REST       | EPR2346Y    | Ab75785   | Abcam              |
| REST       |             | 07-579    | Millipore          |
| pAKT       | Ser473      | 9291      | Cell Signaling     |
| AKT        |             | 9272      | Cell Signaling     |
| SYP        | D4          | Sc-17750  | Santa Cruz Biotech |
| NSE        |             | MAB324    | Millipore          |
| Beta-TRCP  | C6          | Sc-390629 | Santa Cruz Biotech |
| HAUSP      | H-200       | Sc-30164  | Santa Cruz Biotech |
| SCG3       | C19         | Sc-1492   | Santa Cruz Biotech |

**Table 2.3 Antibody Information**

## **2.6 Ubiquitination assays**

*In vivo* ubiquitination assays were performed as previously described (146) with a modification that includes the application of 1% SDS to induce a denatured condition. Cells were transfected with plasmids encoding ubiquitin plus REST and its mutants (S1024A, S1027A, S1030A and S1024/1027/1030A) from Drs. Stephen Elledge, Gail Mandel, and Gerald Thiel. Twenty-four hours post-transfection, cells were treated with 8 $\mu$ M of MG132 with either vehicle or 50 $\mu$ M of LY294002 for 8 hours. Whole cell lysates were extracted using an NETN buffer (50mM of Tris pH8.0, 150mM of NaCl, 1% NP40, 1mM EDTA) plus phosphatase inhibitors (Roche). Lysates were added with 1% SDS and heated at 95 °C for 5 minutes. Protein extracts were then diluted 10 times before being subjected to immunoprecipitation of either REST or ubiquitin. Precipitated proteins were then immunoblotted to detect protein of interest.

## **2.7 Transcriptomic sequencing**

LNCaP cells that were treated with control, AKTi, ARi, or AKTi+ARi. RNA was extracted using the mirVana RNA Isolation Kit (Ambion, Burlington, Canada) according to the manufacturer's protocol. Two independently repeated experiments were performed for each experimental condition. The quantity/purity and quality of the RNA samples were respectively assessed by Nanodrop 2000 and 2100 Bioanalyzer (Caliper Technologies Corp., Canada) before being sent for AmpliSeq Transcriptome Sequencing. Library preparation, sequencing, and primary analyses were performed by the UBC-DMCBH Next Generation Sequencing Centre following the protocol described by Li *et al* (147). In summary, cDNA was synthesized from 100 ng of total RNA using the SuperScript® VILO™ cDNA Synthesis kit and amplified with Ion AmpliSeq™ technology. Barcoded cDNA libraries were diluted to 100pM, equally pooled, and amplified on

Ion Torren OneTouch2 instrument using emulsion PCR. Then, templated libraries were subjected for sequencing of >20,000 RefSeq transcripts using the Ion Torrent Proton™ sequencing system. Primary analysis and normalization were performed using the AmpliSeq RNA plugin available through the Ion Torrent™ suite Software.

Following the primary analysis, Differential Gene Analysis (DEG) and adjusted p-value was performed using the R/Bioconductor package DESeq2 with Ampliseq raw counts. The DESeq2 package is a specially designed algorithm that estimates variance-mean dependence in count data from sequencing assays and test for DEG based on a model using the negative binomial distribution that takes into account of gene-gene interactions (148). Genes with adjusted p-value < 0.1 and fold change > 1.5 were filtered and ranked.

## **2.8 Public dataset and data analyses**

The NEPC patient transcriptomic data published by Beltran et al. 2016 was obtained with permission from their group (50). This set of data contains RNA-seq data from 15 NEPC patient tumors and 30 AdPC patient tumors. These transcriptomic data were obtained from RNA-seq by the HiSeq 2500 to generate  $2 \times 75$ -bp paired-end reads. After procuring the normalized expression levels (fragments per kilobase of transcript per million mapped reads or FPKM) of 20,345 genes, DEG analyses were performed using the two-tailed unpaired T-test after transforming the FPKMs via  $\log_2(\text{FPKM}+1)$ . Benjamini-Hochberg (BH; FDR) multiple hypothesis corrections were performed. Genes with  $\text{FDR} < 0.1$  were ranked according to fold change.

Another public data set was obtained from the GEO database. The GSE51461 dataset contains microarray data from LNCaP cells treated with hormonal depletion or REST siRNA knockdown. Specifically, mRNA expressions of these LNCaP cells under various treatment conditions were probed with the Agilent-028004 SurePrint G3 Human GE 8 \* 60K Microarray chips. Obtained data were normalized expression levels. DEG analyses were performed using the two-tailed unpaired T-test followed by Benjamini-Hochberg (BH; FDR) multiple hypothesis corrections. Genes with  $FDR < 0.1$  were ranked according to fold change.

## **2.9 Gene Set Enrichment Analysis**

Gene Set Enrichment Analysis (GSEA) is a computational analytical method for determining any correlations of a set of gene expression data to a certain groups of genes called gene set (149). It is an open-access software provided by the Broad Institute. In this thesis, a gene set for NEPC patients was manually curated from the Beltran et al. publication (50) by filtering the top 500 upregulated genes with  $padj < 0.05$  according to RNA-seq profiling in 15 NEPC and 30 AdPC samples. A gene set for LNCaP cells with REST knockdown was manually curated from the Svensson publication (138) by filtering the top 100 upregulated genes with  $padj < 0.1$  according to microarray-profiling in 3 REST knockdown and 2 control samples. In addition, three gene sets of top 100 upregulated genes with  $padj < 0.1$  from all Ampliseq-sequenced treatment groups from this project were manually curated. These curated gene sets were added to the default Hallmarks gene set group from the official MSigDB Collections <http://software.broadinstitute.org/gsea/msigdb/collections.jsp>. By comparing the expression data of interest to this gene set group, normalized enrichment score (NES) and FDR were

calculated automatically via predefined algorithms. In general, correlations are considered strong with high NES and lower FDR scores (FDR is recommended to be at least  $< 0.25$ ).

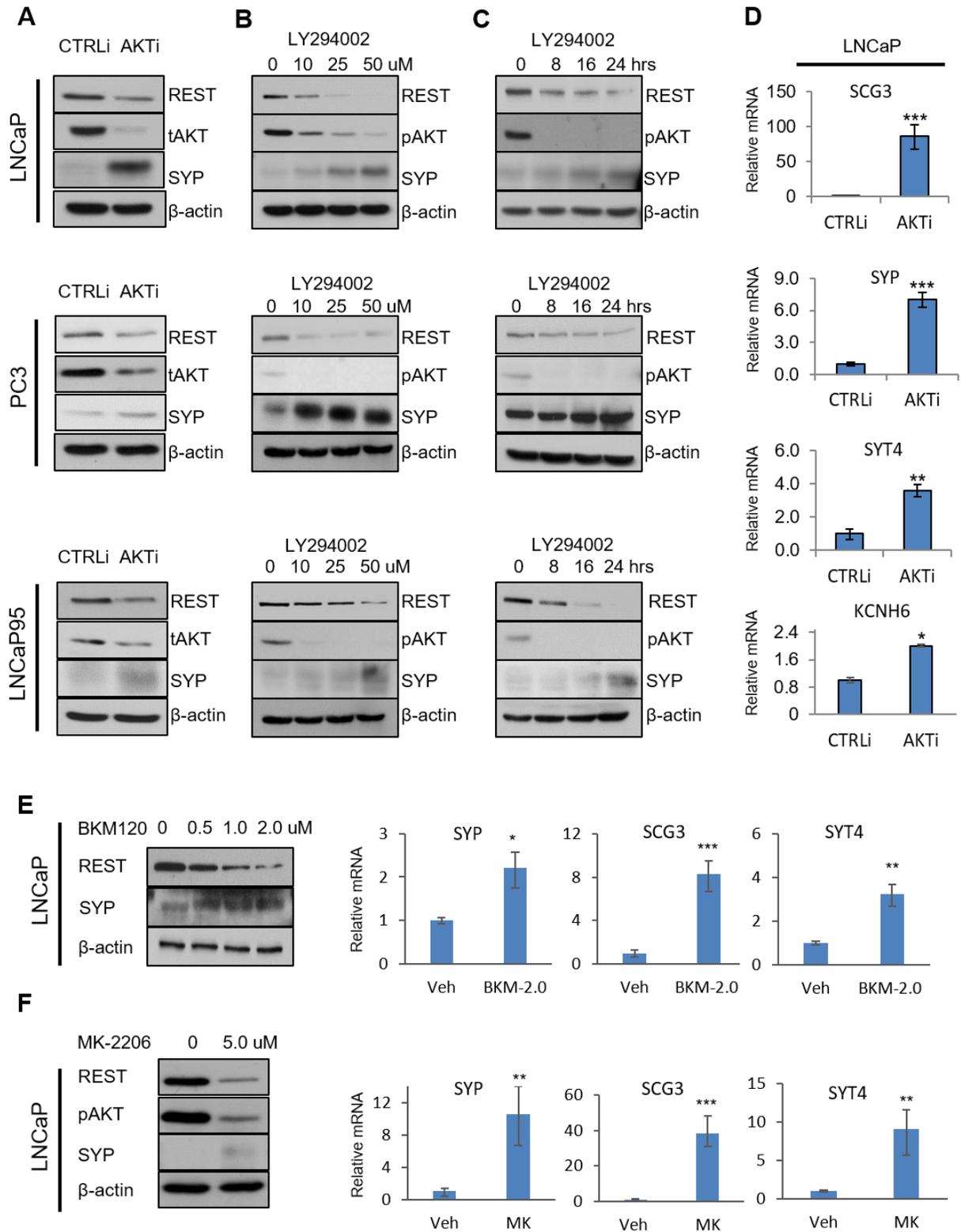
## **2.10 Statistics**

Statistical analyses were carried out using R (version 3.3.2) for parametric (2-tailed student's paired or unpaired t-test, and one-way ANOVA test followed by Tukey' post-hoc test) with statistical significance set at  $p < 0.05$  as \*,  $p < 0.01$  as \*\* and  $p < 0.001$  as \*\*\*, and non-parametric (chi-square test) statistics.

## Chapter 3: Results

### 3.1 PI3K/AKT inhibition on REST and NE marker expressions

Human prostate cancer cell lines such as LNCaP, PC3, and LNCaP 95 cells are PTEN-deficient and have overactive PI3K/AKT signaling as is frequently observed in metastatic PCa. To test whether AKT inhibition can affect REST expression and induce an NE-phenotype in PCa cells, we transiently transfected each of them with control and AKT siRNA. AKT depletion in all cell lines resulted in downregulation of REST protein and upregulation of the NE marker synaptophysin (SYP) (Fig. 3.1A). Likewise, when LNCaP, PC3, and LNCaP95 cells were treated with the PI3K inhibitor, LY294002, expression of REST was suppressed while SYP protein levels increased in both time- and dose-dependent manners (Fig. 3.1B & C). Indeed, AKT depletion by siRNA induced a wide range of NE markers such as SYP, SCG3, SYT4, and KCNH6 ( $p < 0.01$ ) (Fig. 3.1D). Similarly, the pan-PI3K inhibitor BKM120 as well as the AKT allosteric inhibitor MK-2206 also reduced REST and increased NE expressions in LNCaP cells (Fig. 3.1E & F). Collectively, our results show that PI3K/AKT inhibition can downregulate REST protein expression and induce NE markers in PTEN-deficient PCa cells.



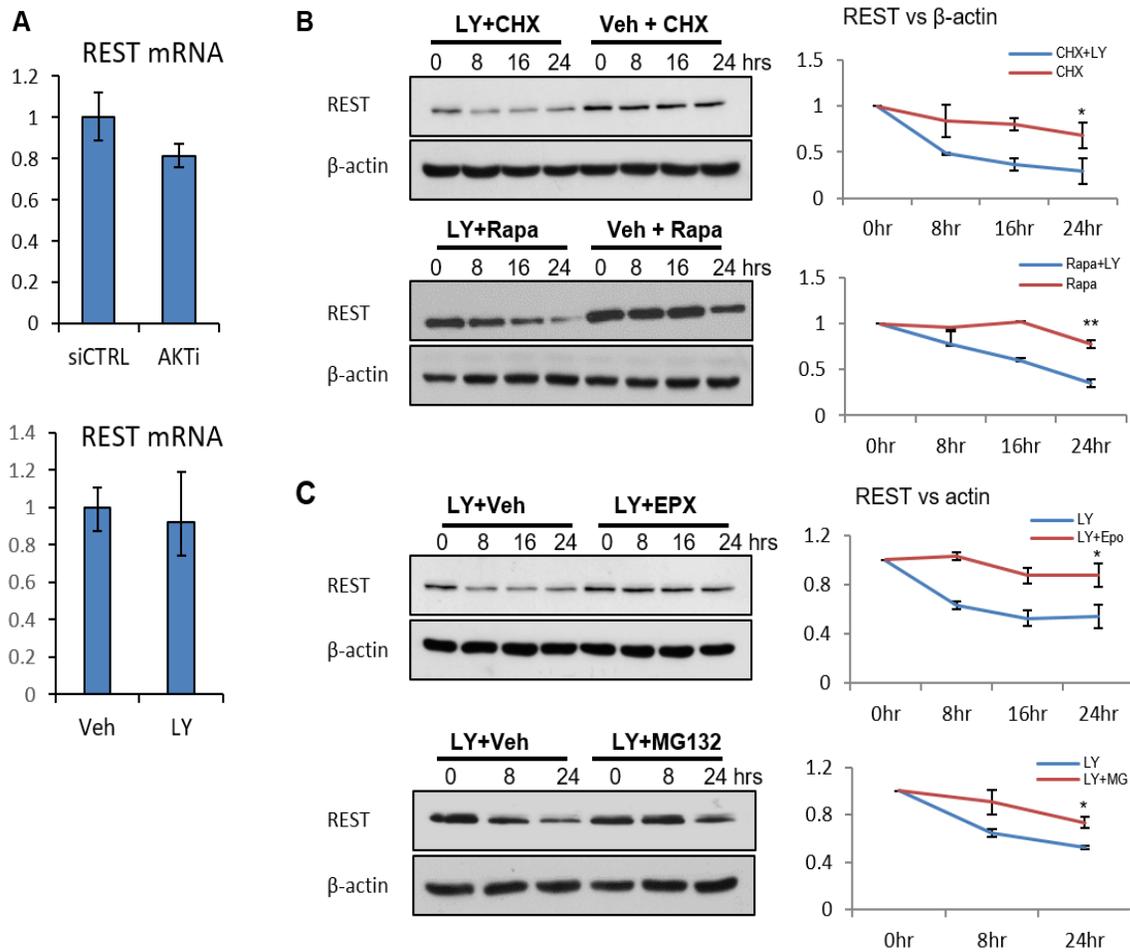
### **Figure 3.1 PI3K and AKT inhibition reduce REST and increase NE marker expressions**

(A) LNCaP, PC3, and LNCaP95 cells were transfected with control siRNA (CTRLi) or siRNA against AKT (AKTi) for 48 hours. Protein levels of REST, total AKT (tAKT), SYP, and  $\beta$ -actin were measured by immunoblotting. (B) LNCaP and LNCaP95 cells were treated with 0, 10, 25, 50  $\mu$ M LY294002 for 24 hours. PC3 cells were treated with 0, 50, 100, 150  $\mu$ M LY294002 for 24 hours. (C) LNCaP and LNCaP95 cells were treated with 50  $\mu$ M LY294002 and PC3 cells were treated with 100  $\mu$ M LY294002 for 0, 8, 16, 24 hours. Cell lysates were immunoblotted with antibodies against REST, pAKT, SYP, and  $\beta$ -actin. (D) Relative mRNA levels of neuroendocrine markers from LNCaP cells treated with CTRLi or AKTi for 48 hours were measured by real-time PCR. (E) Left: LNCaP cells were treated with 0, 0.5, 1.0, 2.0  $\mu$ M BKM120 for 24 hours and collected cell lysates were immunoblotted with REST, SYP, and  $\beta$ -actin. Right: relative mRNA levels of neuroendocrine markers from LNCaP cells treated with vehicle (Veh) or 2.0  $\mu$ M BKM120 for 24 hours were measured by real-time PCR. (F) Left: LNCaP cells were treated with 0, 5.0  $\mu$ M MK-2206 for 24 hours and collected cell lysates were immunoblotted with REST, p-AKT, SYP, and  $\beta$ -actin. Right: relative mRNA levels of neuroendocrine markers from LNCaP cells treated with vehicle (Veh) or 5.0  $\mu$ M MK-2206 for 24 hours were measured by real-time PCR. (E) Left: LNCaP cells were treated with 0, 0.5, 1.0, 2.0  $\mu$ M BKM120 for 24 hours and collected cell lysates were immunoblotted with REST, SYP, and  $\beta$ -actin. Right: relative mRNA levels of neuroendocrine markers from LNCaP cells treated with vehicle (Veh) or 2.0  $\mu$ M BKM120 for 24 hours were measured by real-time PCR. (F) Left: LNCaP cells were treated with 0, 5.0  $\mu$ M MK-2206 for 24 hours and collected cell lysates were immunoblotted with REST, p-AKT, SYP, and  $\beta$ -actin. Right: relative mRNA levels of neuroendocrine markers from LNCaP cells treated with vehicle (Veh) or 5.0  $\mu$ M MK-2206 for 24 hours were measured by real-time PCR. Statistical analyses were performed by paired student's t-test with  $p < 0.05$  as \*,  $p < 0.01$  as \*\* and  $p < 0.001$  as \*\*\*.

### **3.2 PI3K/AKT inhibition on REST protein stability**

Although REST protein levels were reduced by AKT knockdown or inhibition, mRNA levels for REST were unchanged, suggesting that suppression of REST expression by PI3K/AKT inhibition is at the post-transcriptional level (Fig. 3.2A). While treatment of LNCaP cells with

translation inhibitors including cycloheximide (CHX) or rapamycin (Rapa) (150) for up to 24h did not significantly reduce REST protein levels, the addition of LY294002 did accelerate REST protein reduction, indicating that the effect of PI3K/AKT blockade is likely at the post-translational level (Fig. 3.2B). To test whether REST protein reduction by PI3K/AKT inhibition is mediated through the proteasome pathway, LNCaP cells were treated with proteasome inhibitors including epoxomicin (EPX) and MG132 (Fig. 3.2C). Additions of these proteasome inhibitors reversed LY294002-induced REST reduction. Together, these results suggest that PI3K/AKT inhibition affects REST protein stability via a proteasome-mediated pathway.



### Figure 3.2 PI3K/AKT inhibition affects REST protein stability

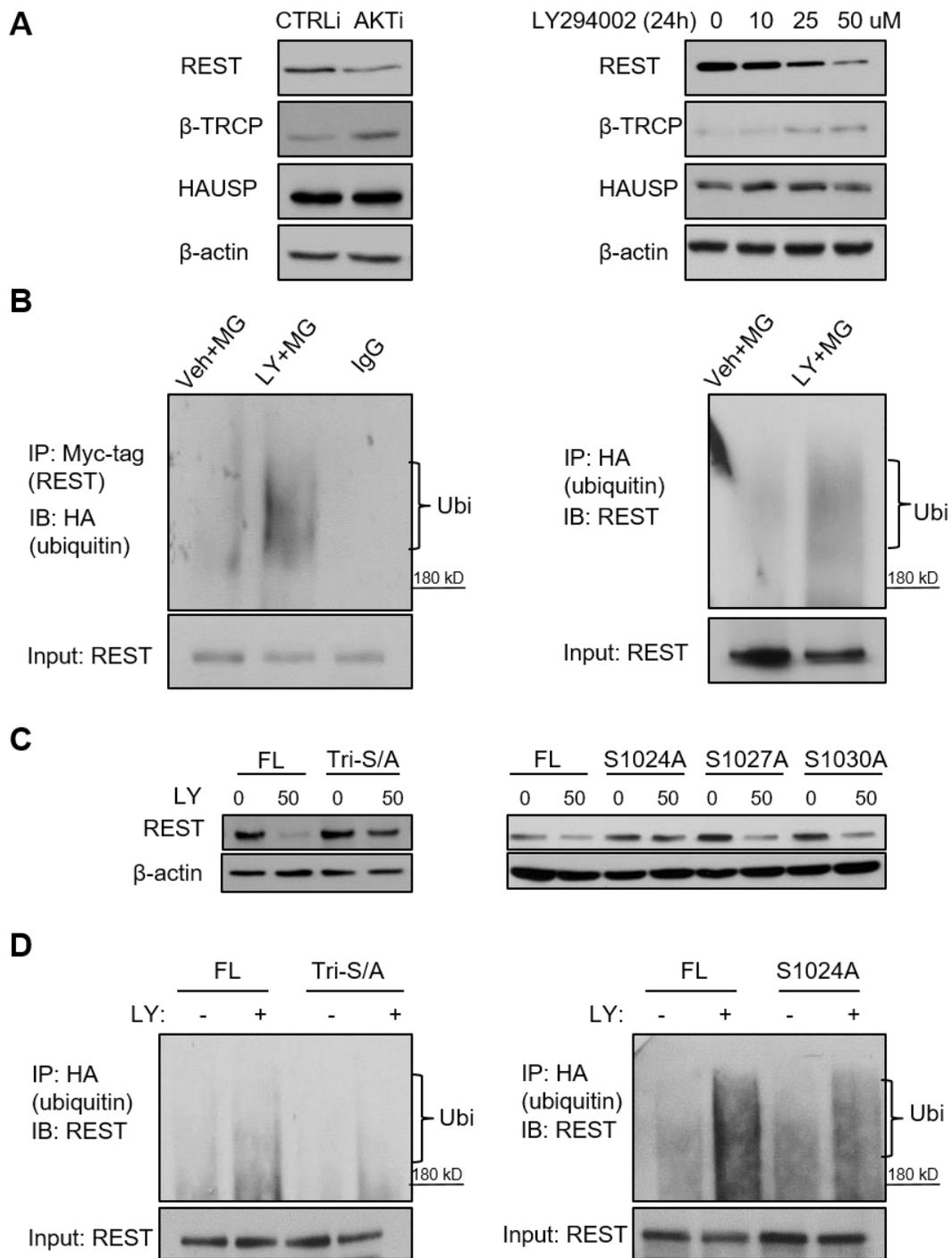
(A) LNCaP cells were transfected with CTRLi or AKTi for 48 hours (top) and vehicle (Veh) or 50  $\mu$ M LY294002 (LY) for 24 hours (bottom). Relative REST mRNA levels were measured by real-time PCR. (B) LNCaP cells were treated with 5  $\mu$ g/ $\mu$ l cyclohexamide (CHX) or 200 nM rapamycin (Rapa) in the condition of vehicle or LY294002 for 0, 8, 16, 24 hours. (C) LNCaP cells were treated with 50  $\mu$ M LY294002 in the condition of vehicle or 100 nM epoxomicin (EPX) for 0, 8, 16, 24 hours. LNCaP cells were also treated with 50  $\mu$ M LY294002 plus vehicle or 8  $\mu$ M MG132 for 0, 8, 16 hours. Cell lysates were immunoblotted with antibodies against REST and  $\beta$ -actin. Experiments were repeated at least three times and one set of the representative blots was shown. Densitometry analyses of REST to  $\beta$ -actin ratios were performed by the Image J software and plotted as mean+SEM. Statistical analyses were performed by one-way ANOVA followed by *Tukey's* post-hoc test with  $p < 0.05$  as \* and  $p < 0.01$  as \*\*.

### 3.3 PI3K/AKT inhibition on REST protein ubiquitination

$\beta$ -TRCP and HAUSP are the most well-described E3-ubiquitin ligase and deubiquitinase that determines REST protein stability (141). Western blot results showed that AKT siRNA and LY294002 both increased  $\beta$ -TRCP expression, but did not affect HAUSP protein levels (Fig. 3.3A). These results suggest that increased  $\beta$ -TRCP E3-ligase expression upon PI3K/AKT inhibition may induce REST ubiquitination and subsequent protein degradation. To test this hypothesis, we transfected LNCaP cells with vectors encoding myc-tagged REST and HA-tagged ubiquitin and treated the cells with MG132 and LY294002. *In vitro* ubiquitination assays showed that REST ubiquitination was increased by LY294002 (Fig. 3.3B). To test whether phosphorylation of REST regulates its protein degradation by PI3K/AKT inhibition, we transfected LNCaP cells with vectors encoding wild type REST or a mutated REST cDNA (tri-serine mutation and single mutations at serine 1024, 2017 and 1030) (Fig. 3.3C). These mutations suppressed the ability of  $\beta$ -TRCP to ubiquitinate REST protein (142).

Immunoblotting showed that REST protein degradation was largely rescued by the triple

mutation and was partially rescued by the S1024A mutation. *In vitro* ubiquitination assays further confirmed that LY294002-mediated REST ubiquitination was reduced by the REST triple mutation, and to a lesser extent by the REST S1024A single mutation (Fig. 3.3D). These findings support the idea that reduction of REST by PI3K/AKT inhibition require phosphorylations within the 1024-1030 region, followed by  $\beta$ -TRCP mediated REST ubiquitination and protein degradation.



### **Figure 3.3 REST protein ubiquitination at the degron region**

(A) LNCaP cells were transfected with CTRLi or AKTi for 48 hours (left) and treated with 0, 10, 25, 50 uM LY294002 for 24 hours. Cell lysates were collected and REST,  $\beta$ -TRCP, HAUSP and  $\beta$ -actin protein levels were measured by immunoblotting. (B) On the left: LNCaP cells were transfected with myc-tagged REST and HA-tagged ubiquitin for 48 hours followed by 8 uM MG132 plus vehicle or 50uM LY294002 treatment for 8 hours. Cell lysates were immunoprecipitated with the myc-tag antibody followed by immunoblotting of HA-tags for ubiquitinated-REST detection. On the right: LNCaP cells were transfected with HA-tagged ubiquitin for 48 hours followed by the 8 uM MG132 plus vehicle or LY294002 treatment for 8 hours. Cell lysates were immunoprecipitated with the HA-tag antibody and immunoblotted with the anti-REST antibody. (C) LNCaP cells were transfected with the flag-tagged full-length REST (FL) or the flag-tagged REST with the S1024/1027/1020A triple mutation (Tri-S/A) for 24 hours, then treated in the condition of vehicle or 50 uM LY294002 for 24 hours (left). LNCaP cells were also transfected with REST (FL) or REST mutant (either S1024A, S1027A, or S1030A) for 24 hours followed by the treatment of vehicle or 50 uM LY294002 for 24 hours (right). Cell lysates were immunoblotted with antibodies against REST and  $\beta$ -actin. (D) LNCaP cells transfected with full-length REST (FL) or REST (Tri-S/A) (left) and full-length REST (FL) or REST (S1024A) (right) for 48 hours followed by treatment of 8 uM MG132 plus 50 uM LY294002 or vehicle for 8 hours. Cell lysates were immunoprecipitated with the HA-tag antibody and immunoblotted with the REST antibody.

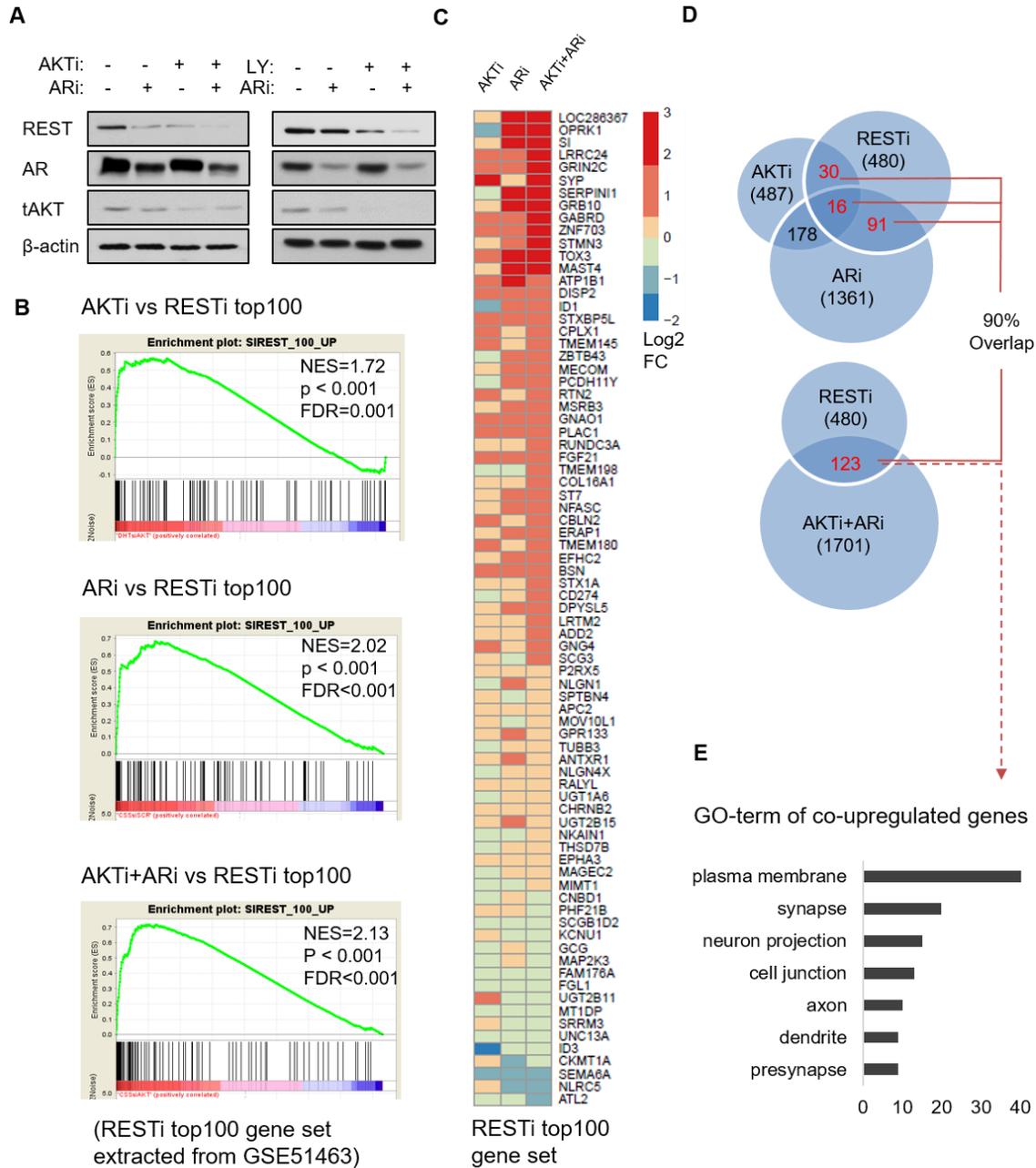
### **3.4 Co-inhibition of AR and PI3K/AKT pathway on REST**

Previous studies have shown that REST protein expression can be reduced by AR inhibition (87,136,138) and we confirmed this in LNCaP cells (Fig. 1.7). As we have shown that PI3K/AKT inhibition can reduce REST via protein degradation, we tested the effects of combination AR/AKT inhibitory treatment. LNCaP cells were transfected with AKT siRNA (AKTi), cultured in androgen depletion condition as in phenol red-free RPMI medium containing 5% CSS (ARi), or AKTi+ARi. Western blots showed that while each treatment separately suppressed REST protein expression, combination treatment resulted in a much more effective

depletion of REST protein (Fig. 3.4A). Combined ARi treatment with LY294002 had the same effect.

We then profiled the transcriptomes of LNCaP cells treated with vehicle, AKTi, ARi or AKTi+Ari using the Ampliseq Transcriptome Analysis (147) as described in Materials and methods. Comparison of the gene profiles of the treatment groups to the transcriptome of LNCaP cells treated with REST siRNA (GEO database GSE51463) using Gene Set Enrichment Analysis (GSEA) (149) revealed that both the AKTi and ARi treated transcriptomes were highly correlated with the top ranked 100 genes regulated by REST (FDR=0.001 and <0.001 respectively) (Fig.3.4B). Heatmapping showed that the combination treatment (AKTi+ARi) not only increased the diversity, but also the fold changes of REST-regulated genes (Fig. 3.4C). For example, some genes, exemplified by OPRK1 were upregulated by ARi but not by AKTi whereas some genes, exemplified by SYP were upregulated by AKTi but not ARi. These types of discordant genes were, however, all upregulated by the combination of AKTi+ARi. Finally, genes such as LRRC24, GRIN2C, GABRD showed stronger fold changes upon AKTi+ARi treatment compared to single treatment conditions. Similarly, among the significantly upregulated genes (fold change>1.5 and padj<0.1), the AKTi+ARi group had more co-upregulated genes with the RESTi group (n=123) than the AKTi (n=46) or ARi (n=107) groups (Fig. 3.4D). Interestingly, 90% of the co-upregulated genes shared by RESTi with each of the single treatments were also co-upregulated by RESTi and AKTi+ARi. Gene Ontology (GO) analyses showed that the 123 genes co-targeted by AKT, AR and REST are associated with cellular functions such as plasma membrane, synapse, neuron projection and cell junction (Fig. 3.4E), suggesting that these genes may indicate the t-NEPC transdifferentiation in cells treated by AKT and AR inhibitions. In summary, these results suggest that AKTi alone can induce NE

phenotypes through REST down-regulation, and the combination of AKTi and ARi can more stringently reduce REST protein levels with concomitant increased expression of REST-regulated genes in LNCaP cells.



**Figure 3.4 REST degradation with AKTi and ARi combination treatment**

(A) On the left: LNCaP cells were cultured in RPMI containing FBS for 48 hours followed by transfections of control siRNA or siRNA against AKT (AKTi) for 48 hours. LNCaP cells were also cultured in androgen depletion condition in RPMI containing CSS for 48 hours and then transfected with control siRNA (ARi) or siRNA against

AKT for 48 hours (AKTi+ARi). On the right: LNCaP cells were cultured in RPMI containing FBS or CSS for 72 hours followed by 50 uM LY294002 or vehicle treatment for 24 hours (Right). Cell lysates were immunoblotted for antibodies against REST, AR, tAKT, pAKT, and  $\beta$ -actin. **(B)** Transcriptomes of LNCaP cells treated with control, AKTi, ARi, and AKTi+ARi were analysed by Ampliseq Transcriptome Analysis. Differential gene expressions of AKTi, ARi, and AKTi+ARi were analyzed by the DESeq2 package in R and compared to the control. GSEA enrichment plots showing the correlations of AKTi, ARi, or AKTi+ARi with the top 100 upregulated genes from the LNCaP cells with REST silencing (RESTi) obtained from the GEO database (GSE51463). Top 100 upregulated genes were ranked according to the log2 fold change and filtered by  $\text{padj} < 0.1$  after Benjamini-Hochberg multiple testing correction. **(C)** A heatmap representing expression changes of genes in the RESTi top100 gene set after treatment of AKTi, ARi, or AKTi+ARi **(D)** A Venn diagram depicting the co-upregulated genes (fold change  $> 1.5$  and  $\text{padj} < 0.1$ ) between each of the AKTi, ARi, or AKT+ARi treatment and RESTi. 90% of co-upregulated genes between RESTi and each of the single treatment were also found within the co-upregulated genes between RESTi and AKTi+ARi. **(E)** The co-upregulated genes between AKTi+ARi and RESTi were analysed by DAVID (version 6.7). Top ranked GO\_TERM sorted gene groups were listed.

### **3.5 Clinical implications of co-inhibition of AR and PI3K/AKT**

We further compared the transcriptomes of AKTi, ARi, or AKTi+ARi with the top 200 upregulated genes (ranked by fold change and filtered with  $\text{padj} < 0.05$ ) specific to t-NEPC patients from the Beltran 2016 cohort (50). GSEA analyses showed that the transcriptome of AKTi alone did not significantly correlate with these upregulated genes specific to t-NEPC patients (FDR=0.715), while the transcriptomes of ARi (FDR=0.004) and AKTi+ARi (FDR=0.002) did (Fig. 3.5A). Interestingly, GO term categorization of the positively and negatively correlated genes within the t-NEPC gene set by Ingenuity Pathway analysis (IPA) revealed that the positively correlated genes were mostly related to neurogenesis, while the negatively correlated genes were mainly related to cell proliferation across the AKTi, ARi,

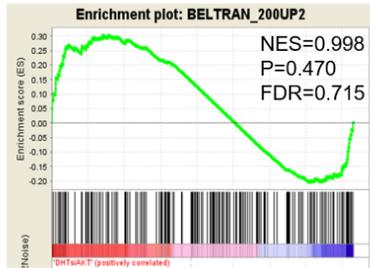
AKTi+ARi groups (Fig. 3.6). For example, the leading edge subset genes (n=43, defined as the core subset of genes responsible for the enrichment score calculation (149)) from the AKTi+ARi GSEA analysis (Group B\_LEAD) were mainly associated with synapse, neurodevelopment, and molecular transport (Fig. 3.5B). In contrast, the strongly negatively correlated core subset of genes (n=14) (Group B\_DOWN) were mainly related to cell growth and proliferation. Consistent with what we have observed in the LNCaP models, combination inhibitions of AKTi and ARi not only increase the diversity but also the fold changes of t-NEPC specific genes. For example, genes such as EYV5, SYP, SYT4, and DCC were mainly upregulated by either AKTi or ARi alone. Genes such as DLGAP3, CCDC151, and HCN3 were more strongly upregulated by the combination AKTi+ARi treatment compared to each of the single treatments (Fig. 3.5B). These findings indicate that combination of AKTi and ARi contributes to NE phenotypes of t-NEPC, but not the highly proliferative properties of t-NEPC.

The significance of REST for t-NEPC progression is evidenced by the fact that there are 29 REST-regulated genes within the top ranked 200 upregulated genes specific to t-NEPC patients. All 29 genes were upregulated by AKTi plus ARi, while only 20 genes were upregulated by AKTi and 26 were upregulated by ARi alone (Fig. 3.5C, left). Genes such as DISP2, AP3B3 and MANEAL were more strongly upregulated by combination treatment of AKTi and ARi. In addition, within the leading edge subgroup of the ARi vs t-NEPC GSEA analysis (Group A\_LEAD, Fig. 3.5C, right), 22% (12/53) genes were regulated by REST. In comparison, 49% (21/43) genes in the leading edge subgroup of the ARi+AKTi vs t-NEPC GSEA analysis (Group B\_LEAD) were regulated by REST ( $p=0.0135$ ) (Fig. 3.5C, right). Together, these findings from

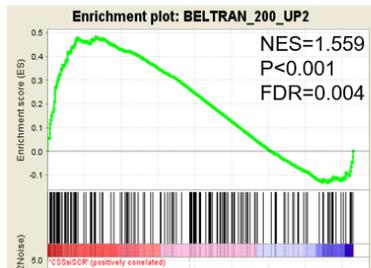
clinical t-NEPC patient samples suggest that AKTi can further enhance ARI induced t-NEPC development via abolishing the suppressive functions of REST.

**A**

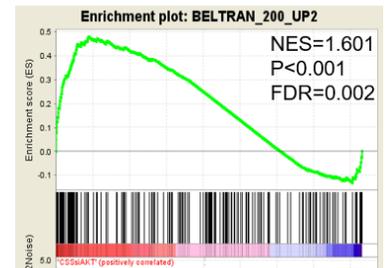
AKTi vs t-NEPC gene set



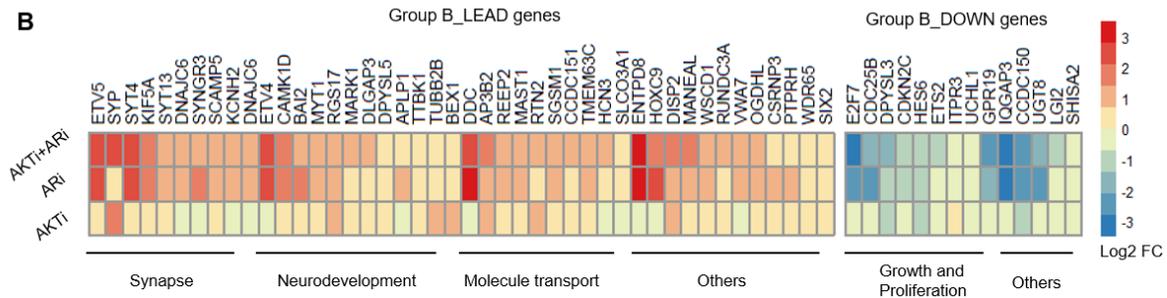
ARI vs t-NEPC gene set



AKTi+ARI vs t-NEPC gene set

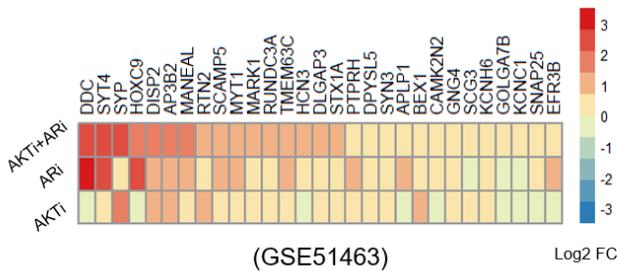


**B**

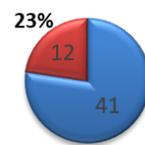


**C**

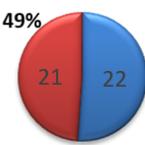
REST-regulated genes in t-NEPC gene set



Group A\_LEAD (ARI)



Group B\_LEAD (AKTi+ARI)

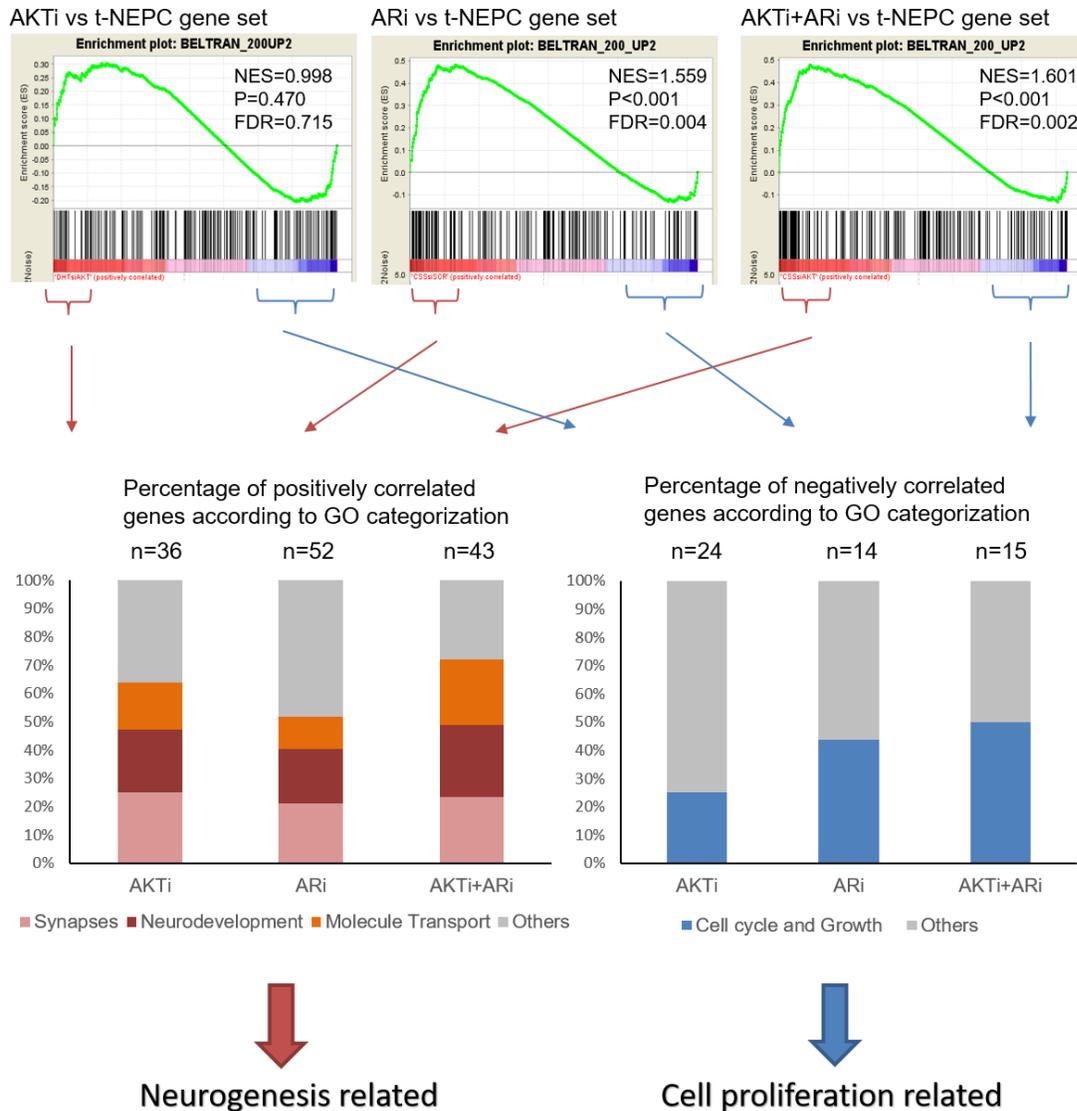


**P=0.0135**

■ REST-Regulated  
■ Non-REST-Regulated

### **Figure 3.5 AKTi, ARi, and AKTi+ARi treatment in relation to t-NEPC patients**

(A) GSEA enrichment plots showing the correlations of AKTi, ARi, or AKTi+ARi with the t-NEPC gene set that consisted of the top 200 upregulated genes from t-NEPC patients from the Beltran cohort (50). Top 200 upregulated genes were ranked according to the log<sub>2</sub> fold change and filtered by padj < 0.05 after Benjamini-Hochberg multiple testing correction. (B) The strongly positively correlated leading edge group genes (n=43) as well as the strongly negatively correlated genes (n=14) from the AKTi+ARi vs. t-NEPC analysis were stratified and analyzed by IPA for GO categorizations. Differential expressions of the stratified genes were presented in the heatmap. (C) Within the t-NEPC gene set, REST-regulated genes (fold change > 1.5 and padj < 0.1) were stratified and their differential expressions were shown in the heatmap (left). The number of REST-regulated and non-REST regulated genes from Group A\_LEAD and Group B\_LEAD of the GSEA enrichment plots were presented as pie charts, respectively. Difference of the proportions of REST-regulated genes within the leading-edge groups was calculated by chi-square test.



**Figure 3.6 GO term analyses of the t-NEPC gene set**

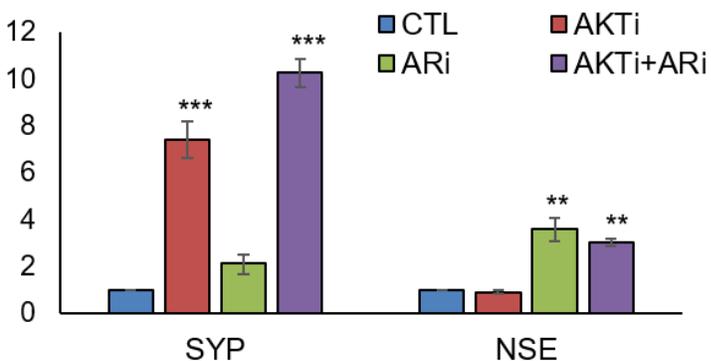
The positively correlated leading-edge group and negatively correlated genes from the GSEA analyses including AKTi vs t-NEPC, ARi vs t-NEPC, and AKTi+ARi vs t-NEPC shown in Fig. 5A were stratified and categorized by DAVID GO\_TERM analyses (version 6.7, <http://david.abcc.ncifcrf.gov/>). For positively correlated genes, groups of synapse, neurodevelopment, molecule transport, and others were presented as the percentage of the total numbers (n) of genes being sorted. Similarly, for negatively correlated genes, cell cycle and proliferation-related genes were represented as a percentage of the total numbers (n) of genes being sorted.

### 3.6 Discussion

Loss of REST is a key factor for prostate adenocarcinoma cells to gain NE phenotypes under various conditions (73,76,151), including AR inhibition (138). Because of the reciprocal activation mechanism between PI3K/AKT and AR signaling pathways (111), we originally hypothesized that PI3K/AKT inhibition would increase AR function in PTEN-deficient cells, thereby stabilizing REST expression to prevent neuroendocrine differentiation. On the contrary, we found that PI3K/AKT inhibition by either AKT siRNA, PI3K inhibitors (LY294002 and BKM120), and AKT inhibitors (MK-2206) also downregulated REST protein levels. Our findings also indicate that REST depletion by PI3K/AKT inhibition is independent of AR activity. REST depletion by PI3K/AKT inhibition relies on the serine phosphorylation of REST, indicating that these serine residuals are not directly targeted by AKT. It is possible that other kinases such as CK1 are activated by AKTi to subsequently phosphorylate REST (143). We have also shown that PI3K/AKT inhibition can induce  $\beta$ -TRCP expression, which in turn recognizes phosphorylated forms of REST for ubiquitination and proteasome degradation. Together, these findings led us to conclude that REST is a novel downstream effector of the PI3K/AKT signaling and that blocking the PI3K/AKT signaling confers PCa cells an NE phenotype at least in part via REST protein degradation.

Both PI3K/AKT inhibition and ARi alone downregulated REST expression, but the combination treatment resulted in additive suppression of REST protein levels and induction of REST-regulated NE genes. Although AKTi and ARi all exert their actions through  $\beta$ -TRCP, our results (Fig. 1.6) as well as others (138) showed that REST reduction by ARi requires at least 96 hours, while PI3K/AKT inhibition induced a more rapid reduction of REST in 8-48 hours. These

findings suggest that PI3K/AKT inhibition and ARi may utilize different mechanisms to trigger  $\beta$ -TRCP to mediate REST ubiquitination. One possibility is that PI3K/AKT inhibition may enhance serine phosphorylation of REST that promotes REST ubiquitination. Combined with upregulation of  $\beta$ -TRCP that we observed, then, PI3K/AKT inhibition could induce more rapid REST degradation than ARi does. In addition, PI3K/AKT inhibition and ARi may exert different but complex impacts on REST functions because AR is a nuclear transcriptional factor that can form a protein complex with REST and regulate REST transcriptional activities (138) whereas AKT is a cytoplasmic kinase that mediates signal cascades and may indirectly affect REST functions. This was evident in that although REST siRNA knockdown upregulates both SYP and NSE (Fig. 1.7), AKTi only upregulates SYP while ARi induces NSE despite both treatments reduce REST expression (Fig. 3.7). These different impacts on REST functions by AKTi and ARi, as a result, may explain why AKTi+ARi upregulates a broader spectrum of REST-regulated genes compared to single treatments both in the LNCaP cell model (Fig. 3.4C) and in the context of t-NEPC patient tumors (Fig. 3.5C). Regardless of the differences, these findings all support that the combination treatment of AKTi and ARi will result in a stronger NE phenotype of PCa cells.



### **Figure 3.7 LNCaP cells under AKTi and/or ARi treatment**

LNCaP cells were treated with either control (CTL), AKTi, ARi, or AKTi+ARi. Real-time PCR results of the relative mRNA expressions of SYP and NSE were shown. Statistical analyses were performed by one-way ANOVA followed by Tuckey's post-hoc test with  $p < 0.01$  as \*\* and  $p < 0.001$  as \*\*\*.

Although the transcriptomes of AKTi and ARi were highly associated with REST siRNA knockdown in the LNCaP cell model, the AKTi transcriptome was not significantly correlated with the genes specific to t-NEPC patients (Fig. 3.5A). Patients in the t-NEPC cohort had not received PI3K/AKT treatment and 33.3% of these patients also had PTEN deletions that resulted in overactive AKT (50), let alone the reciprocal activation of the PI3K/AKT pathway under anti-AR therapies. In contrast, the positive correlation of the ARi transcriptome with the t-NEPC gene set (Fig. 3.5A) is consistent with the fact that these t-NEPC tumors had undergone anti-AR therapies and are likely therapy-induced. However, AKTi plus ARi induced a broader and stronger t-NEPC specific gene changes (Fig. 3.5B) and REST-regulated gene expressions (Fig. 3.5C), suggesting that treatment of PI3K/AKT inhibition to a PCa patient may facilitate the progress of ARi-induced t-NEPC tumor development. Furthermore, since AKTi and ARi mainly regulate different transcriptomes (Fig. 3.4D), we expect that the combination of AKTi with ARi could potentially induce uncharacterized subtypes of t-NEPC under the selection pressure that may be different from the 6 proposed subtypes of t-NEPC tumors (47).

While the traditional role of PI3K/AKT signaling pathway was to promote cell survival and proliferation, overexpression of AKT has been implicated in t-NEPC development. For example, recent reports have shown that N-Myc/AKT overexpression and Rb1/PTEN knockdown can

induce neuroendocrine tumors in transgenic mice and xenografts (56,59,152). Interestingly, the NE tumors developed from the Lee et al. study were derived from basal epithelial cells, where AR-negative basal and neuroendocrine cells reside (152). The NE-phenotype in tumors developed from the Darleen et al. group required N-Myc overexpression while AKT overexpression alone was not sufficient (59). Similarly, PTEN knockdown initiated only metastatic adenocarcinomas but not NEPC as reported by Ku et al (56). Consistent with these findings, Zou et al. observed NEPC tumors only in mice with PTEN and TP53 dual knockouts (153). Based on these findings, we propose that depending on the initial phenotype (luminal epithelial or neuroendocrine) of PCa cells, gain-of-function of AKT can stimulate cell proliferation that drives either AdPC or NEPC tumor formation. On the other hand, AKT blockade in PTEN-deficient PCa cells not only suppresses proliferation, but also induces neuroendocrine transdifferentiation through down-regulating REST expressions.

Recent findings support that t-NEPC is likely derived from adenocarcinoma (AdPC) through coordinated gain of lineage plasticity, neuroendocrine differentiation, and cell proliferation processes under the selection pressure of ARPI (Fig. 1.4). According to this hypothesis, our observations in this study suggest that PI3K/AKT inhibition may provide an opportunity for PTEN-deficient PCa cells to gain an NE-phenotype by downregulating REST (Fig. 3.5B) while inhibiting cell growth and proliferation. Our findings indicate that PI3K/AKT inhibition plays an important role in initiating neuroendocrine differentiation, one of the necessary events for t-NEPC tumor establishment.

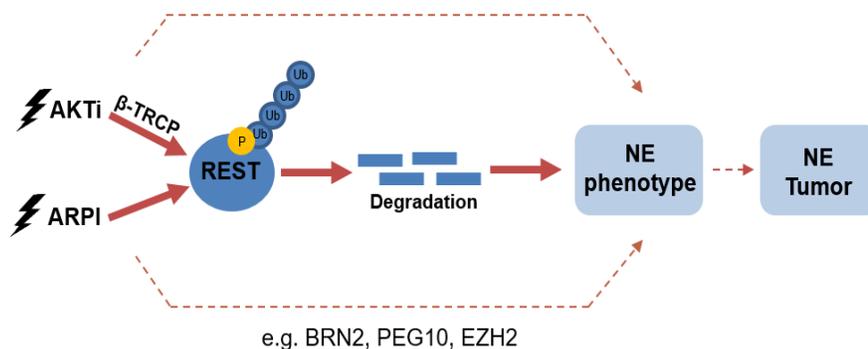
## Chapter 4: Conclusions

### 4.1 Summary of findings

Although NEPC is rare in untreated PCa patients, the stringent hormone therapies now used for advanced PCa/CRPC are associated with a significantly increased risk for the development of t-NEPC (154). In fact, some estimate that up to 25% of patients treated with enzalutamide or abiraterone will develop t-NEPC (49). NEPC is highly aggressive and is particularly difficult to treat. Current strategies for treatment of NEPC are based on the use of a platinum-based agent in conjunction with etoposide (155). This therapy, unfortunately, only provides palliative relief.

Given the increasing rate of occurrence of t-NEPC in these cohorts, it would be prudent to assess whether novel therapeutic agents used to treat CRPC might contribute to the development of t-NEPC. In chapter 3, we assessed the possibility that novel PI3K/AKT-targeted therapies for PCa might also contribute to the development of t-NEPC. We identified REST as a novel downstream effector of PI3K/AKT signaling (Fig. 4.1) and showed that inhibitions of the PI3K/AKT pathway (siRNA against AKT, LY294002, BKM-120, MK-2206) consistently reduced expression of REST protein in androgen-sensitive and -insensitive PCa cells. PI3K/AKT inhibitions do not affect REST mRNA transcription but protein stability. We further discovered that PI3K/AKT inhibition enhanced REST protein degradation through a  $\beta$ -TRCP mediated proteasome pathway that involves the REST 1024/1027/1030 phosphodegron region. Finally, we showed that the combination of AKTi and ARi can further aggravate REST protein depletion and promote NE transdifferentiation of PCa cells from Western blot and real-time PCR. The results from the transcriptomic analyses (in comparison with previous *in vivo* transcriptomic data and patient data) further corroborated the third initial hypothesis, which describes the potential additive effect of the combination therapy on REST reduction and NE differentiation.

### Schematic summary



**Figure 4.1 Schematic thesis project summary**

## **4.2 Limitations**

One limitation of this thesis work is the treatment period of PI3K/AKT drugs to PCa cells. Since the time period of PI3K/AKT inhibition treatment did not exceed 48 hours, the reduction of REST protein levels and NE marker expressions observed in this work may represent transient stress response from PCa cells. However, the development of t-NEPC most likely requires an extended period of time of therapeutic intervention allow tumor cells to gain lineage plasticity, NE differentiation, and turn on the “proliferative switch” of differentiated tumor cells (50,51). Long term drug treatment studies were not designed in the *in vivo* studies in this project due to concerns of excessive cell death in PCa cells as they rely heavily on this signaling pathway for survival and proliferation. A potential solution addressing this issue links to another limitation, which is the lack of 3D culturing, organoids and *in vivo* studies, as will be discussed in the following section **4.3**.

In this thesis work, a majority of studies utilized the drug LY294002. As one of the earliest pan-PI3K inhibitors, this drug was shown to have multiple off-target effects such as Pim-1 (156), suggesting a potentially non-specific inhibitory and toxicity effect (157). In fact, a previous study has shown that LY294002 could induce NE-features in PCa cells via HER2 activation (158), implying the potential existence of a PI3K-dependent and AKT-independent pathway towards NE marker induction in PCa cells. In order to address these problems, the AKT siRNA, pan-PI3K drug BKM-120, and AKT allosteric inhibitor MK-2206 were also used to validate the findings obtained upon treating LY294002 to PCa cells (Fig 3.1). The consistent observations of REST protein reduction and NE marker expression in all of these treatments support that LY294002 at least in part induce NE marker expression in PCa cells via accelerated REST protein degradation.

Although Ampliseq sequencing and its subsequent bioinformatics analyses have provided valuable insights into the global transcriptomic changes of PCa cells upon AKTi and/or ARi treatments, the small sample size of each treatment condition (duplicates) limit the statistical calculation of significantly changed gene expressions. Consequently, upon DESeq2 analyses of the raw transcriptomic data, some changes of gene expressions may have been missed according to our criterion  $\text{padj} < 0.1$ , resulting in possible false negatives. Additionally, this sequencing method only profiles the transcriptome, but multiple studies have shown low correlations between the transcriptome and proteome possibly due to different half-lives and post transcription machinery. In fact, correlations coefficients between transcriptomes and proteomes have been reported in the range of  $0.16 < r < 0.66$  depending on the analysis platforms and

model organisms (159–161). These findings strongly argue for the need of proteomic validations of significantly changed genes identified from transcriptomic studies.

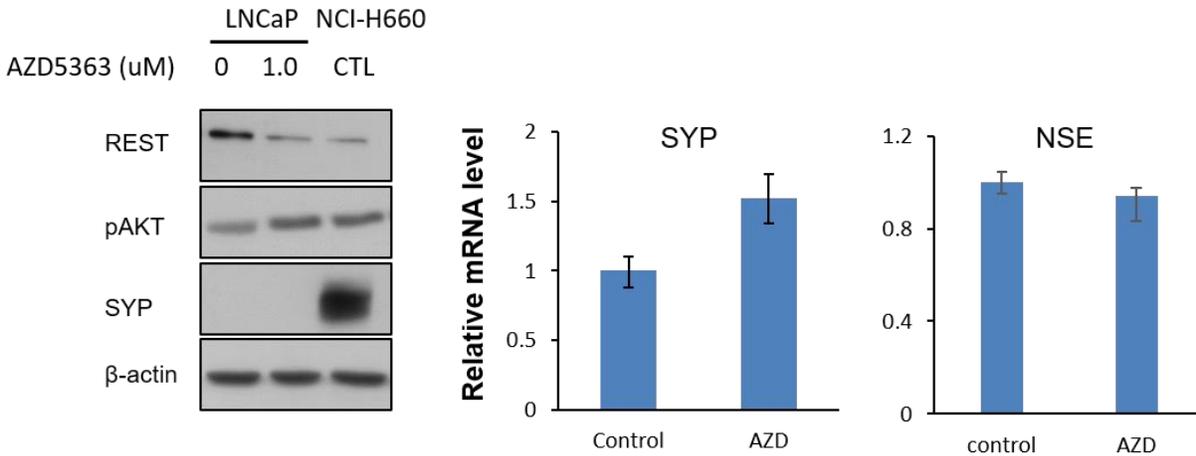
Another limitation of this project is the undetermined status of REST degron phosphorylation. Findings so far indicate that the REST S1024/1027/1030 phosphodegron region is a necessary component of PI3K/AKT inhibition-mediated REST degradation. However, due to the limitations of the available REST antibodies that prevent efficient co-immunoprecipitation experiments, whether phosphorylation status changes at this degron region remains to be answered. Identifying REST degron phosphorylation status can be critical in understanding the time difference between AKTi- and ARi-mediated REST ubiquitination and degradation.

### **4.3 Future direction**

Since it is yet unknown that whether PI3K/AKT inhibition can induce t-NEPC tumors or only transient NE-differentiation in PCa cells, it is critical to investigate the long term effect of this therapeutic intervention on t-NEPC development. In order to study the long term effects, 3D/organoid culture and animal studies will be necessary as PCa cells growing in these conditions could last longer and be more resilient to the toxicity caused by the PI3K/AKT drugs. In addition, co-treatment of PI3K/AKT drugs and ARPI in animal models such as the LTL331 line (51) could answer whether this novel approach can accelerate t-NEPC development in PCa. Moreover, the potential implication of PI3K/AKT inhibition on t-NEPC development argues for retrospective studies upon the completion of preclinical and clinical investigation of new PI3K/AKT inhibitors. To date, studies of novel PI3K/AKT inhibitors only measured tumor size/proliferation rate in preclinical models and progression-free survival as the primary outcome

in patient cohorts. Whether animal and/or patient tumor samples treated with PI3K/AKT inhibitors show reduced REST protein expression and increased NE expression upon immunohistochemistry (IHC) staining warrant further investigations.

In order to comprehensively understand the impact of PI3K/AKT inhibitors on REST protein stability and NE marker expression, comparative studies of different drugs and to a lesser extent, different cell lines, will be necessary. As Liang et al. previously showed that different PI3K/AKT inhibitors showed varied impact on with AR protein stability and mRNA expression (162), these drugs may also exert differential effects on REST and NE marker expressions due to varied inhibitory mechanisms as well as off-target effects. Interestingly, the AKT competitive inhibitor AZD5363 potently reduced REST protein expression but did not induce NE marker expressions (Figure 4.2), further supporting the complex cellular interactions involved upon the treatment of PI3K/AKT drugs. Consequently, future investigations can focus on 1) the impact of newly developed or currently studied PI3K/AKT inhibitors on REST and NE markers and 2) the potential mechanism by which AZD5363 suppress NE marker expression in REST-depleted conditions. Findings from the latter part may provide new insights into preventing NE differentiation in PCa cells.



**Figure 4.2 Impact of AZD5363 on REST and NE marker expressions**

Right: LNCaP cells were treated with 0 and 1 uM AZD5363 for 24 hours. Cells lysates were collected along with NCI-H660 lysates as the positive NE marker control. The lysates were immunoblotted for REST, pAKT, SYP, and  $\beta$ -actin. Left: relative mRNA levels of SYP and NSE from LNCaP cells treated with control or 1.0 uM AZD5363 for 24 hours were measured by real-time PCR. Statistical analyses were performed by paired student's t-test with  $p < 0.05$  as \*,  $p < 0.01$  as \*\* and  $p < 0.001$  as \*\*\*.

The Polycomb group protein Enhancer of zeste 2 (EZH2) is a methyltransferase that plays an important role in cellular epigenetic regulations in part via its ability to trimethylate lysine 27 in histone H3 (H3K27). Elevated EZH2 was reported in t-NEPC patients (50). EZH2 overexpression was also observed in NEPC-like tumors from animal models and inhibition of this protein reduced the growth of NCI-H660 cells as well as NEPC-like organoids but not AdPC cells (56,59). Interestingly, AKT has been reported to phosphorylate EZH2 at serine 21 to suppress its methylation abilities at H3K27 in 293T cells (163). Given the important roles of EZH2 in t-NEPC development and progression, it would be worthwhile to investigate whether PI3K/AKT inhibitors can enhance EZH2 activities in PCa cells. If true, this AKT-EZH2 axis would represent another mechanism by which PI3K/AKT inhibition utilizes to promote t-NEPC.

#### **4.4 Overall significance**

In summary, we report a novel finding that blocking the PI3K/AKT signaling pathway can reduce REST and induce NE phenotypes in PTEN-deficient PCa cells. Co-targeting PI3K/AKT and AR resulted in more REST depletion and stronger neuroendocrine differentiation of PCa cells. These findings indicate a potential implication of PI3K/AKT inhibition in PCa and provide a caution for the development of this therapeutic strategy.

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<http://www.ncbi.nlm.nih.gov/pubmed/16224021>

## Appendix

I have the fortunate opportunity to participate in several projects during my M.Sc. studies.

Manuscripts from these projects are listed below.

List of Publications (during MSc study):

1. Li Y, Li H, Xie N, **Chen R**, Lee A, Slater D, Lye S, Dong X. HoxA10 and HoxA11 Regulate the Expression of Contraction-Associated Proteins and Contribute to Regionalized Myometrium Phenotypes in Women. *Reproductive Sciences*. In Press, 2017
2. Li H, Xie N, **Chen R**, Verreault M, Fazli L, Gleave ME, Barbier O, Dong X. UGT2B17 expedites progression of castration-resistant prostate cancer by promoting ligand-independent AR signaling. *Cancer Research*. Sep 2016. PMID: 27659047.
3. **Chen R**, Yu Y, Dong X. Progesterone receptor in the prostate: A potential suppressor for benign prostatic hyperplasia and prostate cancer. *Journal of Steroid Biochemistry and Molecular Biology*. Apr 2016. PMID: 27125450.

Manuscript under review:

1. **Chen R**, Li Y, Buttyan R, Dong X. Implications of PI3K/AKT inhibition on REST protein stability and neuroendocrine phenotype acquisition in prostate cancer cells. Manuscript in preparation for resubmission.
2. **Chen R**, Dong X. Molecular model of neuroendocrine prostate cancer development and progression. Manuscript in preparation for submission.