National and Kapodistrian University of Athens

School of Health Sciences

Medical School, Department of Basic Medical Sciences

Laboratory of Histology-Embryology

Professor-Director: Vassilis G. Gorgoulis

PhD Thesis

Διερεύνηση της ογκογόνου δράσης του παράγοντα αδειοδότησης της αντιγραφής CDC6 στον καρκίνο του προστάτη

Ioanna Mourkioti

Biologist BSc, MSc

Athens

November 2023

National and Kapodistrian University of Athens

School of Health Sciences

Medical School, Department of Basic Medical Sciences

Laboratory of Histology-Embryology

Professor-Director: Vassilis G. Gorgoulis

PhD Thesis

Investigation of the oncogenic role of the replication licensing factor CDC6 in Prostate Cancer

Ioanna Mourkioti

Biologist BSc, MSc

Athens

November 2023

PhD candidate Date of Application: 20th of October 2017

3-member advisory committee Date of appointment: 30th of November 2017

3-member advisory committee

1. **Mirsini Kouloukoussa:** Emeritus Professor, Department of Histology-Embryology, Medical School, NKUA (Supervisor)
2. **Vassilis G. Gorgoulis:** Professor-Director, Department of Histology-Embryology, Medical School, NKUA
3. **Athanassios Kotsinas:** Associate Professor, Department of Histology-Embryology, Medical School, NKUA

Title of PhD Thesis Date of Selection: 9th of February 2018

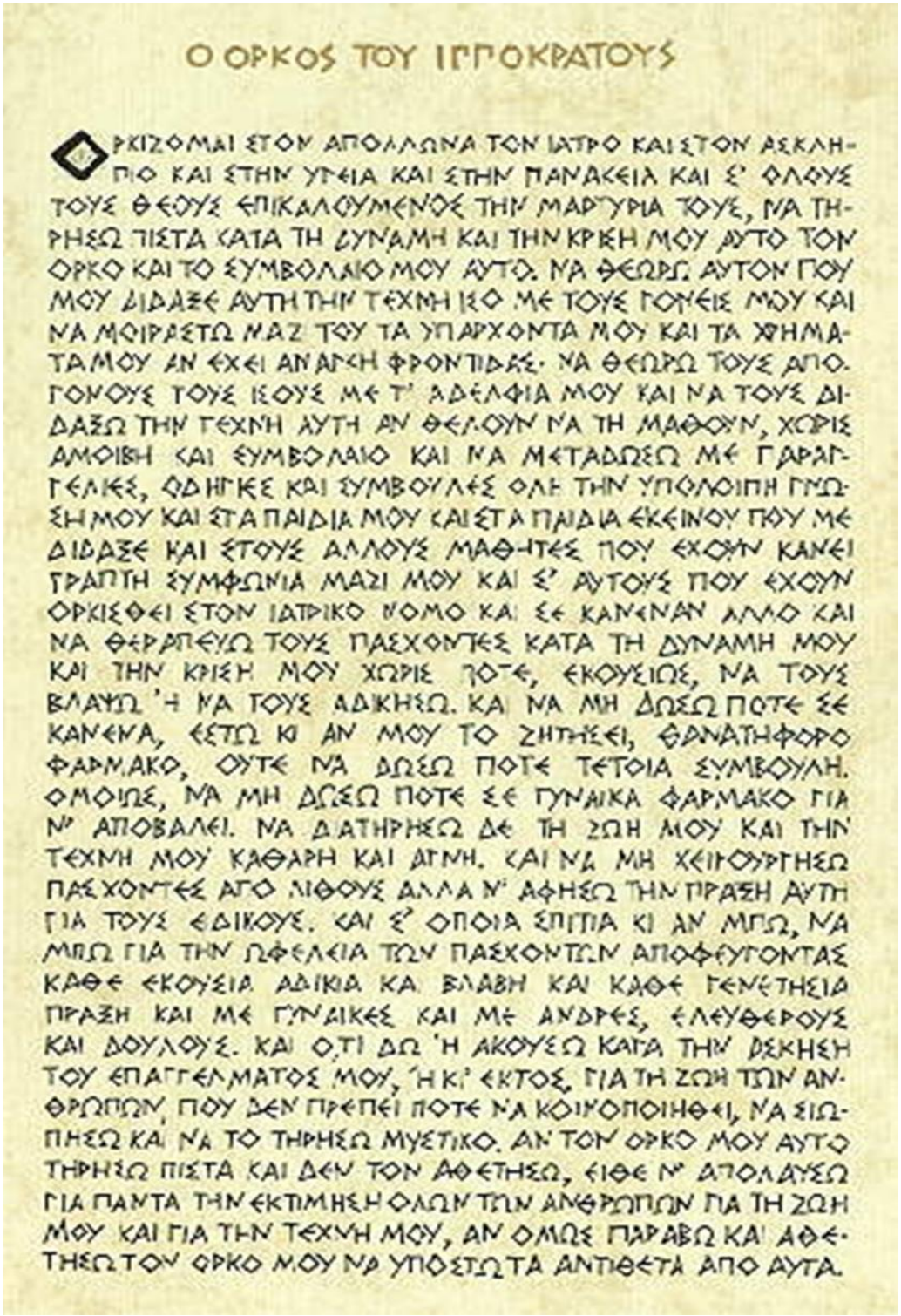
PhD Thesis Date of Submission: 6th of November 2023

7-member advisory committee

1. **Mirsini Kouloukoussa:** Emeritus Professor, Department of Histology-Embryology, Medical School, NKUA (Supervisor)
2. **Vassilis G. Gorgoulis:** Professor-Director, Department of Histology-Embryology, Medical School, NKUA
3. **Athanassios Kotsinas:** Associate Professor, Department of Histology-Embryology, Medical School, NKUA
4. **Konstantinos Evangelou:** Associate Professor, Department of Histology-Embryology, Medical School, NKUA
5. **Sofia Havaki:** Associate Professor, Department of Histology-Embryology, Medical School, NKUA
6. **Nefeli Lagopati:** Assistant Professor, Department of Biology, Medical School, NKUA
7. **Emmanouil Vardas:** Assistant Professor, Hospital Dentistry, School of Dentistry NKUA

PhD Thesis Date of Defense: 20th of December 2023

Grade of PhD Thesis: Excellent (10/10)



Ο ΟΡΚΟΣ ΤΟΥ ΙΠΠΟΚΡΑΤΟΥΣ

ΟΡΚΙΖΟΜΑΙ ΣΤΟΝ ΑΠΟΛΛΩΝΑ ΤΟΝ ΙΑΤΡΟ ΚΑΙ ΣΤΟΝ ΑΣΚΛΗΠΙΟ ΚΑΙ ΣΤΗΝ ΥΓΕΙΑ ΚΑΙ ΣΤΗΝ ΠΑΝΑΚΕΙΑ ΚΑΙ Σ' ΟΛΟΥΣ ΤΟΥΣ ΘΕΟΥΣ ΕΠΙΚΑΛΟΥΜΕΝΟΣ ΤΗΝ ΜΑΡΤΥΡΙΑ ΤΟΥΣ, ΝΑ ΤΗΡΗΣΩ ΠΙΣΤΑ ΚΑΤΑ ΤΗ ΔΥΝΑΜΗ ΚΑΙ ΤΗΝ ΚΡΙΣΗ ΜΟΥ ΑΥΤΟ ΤΟΝ ΟΡΚΟ ΚΑΙ ΤΟ ΣΥΜΒΟΛΑΙΟ ΜΟΥ ΑΥΤΟ. ΝΑ ΘΕΩΡΩ ΑΥΤΟΝ ΠΟΥ ΜΟΥ ΔΙΔΑΞΕ ΑΥΤΗ ΤΗΝ ΤΕΧΝΗ ΙΣΟ ΜΕ ΤΟΥΣ ΓΟΝΕΙΣ ΜΟΥ ΚΑΙ ΝΑ ΜΟΙΡΑΣΤΩ ΜΑΖΙ ΤΟΥ ΤΑ ΥΠΑΡΧΟΝΤΑ ΜΟΥ ΚΑΙ ΤΑ ΧΡΗΜΑΤΑ ΜΟΥ ΑΝ ΕΧΕΙ ΑΝΑΓΚΗ ΦΡΟΝΤΙΔΑΣ· ΝΑ ΘΕΩΡΩ ΤΟΥΣ ΑΠΟΓΟΝΟΥΣ ΤΟΥΣ ΙΣΟΥΣ ΜΕ Τ' ΑΔΕΛΦΙΑ ΜΟΥ ΚΑΙ ΝΑ ΤΟΥΣ ΔΙΔΑΞΩ ΤΗΝ ΤΕΧΝΗ ΑΥΤΗ ΑΝ ΘΕΛΟΥΝ ΝΑ ΤΗ ΜΑΘΟΥΝ, ΧΩΡΙΣ ΑΜΟΙΒΗ ΚΑΙ ΣΥΜΒΟΛΑΙΟ ΚΑΙ ΝΑ ΜΕΤΑΔΩΣΩ ΜΕ ΓΡΑΦΕΣ, ΟΔΗΓΙΕΣ ΚΑΙ ΣΥΜΒΟΥΛΕΣ ΟΛΗ ΤΗΝ ΥΠΟΛΟΙΠΗ ΓΝΩΣΗ ΜΟΥ ΚΑΙ ΣΤΑ ΠΑΙΔΙΑ ΜΟΥ ΚΑΙ ΣΤΑ ΠΑΙΔΙΑ ΕΚΕΙΝΟΥ ΠΟΥ ΜΕ ΔΙΔΑΞΕ ΚΑΙ ΣΤΟΥΣ ΑΛΛΟΥΣ ΜΑΘΗΤΕΣ ΠΟΥ ΕΧΟΥΝ ΚΑΝΕΙ ΓΡΑΠΤΗ ΣΥΜΦΩΝΙΑ ΜΑΖΙ ΜΟΥ ΚΑΙ Σ' ΑΥΤΟΥΣ ΠΟΥ ΕΧΟΥΝ ΟΡΚΙΣΘΕΙ ΣΤΟΝ ΙΑΤΡΙΚΟ ΝΟΜΟ ΚΑΙ ΣΕ ΚΑΝΕΝΑΝ ΑΛΛΟ ΚΑΙ ΝΑ ΘΕΡΑΠΕΥΩ ΤΟΥΣ ΠΑΣΧΟΝΤΕΣ ΚΑΤΑ ΤΗ ΔΥΝΑΜΗ ΜΟΥ ΚΑΙ ΤΗΝ ΚΡΙΣΗ ΜΟΥ ΧΩΡΙΣ ΠΟΤΕ, ΕΚΟΥΣΙΩΣ, ΝΑ ΤΟΥΣ ΒΛΑΨΩ Ή ΝΑ ΤΟΥΣ ΑΔΙΚΗΣΩ. ΚΑΙ ΝΑ ΜΗ ΔΩΣΩ ΠΟΤΕ ΣΕ ΚΑΝΕΝΑ, ΕΣΤΩ ΚΙ ΑΝ ΜΟΥ ΤΟ ΖΗΤΗΣΕΙ, ΘΑΝΑΤΗΦΟΡΟ ΦΑΡΜΑΚΟ, ΟΥΤΕ ΝΑ ΔΩΣΩ ΠΟΤΕ ΤΕΤΟΙΑ ΣΥΜΒΟΥΛΗ. ΟΜΟΙΩΣ, ΝΑ ΜΗ ΔΩΣΩ ΠΟΤΕ ΣΕ ΓΥΝΑΙΚΑ ΦΑΡΜΑΚΟ ΓΙΑ Ν' ΑΠΟΒΑΛΕΙ. ΝΑ ΔΙΑΤΗΡΗΣΩ ΔΕ ΤΗ ΖΩΗ ΜΟΥ ΚΑΙ ΤΗΝ ΤΕΧΝΗ ΜΟΥ ΚΑΘΑΡΗ ΚΑΙ ΑΓΝΗ. ΚΑΙ ΝΑ ΜΗ ΧΕΙΡΟΥΡΓΗΣΩ ΠΑΣΧΟΝΤΕΣ ΑΓΩ ΛΙΘΟΥΣ ΑΛΛΑ Ν' ΑΦΗΣΩ ΤΗΝ ΠΡΑΞΗ ΑΥΤΗ ΓΙΑ ΤΟΥΣ ΕΙΔΙΚΟΥΣ. ΚΑΙ Σ' ΟΠΟΙΑ ΣΠΙΤΙΑ ΚΙ ΑΝ ΜΠΩ, ΝΑ ΜΠΩ ΓΙΑ ΤΗΝ ΩΦΕΛΕΙΑ ΤΩΝ ΠΑΣΧΟΝΤΩΝ ΑΠΟΦΕΥΓΟΝΤΑΣ ΚΑΘΕ ΕΚΟΥΣΙΑ ΑΔΙΚΙΑ ΚΑΙ ΒΛΑΒΗ ΚΑΙ ΚΑΘΕ ΓΕΝΕΤΗΣΙΑ ΠΡΑΞΗ ΚΑΙ ΜΕ ΓΥΝΑΙΚΕΣ ΚΑΙ ΜΕ ΑΝΔΡΕΣ, ΕΛΕΥΘΕΡΟΥΣ ΚΑΙ ΔΟΥΛΟΥΣ. ΚΑΙ ΟΤΙ ΔΩ Ή ΑΚΟΥΣΩ ΚΑΤΑ ΤΗΝ ΔΕΚΗΣΗ ΤΟΥ ΕΠΑΓΓΕΛΜΑΤΟΣ ΜΟΥ, Ή ΚΙ' ΕΚΤΟΣ, ΓΙΑ ΤΗ ΖΩΗ ΤΩΝ ΑΝΘΡΩΠΩΝ, ΠΟΥ ΔΕΝ ΠΡΕΠΕΙ ΠΟΤΕ ΝΑ ΚΟΙΝΟΠΟΙΗΘΕΙ, ΝΑ ΣΙΩΠΗΣΩ ΚΑΙ ΝΑ ΤΟ ΤΗΡΗΣΩ ΜΥΣΤΙΚΟ. ΑΝ ΤΟΝ ΟΡΚΟ ΜΟΥ ΑΥΤΟ ΤΗΡΗΣΩ ΠΙΣΤΑ ΚΑΙ ΔΕΝ ΤΟΝ ΑΘΕΤΗΣΩ, ΕΙΘΕ Ν' ΑΠΟΛΑΥΣΩ ΓΙΑ ΠΑΝΤΑ ΤΗΝ ΕΚΤΙΜΗΣΗ ΟΛΩΝ ΤΩΝ ΑΝΘΡΩΠΩΝ ΓΙΑ ΤΗ ΖΩΗ ΜΟΥ ΚΑΙ ΓΙΑ ΤΗΝ ΤΕΧΝΗ ΜΟΥ, ΑΝ ΟΜΩΣ ΠΑΡΑΒΩ ΚΑΙ ΑΘΕΤΗΣΩ ΤΟΝ ΟΡΚΟ ΜΟΥ ΝΑ ΥΠΟΣΤΩ ΤΑ ΑΝΤΙΘΕΤΑ ΑΠΟ ΑΥΤΑ.

AKNOWLEDGEMENTS

The present Ph.D. Thesis on "Investigation of the oncogenic role of the replication licensing factor CDC6 in Prostate Cancer" was carried out in the period 2017-2023 in the Histology and Embryology laboratory of Medical School of the University of Athens under the supervision of Professor-Director Vassilis G. Gorgoulis.

Several people contributed to the completion of this work to whom I owe my deepest "thank you". First of all, I would like to thank Professor Vassilis G. Gorgoulis for giving me the opportunity to become a member of his research group, as I so desired, and to work alongside him. I am extremely grateful for the unwavering guidance, support, nurturing and confidence he has shown in me all these years.

I sincerely thank the Emeritus Professor Mirsini Kouloukoussa for the honor she gave me to undertake, initially, the supervision of my Ph.D. and to be a member of my Advisory Committee.

In addition, I would like to especially thank Associate Professor Athanasios Kotsinas, member of my Advisory Committee and cornerstone of the Molecular Carcinogenesis Group for his support and help over the years.

Next, I would like to thank Associate Professor Konstantinos Evangelou, Associate Professor Sophia Havaki, Assistant Professor Nefeli Lagopati and Assistant Professor Emmanouil Vardas for honoring me by taking part in the Seven-Member Examining Committee of this Thesis. Thank you all for your support and guidance. I am deeply indebted to Assistant Professor Nefeli Lagopati for her cooperation, invaluable help and relentless support.

I would also like to extend my deepest gratitude to Assistant Professor Angelos Papaspyropoulos. The completion of this work would not have been possible without the guidance and constructive advice that Angelos Papaspyropoulos provided me.

I would be remiss if I did not thank all the members, past and present, of the Molecular Carcinogenesis Group for their support and collaboration. Special thanks to Andriani Angelopoulou for her cooperation, encouragement and patience she provided me during our common research journey. I sincerely thank Dimitris Veroutis and George Theocharous for our great collaboration, and their help with the senescence detection experiments as well as Dr. Aikaterini Polyzou for the bioinformatic analysis that was crucial for the completion of this work.

Several of the experiments presented in this work were carried out in collaboration with other laboratories in Greece. For this, I thank Researcher Dr. Dimitrios Kletsas at the NCSR “Demokritos” as well as the Associate Professor Dimitrios Stellas at National Hellenic Research Foundation.

I would also like to extend my sincere thanks to MD Anderson Cancer Center in Houston, Texas of USA and especially to Professor Christopher J. Logothetis for giving me the opportunity to be part of this top ranked Cancer Center worldwide for 2 and a half years. I would like to acknowledge

the valuable advice and support Professor Logothetis very kindly provided me as well as his profound belief in my work and in my abilities.

Of course, I wouldn't be what I am and I wouldn't have been able to complete my studies if I didn't have the support and unlimited love of my family. So, I would like to express my deepest appreciation and to dedicate my Ph.D. thesis to my parents Giannis and Krinio and my brother Nikos, who have been such a constant for me during the emotionally stressful and intellectually challenging years of my Ph.D. Thank you endlessly from the bottom of my heart.

TABLE OF CONTENTS

CURRICULUM VITAE

Ioanna Mourkioti

Biologist, BSc, MSc, PhD candidate

PRESENT TITLE AND AFFILIATION

Primary Appointment

PhD Candidate, School of Medicine, Department of Histology and Embryology, National and Kapodistrian University of Athens

CITIZENSHIP

Greek

EDUCATION

May 2018 – July 2020: Graduate Student–non-UTHSCH

Department of Genitourinary Medical Oncology, MD Anderson Cancer Center, Houston, TX

Supervisor: Christopher J. Logothetis, Professor, Department of Genitourinary Medical Oncology

Director, The David H. Koch Center for Applied Research of Genitourinary Cancers

Nov. 2017 – Present: PhD candidate

Department of Histology and Embryology, School of Medicine, National and Kapodistrian University of Athens

Supervisor: Vassilis G. Gorgoulis, Professor and Director, Department of Histology-Embryology, Athens Medical School

Oct. 2015 – Oct. 2017: Master's Degree in Science. Applications of Biology in Medicine, collaboration between the Faculty of Biology & the School of Medicine, National and Kapodistrian University of Athens

Mark: 9.37/10

Master's Thesis: *The role of the replication factor Cdc6 in carcinogenesis*. Graded with distinction (10/10)

Supervisor: Vassilis G. Gorgoulis, Professor and Director, Department of Histology-Embryology, Athens Medical School

Sep. 2009 – Oct. 2015: Bachelor's Degree in Biology, National and Kapodistrian University of Athens

Mark: 6.6/10

Experimental Thesis: *Relative gene amplification of MET gene in non-small cell lung cancer*. Graded with distinction (10/10)

Supervisor: Angelica A. Saetta, Associate Professor, School of Medicine, National and Kapodistrian University of Athens

Sept. 2006 – June 2009: High School Diploma

Mark: 19.5/20

PUBLICATIONS

Peer-Reviewed Publications

1. **Mourkioti I**, Polyzou A, Veroutis D, Theocharous G, Lagopati N, Gentile E, Stravokefalou V, Thanos DF, Havaki S, Kletsas D, Panaretakis T, Logothetis CJ, Stellas D, Petty R, Blandino G, Papaspyropoulos A, Gorgoulis VG. A GATA2-CDC6 axis modulates androgen receptor blockade-induced senescence in prostate cancer. J Exp Clin Cancer Res. 2023 Jul 29;42(1):187. doi: 10.1186/s13046-023-02769-z.
2. **Mourkioti I**, Angelopoulou A, Belogiannis K, Lagopati N, Potamianos S, Kyrodimos E, Gorgoulis V, Papaspyropoulos A. Interplay of Developmental Hippo-Notch Signaling Pathways with the DNA Damage Response in Prostate Cancer. Cells. 2022 Aug 7;11(15):2449. doi: 10.3390/cells11152449.
3. Papaspyropoulos A, Angelopoulou A, **Mourkioti I**, Polyzou A, Pankova D, Toskas K, Lanfredini S, Pantazaki AA, Lagopati N, Kotsinas A, Evangelou K, Chronopoulos E, O'Neill E, Gorgoulis V. RASSF1A disrupts the NOTCH signaling axis via SNURF/RNF4-mediated ubiquitination of HES1. EMBO Rep. 2022 Feb 3;23(2):e51287. doi: 10.15252/embr.202051287.
4. Zampetidis CP, Galanos P, Angelopoulou A, Zhu Y, Polyzou A, Karamitros T, Kotsinas A, Lagopati N, **Mourkioti I**, Mirzazadeh R, Polyzos A, Garnerone S, Mizi A, Gusmao EG, Sofiadis K, Gál Z, Larsen DH, Pefani DE, Demaria M, Tsirigos A, Crosetto N, Maya-Mendoza A, Papaspyropoulos A, Evangelou K, Bartek J, Papantonis A, Gorgoulis VG. A recurrent chromosomal inversion suffices for driving escape from oncogene-induced senescence via subTAD reorganization. Mol Cell. 2021 Dec 2;81(23):4907-4923.e8. doi: 10.1016/j.molcel.2021.10.017.
5. Papaspyropoulos A, Lagopati N, **Mourkioti I**, Angelopoulou A, Kyriazis S, Liontos M, Gorgoulis V, Kotsinas A. Regulatory and Functional Involvement of Long Non-Coding RNAs in DNA Double-Strand Break Repair Mechanisms. Cells. 2021 Jun 15;10(6):1506. doi: 10.3390/cells10061506.
6. Lagopati N, Tsioli P, **Mourkioti I**, Polyzou A, Papaspyropoulos A, Zafiropoulos A, Evangelou K, Sourvinos G, Gorgoulis VG. Sample pooling strategies for SARS-CoV-2 detection. J Virol Methods 2021 Mar;289:114044. doi: 10.1016/j.jviromet.2020.114044
7. Koinis F, Corn P, Parikh N, Song J, Vardaki I, **Mourkioti I**, Lin SH, Logothetis C, Panaretakis T, Gallick G. Resistance to MET/VEGFR2 Inhibition by Cabozantinib Is Mediated by YAP/TBX5-Dependent Induction of

FGFR1 in Castration-Resistant Prostate Cancer. *Cancers* 2020, *12*(1), 244; https://doi.org/10.3390/cancers12010244

8. Komseli E-S, Pateras IS, Krejsgaard T, Stawiski K, Rizou SV, Polyzos A, Roumelioti F-M, Chiourea M, **Mourkioti I**, Paparouna E, Zampetidis CP, Gumeni S, Trougakos IP, Pefani DE, O'Neill E, Gagos S, Eliopoulos AG, Fendler W, Chowdhury D, Bartek J, Gorgoulis VG. A prototypical non-malignant epithelial model to study genome dynamics and concurrently monitor micro-RNAs and proteins in situ during oncogene-induced senescence. BMC Genomics. 2018 Jan 10;19(1):37. doi: 10.1186/s12864-017- 4375-1.
9. Psaraki AA, Chatziandreou I, **Mourkioti I**, Sakellariou S, Korkolopoulou P, Patsouris E, Saetta AA. Detection of FGFR1 and MET gene amplification in squamous cell carcinomas of lung. Honorary Tome of Cardiology Professor C. Stefanadis, Medical School of Athens, 2016.
10. Chatziandreou I, Tsioli P, Sakellariou S, **Mourkioti I**, Giannopoulou I, Levidou G, Korkolopoulou P, Patsouris E, Saetta AA. Comprehensive molecular analysis of NSCLC; clinicopathological associations. PloS One. 2015;10(7):e0133859. doi: 10.1371/journal.pone.0133859.

Presentations at National or International Conferences

1. **Mourkioti I**, Chatziandreou I, Tsikalakis S, Svolaki I, Papasideri I, Patsouris E, Saetta AA. ''Relative gene amplification of MET gene in Non-Small Cell Lung cancer''. 37ο Annual Scientific Conference of the Hellenic Society for Biological Sciences, May 2015, Volos**,** Greece (poster and oral presentation).
2. Psaraki AA, Chatziandreou I, **Mourkioti I**, Korkolopoulou P, Patsouris E, Saetta AA. ''Relative gene amplification analysis of FGFR1, MET, DCUN1D1 and BCL9 gene in squamous cell carcinomas of the lung,'' 12th Meeting of the European Human Genetics Societies, May 2016, Barcelona, Spain (poster).
3. **Mourkioti I**, Papaspyropoulos A, Gorgoulis VG, Lagopati N. ''The role of senescence in Prostate Cancer''. 1st Panhellenic Conference of Natural Sciences in Health: Innovations and Prospects. September 2023, Athens, Greece (poster)

ABSTRACT

TITLE: ''Investigation of the oncogenic role of the replication licensing factor CDC6 in Prostate Cancer''

Background: Prostate cancer is a hormone-dependent type of cancer that represents a leading cause of cancer morbidity and mortality in men around the world. Androgen deprivation therapy (ADT) remains the mainstay of therapy for patients with prostate cancer, but has proven efficient only in early-stage androgen-responsive disease state. Unfortunately, prostate cancer gradually progresses into an androgen-irresponsive and metastatic disease state for a great number of patients. Based on our previously presented oncogene-induced model for cancer progression, senescence has been established as a very important tumor-barrier mechanism. However, the implication of senescence in the progression of early-stage androgen-dependent to highly aggressive and metastatic castration-resistant prostate cancer (CRPC) should be further investigated.

Materials & Methods: In this study we implemented androgen-responsive (LNCaP) and –irresponsive (C4-2B and PC-3) prostate cancer cells, treated or not with enzalutamide, an Androgen Receptor (AR) inhibitor. In order to identify potential senescence regulators upon treatment with enzalutamide we conducted RNA sequencing and pathway analyses in LNCaP cells. Moreover, we continued with evaluation of the invasive potential of cells and senescence status upon AR signaling inhibition via enzalutamide and/or RNAi-mediated depletion of selected targets in all cell lines, accompanied by bioinformatics analyses on a broad range of *in vitro* and

in vivo datasets. The most important findings were also confirmed in LNCaP and C4-2B mouse xenografts. Assessment of senescence status was performed using the state-of-the-art GL13 staining by immunocytochemical staining and confocal microscopy.

Results: In this study we found that enzalutamide treatment promotes induction of senescence in androgen-dependent cells through downregulation of the replication licensing factor CDC6. Mechanistically, we show that enzalutamide in androgen-dependent cells activates the endogenous levels of GATA2 transcription factor, that functions as a *CDC6* repressor, thus resulting in CDC6 downregulation. Intriguingly, in enzalutamide-resistant cells we observed a decrease in GATA2 levels, and a consequent CDC6 stabilization followed by enhanced activation of Epithelial-To-Mesenchymal Transition (EMT) markers and absence of senescence. We demonstrate that CDC6 depletion can converse the oncogenic status and establish senescence irrespective of treatment responsiveness, thereby highlighting the importance of CDC6 for the regulation of prostate cancer progression.

Conclusions: We identify a crucial senescence-mediated GATA2-CDC6 signaling axis which is conversely regulated in enzalutamide-responsive and -irresponsive prostate cancer environments. Upon acquired resistance, GATA2 repression stabilizes CDC6, with harmful consequences in prostate cancer progression through EMT worsening and senescence abrogation. However, bypassing the GATA2-CDC6 axis by experimental silencing of CDC6 is sufficient to reverse oncogenic characteristics and induce senescence, offering a therapeutic window even upon acquiring therapy resistance.

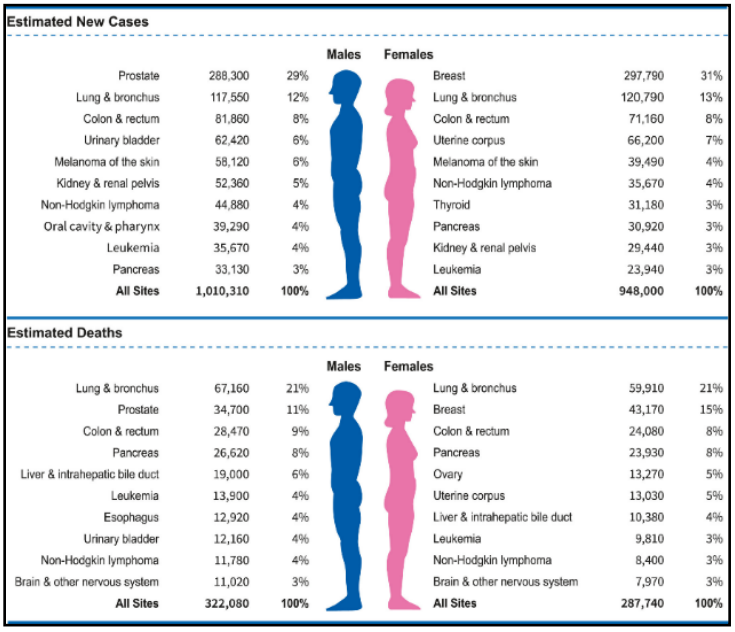
CHAPTER 1

INTRODUCTION

1.1 PROSTATE CANCER

Prostate cancer belongs in the family of hormone-dependent cancers and represents a leading cause of cancer morbidity and mortality in men worldwide. In 2023, prostate cancer was ranked as the primary cancer type for the estimated new diagnosed cases and as the second most common cause of cancer-related death, resulting in around 34,700 deaths in men in the USA (1).

Prostate is a small, about the size of a walnut, gland of the male reproductive system. It produces semen which mixes with sperm produced by the testes. Semen is required for the transport and survival of the sperm. Prostate needs androgens in order to grow and function normally (2). Androgens are sex hormones that regulate the development and maintenance of the male reproductive system as well as the secondary sexual characteristics. In men the most abundant androgens are testosterone and its potent metabolite, dihydrotestosterone (DHT) (2).



Estimated New Cases

Males			Females		
Prostate	288,300	29%	Breast	297,790	31%
Lung & bronchus	117,550	12%	Lung & bronchus	120,790	13%
Colon & rectum	81,860	8%	Colon & rectum	71,160	8%
Urinary bladder	62,420	6%	Uterine corpus	66,200	7%
Melanoma of the skin	58,120	6%	Melanoma of the skin	39,490	4%
Kidney & renal pelvis	52,360	5%	Non-Hodgkin lymphoma	35,670	4%
Non-Hodgkin lymphoma	44,880	4%	Thyroid	31,180	3%
Oral cavity & pharynx	39,290	4%	Pancreas	30,920	3%
Leukemia	35,670	4%	Kidney & renal pelvis	29,440	3%
Pancreas	33,130	3%	Leukemia	23,940	3%
All Sites	**1,010,310**	**100%**	**All Sites**	**948,000**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	67,160	21%	Lung & bronchus	59,910	21%
Prostate	34,700	11%	Breast	43,170	15%
Colon & rectum	28,470	9%	Colon & rectum	24,080	8%
Pancreas	26,620	8%	Pancreas	23,930	8%
Liver & intrahepatic bile duct	19,000	6%	Ovary	13,270	5%
Leukemia	13,900	4%	Uterine corpus	13,030	5%
Esophagus	12,920	4%	Liver & intrahepatic bile duct	10,380	4%
Urinary bladder	12,160	4%	Leukemia	9,810	3%
Non-Hodgkin lymphoma	11,780	4%	Non-Hodgkin lymphoma	8,400	3%
Brain & other nervous system	11,020	3%	Brain & other nervous system	7,970	3%
All Sites	**322,080**	**100%**	**All Sites**	**287,740**	**100%**

Figure 1.1: The 10 most common cancer types diagnosed in men and women in United States in 2023. Prostate cancer is the first most common cancer type for the estimated new cases and the second most common cause of cancer-related mortality of men in US in 2023 (1).

1.2. CELLULAR PROGRESSION OF PROSTATE CANCER

Luminal cells of the normal prostate epithelium have been characterized as origin cells of Prostate cancer. In early stages, premalignant alterations, known as prostatic intraepithelial neoplasia (PIN), occur, and among them, only the ones that are characterized as high-grade PIN (HGPIN), turn into malignant invasive prostatic adenocarcinoma and finally lead to metastasis in the lymph nodes, bone, liver, and lung through the circulation system. A variety of molecular changes have been identified during prostate cancer progression (**Figure 1.2**). For example, in HGPIN, BCL2, GSTP1 upregulation, MYC overexpression, PTEN, NKX3.1, TMPRSS2-ERG fusion loss as well as SPOP mutation have been reported. During HGPIN transformation into prostate adenocarcinoma, loss of tumor suppressor genes like PTEN and RB1 and overexpression of specific oncogenes with frequent mutations such as FOXA1, occur. Finally, during prostate adenocarcinoma progression into metastatic prostate adenocarcinoma, many molecular changes occur like overexpression and/or mutations in AR, ATM, ATR, RAD51, and CXCR4, as well as loss of various tumor suppressor genes such as SMAD4 (3).

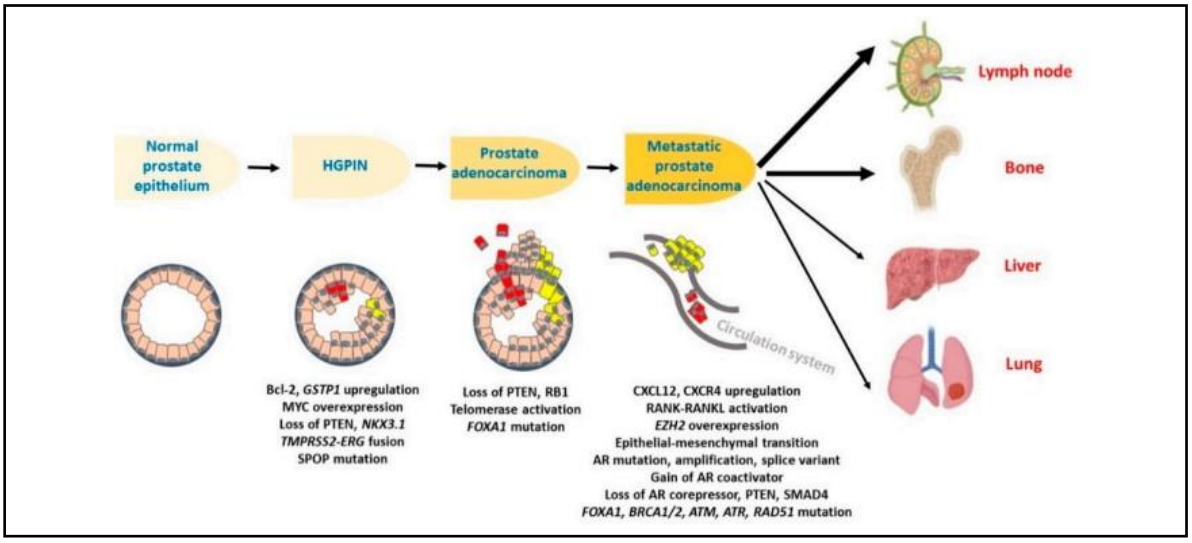


Figure 1.2: Stages of Prostate Cancer progression (3).

1.3. ANDROGEN RECEPTOR (AR): STRUCTURE AND MODEL OF ACTION

Androgen receptor (AR) is a nuclear steroid hormone receptor that functions as a transcription factor and modulates the prostate development and growth. The AR gene is located in chromosome Xq11.2-q12 and consists of 8 exons (4)(**Figure 1.3**). AR consists of four domains: the ligand-binding domain (LBD), the DNA-binding domain (DBD), the N-terminal domain (NTD), and the hinge region (HR) that separates the LBD from the DBD.

- The N -terminal domain (NTD) which is encoded by exon 1 includes the Activating factor 1(AF1). AF1 region functions as the major effector region of NTD. Moreover, the AF1 region consists of 2 transcription activation units, Tau-1 and Tau-5 that are crucial for the transcriptional function of the AR. Tau-1 and Tau-5 motifs, ensure the functional NTD-LBD interaction which is indispensable for the stability of the AR dimer complex (5).

- The DNA-binding domain (DBD) which is encoded by exons 2 and 3, consists of two zinc finger motifs; the α-helix N-terminal zinc finger encoded by exon 2, which interrelates with the nucleotides in the hormone response element in DNA and the zinc finger encoded by exon 3. The nuclear localization signal which is located in the DBD-HR junction is required for the nuclear transport of the AR (6, 7).

- The Ligand binding domain (LBD) which is encoded by exons 4–8, is necessary for the binding of AR to its ligands, testosterone and its metabolite dihydrotestosterone (DHT). The LBD is the most prone domain of AR gene to AR point mutations in prostate cancer (8).

- The Hinge region (HR) which is a short amino acids sequence carries a nuclear localization signal (NLS) required for the nuclear transport of the AR (8).

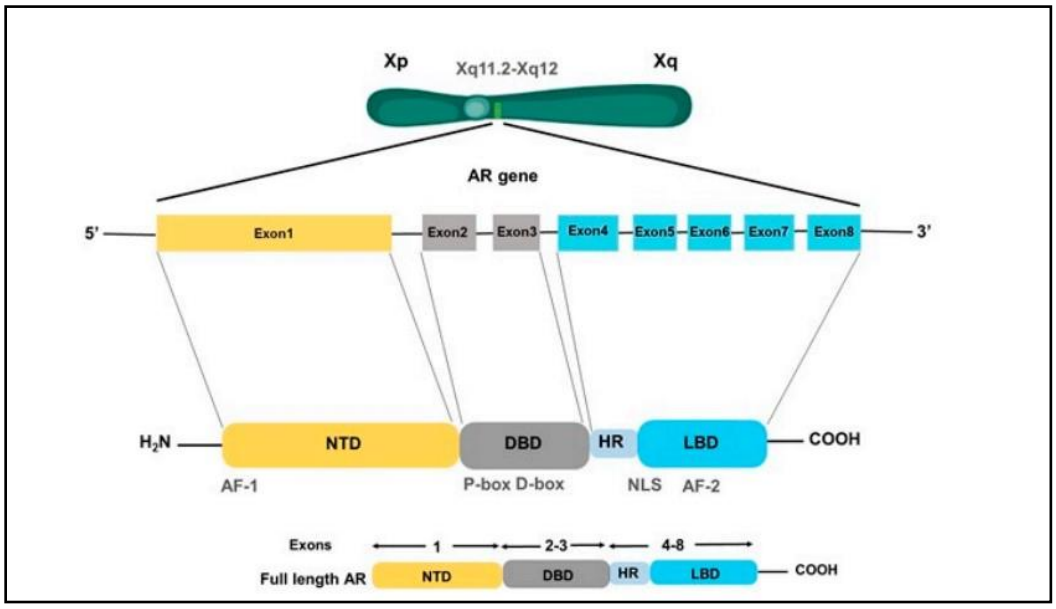


Figure 1.3: Structure of AR gene (3).

Almost all types of early-stage prostate cancer require androgens that activate the androgen receptor (AR) signaling pathway, thereby promoting cell survival (9-12).

There are two models of action of AR; the canonical (Genomic) and the Non-Canonical (Non-genomic) model.

Regarding the AR canonical model of action, before binding to androgens, AR is in a inactivated state in the cytoplasm, where it is sequestered by many chaperone and co-chaperone proteins such as heat-shock proteins, through its ligand-binding domain (13). When androgens bind to the Ligand Binding Domain (LBD) of AR, AR dissociate from the chaperone proteins, undergoes conformational changes which promote its dimerization. AR homodimer interacts with the cytoskeletal protein Filamin A which helps AR transition into the nucleus. Nuclear AR homodimers bind to specific regions in the promoter of downstream target genes, known as androgen response elements (AREs) via their association with various co-regulators (14, 15). These

complexes then, recruit and stabilize the RNA polymerase II machinery to begin gene transcription (3).

Moreover, it has to be mentioned that AR signaling is not only activated by the binding of androgens but also by growth factors like EGF and cytokines such as IL-6. In the absence of androgens, EGF can promote the transcriptional activation of AR via RAS/RAF/MAPK and PI3K/Akt signaling pathways (16-18). Furthermore, binding of IL-6 to IL-6 receptor can transcriptionally activate AR through MAPK and STAT3 pathways (19).

Except from the genomic model of AR action, androgens can also promote many cellular responses independently of the AR-binding to downstream gene promoters. AR also interact with other molecular factors located in the cytoplasm and the inner cell membrane inducing the activation of signaling cascades (20, 21)

Regarding the non-Canonical model of AR action, AR that is either bound in cell membrane via palmitoylation or transported to membrane via Caveolin 1, interacts through its N -terminal domain with the SH3 domain of SRC proto-oncogene (Src). This interaction promotes the unfolding of Src and the activation of its kinase domain and subsequently its autophosphorylation. Activation of Src then promotes the Ras-mediated MAPK/ERK signaling cascade (22). Membrane-bound AR can also promote cytoskeletal reorganizations through activation of FAK/Cdc24/Rac1/PI3K signaling cascade, thereby regulating the adhesion and migration of prostate cancer cells (23, 24).

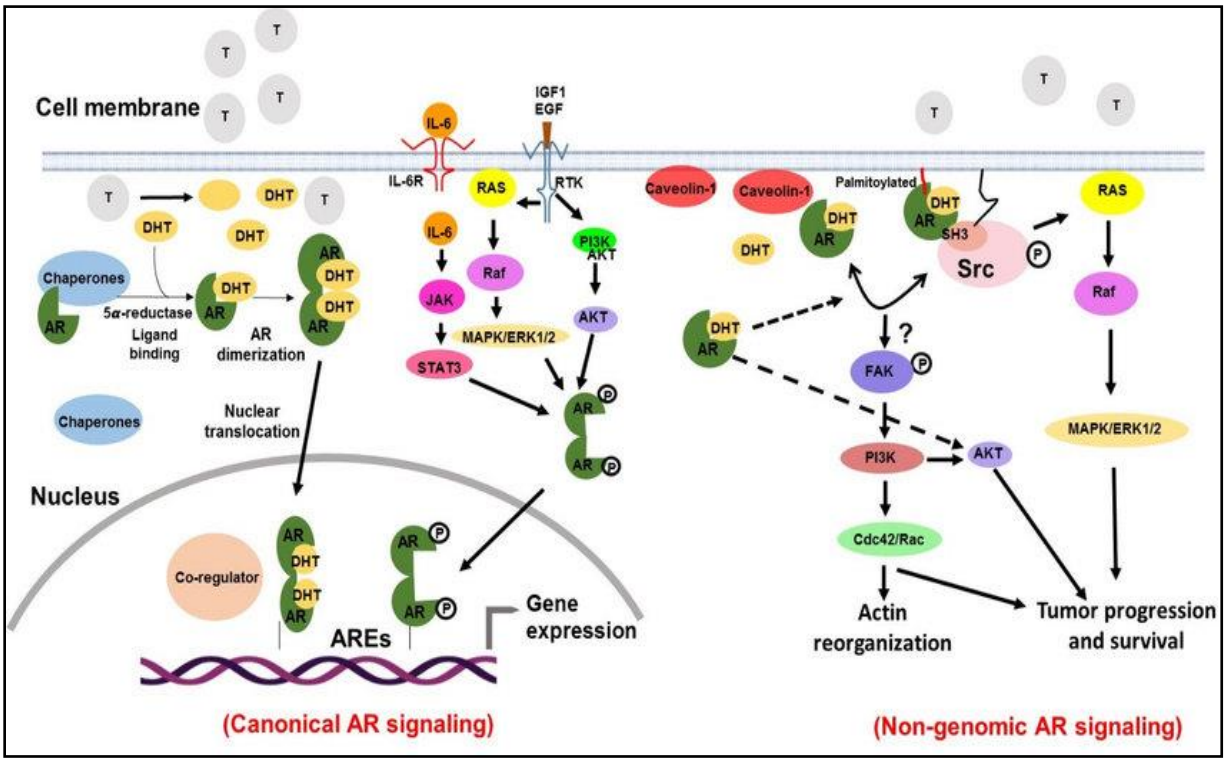


Figure 1.4: Canonical (Genomic) and Non-Canonical (Non-genomic) AR signaling pathway (3).

1.4 TARGETING AR SIGNALING PATHWAY (3)

Since AR signaling pathway is a very crucial regulator of prostate cancer cell growth and development, its targeting has been thoroughly studied for its therapeutic benefits in Prostate Cancer. Targeting of AR signaling node consists of either targeting AR ligands or targeting AR itself. Generally, androgen deprivation therapy (ADT) is applied during early-stage disease as it decreases the levels of testosterone, performed either by surgical or medical castration (25-27). Although ADT has been effective only for the early stages of prostate cancer when there is dependence on androgens, prostate cancer finally progresses to an androgen-independent disease state (28, 29). The mechanisms implicated in the acquired therapy resistance in the field of prostate cancer still remain elusive. Moreover, AR signaling pathway can be indirectly suppressed by targeting its interaction with co-factors.

- **Targeting AR ligands**

Physiologically, hypothalamus regulates androgen biosynthesis and secretion by testes. Hypothalamus releases Luteinizing Hormone-Releasing Hormone (LHRH), which then causes the pituitary gland to release Follicle Stimulating Hormone (FSH) as well as Luteinizing Hormone (LH) (3). LH modulates the conversion of cholesterol to dehydroepiandrosterone and androstenedione on the Leydig cells of testes. Androstenedione is then converted to testosterone through the action of 17-beta-hydroxysteroid dehydrogenase (3). It has to be highlighted that testosterone can be further enzymatically converted to dihydrotestosterone (DHT). On the other hand, FSH promotes the expression of LH receptors on the Sertoli cells within the testes. As a result, LHRH targeting via agonists or antagonists reduces serum androgen levels (3).

LHRH Agonists (3)

LHRH agonists (also known as LHRH analogs) has been a treatment option for Prostate Cancer in the United States since 1984 (30). LHRH agonists are synthetic peptides with structural similarity with LHRH. LHRH agonists bind to LHRH receptor on the pituitary gland, block the release of LH, resulting in reduced stimulation of Leydig cells and consequently decreased androgen synthesis. Leuprolide (Lupron) is one of the currently used LHRH agonists for the treatment of Prostate Cancer. Lupron promotes a continual stimulation of the anterior pituitary that desensitizes the LHRH receptor, leading to an extended reduced release of FSH and LH and therefore suppresses androgen synthesis (31). However, the first administration of LHRH agonists triggers an increase in testosterone levels that can drive a tumor flare reaction in many patients with advanced-stage Prostate Cancer (32, 33). In order to avoid these harmful adverse reactions, one treatment option that has been tested in the field of Prostate Cancer, is the combination of Leuprolide with other agents that also target the AR signaling pathway, such as bicalutamide(34).

LHRH Antagonists (3)

LHRH antagonists regulate more efficiently the testosterone levels compared with LHRH agonists (35). LHRH antagonists directly impede the binding of LHRH to its receptor, leading to immediate LH blocking and as a result in suppressed testosterone and DHT levels (36, 37). Abarelix, which was the first used LHRH antagonist against advanced Prostate Cancer, based on findings from clinical trials, did not promote an increase in testosterone levels like LHRH agonists did (38). However, in some patients, Abarelix was found to cause systemic allergic reaction due to release of histamine that Abarelix tiggered (39). Degarelix is a new generation FDA-approved for the treatment of advanced Prostate Cancer LHRH antagonist (40). Degarelix can effectively reduce testosterone levels without harmful side effects such as testosterone surge or allergic reactions(36, 41).

Androgen Synthesis Inhibitors (3)

One of the reasons that castration therapy can fail is the intra-tumoral synthesis of androgens as well as the adrenal androgens (42, 43). Consequently, during the years, it has been unmet need to develop new therapeutic strategies that would target the biosynthetic enzymes involved in androgen synthesis. Ketokonazole, is a second-line therapeutic agent against mCRPC, which suppresses gonadal and adrenal androgen synthesis through inhibition of the cytochrome P450 (CYP450) (44-46). Abiraterone acetate is the first FDA-approved drug with remarkable antitumor activity in patients with CRPC. Abiraterone acetate suppresses both adrenal and intra-tumoral steroidogenesis through inhibiting the action of Cytochrome P450 (CYP) 17α-hydroxylase and 17,20-lyase (47). Orteronel (TAK-700) is a new drug that inhibits human CYP17A1 (48) and Finasteride, inhibits the action of 5-reductase enzyme and consequently blocks the conversion of testosterone to its metabolite DHT, resulting in reduced DHT synthesis (49).

- Targeting the Receptor

Taking into consideration the drawbacks of the ligand-targeted therapeutic options for Prostate Cancers, the development of new therapeutic strategies that directly target AR, was necessary.

These strategies mostly include steroidal or non-steroidal anti-androgens that inhibit the binding of the ligand to the AR.

Steroidal Anti-Androgens (3)

Cyproterone Acetate (CPA) is the most widely used steroidal antiandrogen for the treatment of Prostate Cancer. CPA derives from hydroxyprogesterone, which has stronger binding affinity for the AR compared to the first-generation non-steroidal antiandrogens (50, 51). Moreover, it has to be stressed that steroidal antiandrogens were also associated with harmful side effects such as hepatotoxicity and cardiovascular diseases (52).

Non-Steroidal Anti-Androgens (3)

Non-steroidal agents have shown to have fewer adverse effects compared to the steroidal anti-androgens. Bicalutamide is a first-generation non-steroidal anti-androgen that binds to AR promoting a conformational change in the co-activator binding site and therefore mediates its transcriptional activity (53). Flutamide blocks the ligand binding to the AR (54). Nilutamide antagonizes with testosterone and DHT for binding to the AR (55). Enzalutamide and Apalutamide are second-generation FDA-approved for mCRPC non-steroidal antiandrogens with very strong AR affinity that inhibit AR nuclear translocation. Some of the harmful effects of Enzalutamide are fatigue and diarrhea (56-58).

- **Targeting AR Interaction with Co-Regulators**

AR signaling node can also be modulated indirectly through targeting AR co-receptors or chaperon proteins. The family of Steroid receptor coactivators (SRCs/p160/NCOA) includes three transcriptional coregulators that associate with steroid receptors in a ligand-dependent manner inducing their transactivation (59, 60). Pharmacological inhibition of SRC is shown to suppress tumor growth (61). Heat shock proteins (HSPs), as it was mentioned above, have a crucial role in AR signaling pathway (62). HSP90 is a part of a multi-chaperone protein assembly that stabilizes

AR in the cytoplasm. After ligand binding to AR, HSP90 dissociates from AR promoting its nuclear translocation (63, 64). A variety of inhibitors that target either HSPs or their co-chaperones, have been developed through years, and regulate the transcriptional activity of AR (65).

1.5 CELLULAR SENESCENCE AND ITS ROLE IN PROSTATE CANCER

Senescence is defined as a cellular state, characterized by stable cell-cycle arrest that can be induced by a variety of stimuli and different mechanisms, not only in vitro but also in vivo, and this is the reason why senescence has been established as an anti-tumor barrier (66). There are different types of cellular senescence and each type is induced by a different type of stressors.

The first type of cellular senescence that was described is the **replicative senescence**, due to telomere length restriction. Telomeres are protein-bound 5'-TTAGGG-3' repeat sequences that cap the ends of chromosomes protecting them from the effects of the DDR pathway and degradation (67). In each round of replication DNA polymerase cannot fully copy the DNA at the ends of chromosomes resulting in their gradual shortening to such an extent that they are no longer protected (68).

These dysfunctional telomeres mimic double strand breaks (DSBs) and trigger the activation of the DDR pathway leading to replicative senescence (67, 69, 70). Cancer cells bypass this barrier through activation of the hTERT enzyme. However, it has to be mentioned that telomerase does not inhibit telomere-independent mechanisms of cellular senescence (71).

Another type of cellular senescence induced by chemotherapeutic drugs is the **therapy-induced senescence**. Radiation therapy and various anti-cancer agents promote DNA damage, mainly DNA double strand breaks (DSBs), driving to senescence in many cell types mainly through activation of p53 pathway with subsequent increase of $p21^{WAF1/CIP1}$ protein (70, 72) or through increase of $p16^{INK4A}$ protein (73, 74). Activation of $p16^{INK4A}$ is necessary for a permanent cell cycle arrest (73).

Normal cells respond to oncogene activation with induction of senescence (**oncogene-induced senescence)**. The phenomenon was first observed in the RAS gene (75) and later extended to other factors of the signaling pathway, such as RAF, MEK, MOS, BRAF (76, 77), as well as other factors associated with proliferation such as E2F1 (78). Oncogenes and the subsequent increase in mitotic potential, trigger DNA damage leading to replicative stress and activation of the DDR pathway (79-81). Furthermore, based on in vivo studies, oncogene-induced cellular senescence that is evident in precancerous lesions, functions as an antitumor barrier (77, 79, 81-85). Additional mutations, often in the *TP53* (p53) or *CDKN2A* ($p16^{INK4A}$) genes, are required in the carcinogenesis process to reverse the growth inhibition (82, 83, 85).

Stress induced by reactive oxygen species or prolonged signaling of cytokines that inhibit cell proliferation, such as interferon β or TGF-β, have been shown to induce cellular senescence (**stress-induced senescence**) (86-88). Similarly, stress that is generated during epithelial cell culture *in vitro*, induces cellular senescence through the $p16^{INK4A}$ pathway, making $p16^{INK4A}$ inactivation necessary for immortalization of normal epithelial cells (89-91).

Finally, alterations in chromatin structure are able to induce cellular senescence. The state of chromatin that determines which genes are active (euchromatin) and which are inactive (heterochromatin) is regulated by histone modifications. Agents that affect histone conformation, such as deacetylase inhibitors, can alter the expression of a variety of genes resulting in cellular senescence(86).

As it was mentioned above, in the field of cancer therapy, radiation therapy and many anti-cancer agents can promote senescence induction in cancer cells (92). Although senescent cells are characterized by an irreversible cell cycle arrest, they often remain metabolically active *in vivo* for months and even for years. Once these cancer cells reenter the cell cycle, cancer can relapse with detrimental effects (66). Moreover, senescent cells that remain dormant can affect their adjacent cells since they secrete high levels of inflammatory cytokines, immune modulators, growth factors, and proteases, which are conjointly termed as senescence associated secretory

phenotype (SASP) (66). In the field of Prostate Cancer, ADT was proven to promote the induction of senescence in prostate cancer cells not only *in vitro* but also *in vivo* (93, 94). Androgen deprivation-induced senescence is characterized by a G1/S cell-cycle arrest, decreased cyclin-dependent kinase activity and hypophosphorylated Rb (95). However, the growth arrest induced by androgen deprivation therapy is not permanent, as cells recover their proliferative ability, when the levels of androgens are restored to normal levels, progressing finally to a state of androgen independence. As a result, androgen deprivation- induced senescence is a temporary cell state that promotes the emergence of CRPC populations supporting the role of senescence as a possible driver of cancer progression (93, 95). The exact involvement of senescence in this process of transformation remains unclear and needs further investigation.

1.6 Cell Division Cycle 6 (CDC6): A CARDINAL FACTOR OF REPLICATION LICENSING MACHINERY

DNA replication is a crucial process in each cell cycle, as it is a necessary condition for the accurate chromosome segregation so that each daughter cell receives a complete and accurate copy of the organism's genome. It is the process by which genetic information is preserved and transmitted as unchanged as possible from cell to cell and from generation to generation. DNA replication of eukaryotic cells occurs only once during S phase of the cell cycle (96).

DNA replication is a tightly controlled process, carried out by huge variety of enzymes that ensure its accuracy and speed. DNA replication is mediated by a dynamic protein complex, termed as replisome, and it consists three stages; initiation, elongation, and termination (97).

The initiation stage of DNA Replication process does not occur randomly within the genome, but in specific regions called origins of replication (ORI). The number of ORI depends on the size of the genome and varies between different species. This stage is the first important step in the sequence of events that responds to mitogenic signals and it is divided into two stages, origin

licensing and origin firing, which take place at different times. These are carefully controlled events determined by periodic fluctuations of CDKs (98).

Replication licensing occurs at the end of M phase and throughout the G1 phase of cell cycle, when pre-replicative complexes (pre-RCs) are formed. Firstly, the hexameric origin recognition complex (ORC) binds to DNA at the ORI and attracts the replication licensing factors (RLFs), CDC6 and CDT1. Then, the RLFs cooperate to recruit to the double-stranded DNA, sequentially, two hexameric minichromosome maintenance 2-7 helicase complexes (MCM 2-7) in an inactive state. It is worth noting that DNA-bound MCMs are always in excess of those that are triggered physiologically in order to be used in case of replicative stress (99).

CDC6 belongs to the family of AAA+ ATP hydrolases (ATPases associated with a variety of cellular activities) and it is related to the protein ORC1 and the proteins ORC4, ORC5 and MCM2-7 (100). Regulation of CDC6 activity plays a key role in the assembly of the pre-replicative complex (pre-RC), during the cell cycle. Studies support that its function depends on its ability to bind and hydrolyze nucleotides. Its binding to nucleotides is associated with morphological changes. In cases of CDC6 inability to bind to ATP, the entry into S phase of cell cycle is affected, while the inability of hydrolysis does not allow the completion of S phase (101).

CDC6 phosphorylation by CDKs in S phase leads to its translocation from the nucleus to the cytoplasm, while CDC6 is degraded by the APC/CCDH1 complex during mitosis (102). More recent data show that CDC6 is also degraded via the CUL4-DDB1-CDT2 axis following its interaction with the proliferating cell nuclear antigen (PCNA) (103), as well as through its binding with the $SCF^{CyclinF}$ protein (104). With these mechanisms, a cell avoids its DNA re-replication (104, 105).

At the C-terminal domain of CDC6 is the Winged-Helix domain, which is common in transcription factors. Possibly, this domain is related to CDC6 binding to ORI or even other regions on DNA. Studies have shown that this specific region mediates interprotein interactions between CDC6 and other AAA+ ATPases of the pre-RC complex forming the substrate for the binding of the MCM2-7 complex (106).

1.7 REPLICATION LICENSING FACTORS AND CANCER

Tight regulation of transcription is crucial for the normal development of multicellular organisms, and its dysregulation has been linked to more than 40 human diseases, including cancer (107). As the transcriptional licensing factors, RLFs, are the first molecules to detect and respond to the mitogenic/oncogenic signals, their deregulation should be one of the most common hallmarks of cancer and may occur from the earliest stages of cancer development, when oncogene overexpression prevails (98).

Based on *in vivo* studies, increased levels of the RLFs, CDC6 and CDT1, have been observed in several types of cancer (108-110). More specifically, high levels of CDC6 occur in 55% of brain cancers, in 50% of malignancies in non-small cell lung cancer, where it is associated with a poor prognosis when there is p53 loss (108), in lymphomas, in cervical cancer, but also in colon, stomach and head and neck cancer (110).

In further analysis of precancerous lesions, it was shown that in all types of cancer studied (lung, colon, head and neck) CDC6 increases from the initial stages of carcinogenesis, specifically at the level of dysplasia (**Figure 1.5**) (109). However, high levels of CDC6 protein does not necessarily mean an increase in the proliferation of cancer cells, since no such connection was observed in non-small cell lung cancer (108).

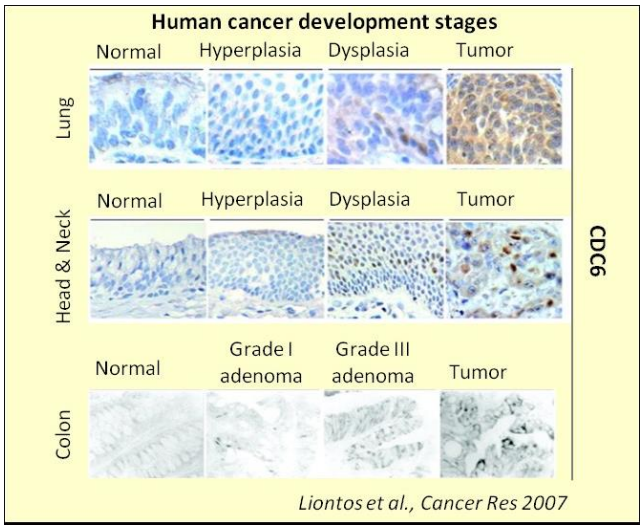


Figure 1.5: Elevation of CDC6 levels from the early stages of carcinogenesis, to the level of dysplasia (109).

1.8 MECHANISMS THAT DYSREGULATE CDC6 EXPRESSION

Since CDC6 is a cardinal molecule of the replication licensing machinery, its overexpression in precancerous and cancerous lesions could be attributed to the increased needs of cells for proliferation.

However, the fact that no correlation between CDC6 and the proliferation marker Ki67 has been found, nor it is detected in hyperplasias and normally dividing tissues (109), indicates CDC6 disturbed expression and/or regulation.

Many molecular events acting either independently or in combination, contribute to CDC6 overexpression in cancer.

a) Overexpression of E2F1/2 transcription factors, that is common in several malignancies due to deregulation of the pRB-E2F pathway, leads to overexpression of CDC6 and CDT1 (111-114).

b) CDC6 and CDT1 gene amplification frequently leads to increased gene expression (109). It is worth noting that CDC6 is located at the 17q21.3 locus in close proximity to the ERBB2

gene locus (encoding HER2) which is amplified in cancer (115-117). Thus, CDC6 amplification could be an independent event or a consequence of ERBB2 amplification.

c) The production of a shorter mRNA isoform due to application of an alternative poly-adenylation site may lead to the accumulation of CDC6 in tumors. This isoform lacks part of the 3' untranslated region, which is the target of several micro-RNAs (miR25, miR541, miR92a/b) responsible for CDC6 degradation, leading to increased transcript stability and higher protein levels (98, 118).

d) The E3 ubiquitin ligase complex CUL4-DDB1-CDT2 leads to degradation of the chromatin-bound RLFs, CDC6 and CDT1, $p21^{WAF1/CIP1}$ protein, as well as other factors during S phase. Under conditions of sustained accumulation of $p21^{WAF1/CIP1}$, degradation is inhibited due to enzyme saturation, resulting in an increase in CDC6 and CDT1 (**Figure 1.6**) (119).

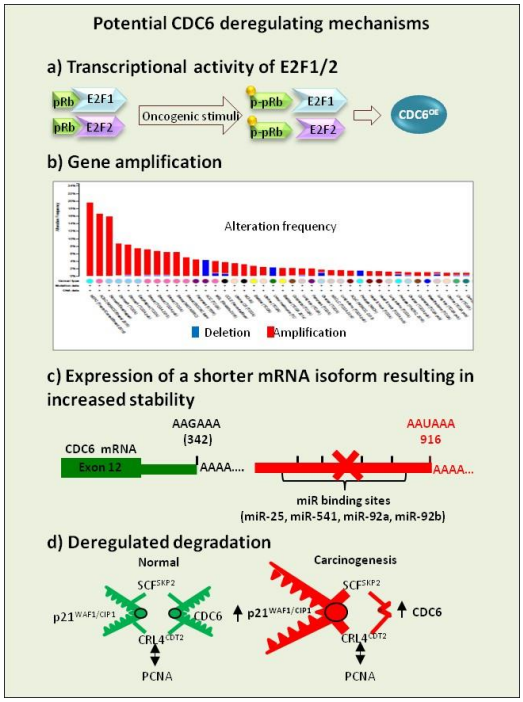


Figure 1.6. Summary of molecular events leading to increased CDC6 expression levels in cancer (Komseli E-S, PhD Thesis, 2018).

With the above mechanisms acting either individually or synergistically, the tight regulation of CDC6 levels, that exhibit characteristic fluctuations during the cell cycle, is disrupted, resulting in abnormal CDC6 accumulation with adverse pathophysiological consequences (98).

1.9 CDC6 ONCOGENIC ACTIVITY

In normal cells, overexpression of CDC6 leads to replication stress, resulting in the induction of the antitumor barrier of senescence, through activation of DDR pathway (79). Experiments in cancerous and precancerous cell lines showed that prolonged overexpression of CDC6 induces re-replication, replicative stress and activation of DDR mechanisms, resulting in genomic instability and selective loss of p53 (109, 110). Corresponding findings were also detected in the case of an indirect increase in RLFs due to the $p21^{WAF1/CIP1}$ overexpression in the absence of p53 (119), while the silencing of RLFs results in DNA re-replication (104).

In summary, deregulated expression of CDC6 has oncogenic characteristics. According to the oncogene-induced model of cancer development, overexpression of oncogenes, such as CDC6, leads to the activation of antitumor barriers of apoptosis or cellular senescence, through induction of the DDR pathway (79, 120). This mechanism is activated by replication stress and by re-replication in the case of CDC6. Prolonged replication stress leads to genomic instability with consequent loss of tumor suppressor genes, such as TP53, bypass of anti-tumor barriers and finally cancer progression (120).

Moreover, CDC6 overexpression in precancerous and cancerous epithelial cells with RAS gene mutations induces the acquisition of mesenchymal cell (EMT) characteristics, with subsequent loss of E-cadherin expression which is a key feature of EMT phenotype (109, 110). Repression of E-cadherin (*CDH1*) gene transcription is achieved through CDC6 binding to the promoter at specific regions called E-boxes. This binding leads to the removal of the transcriptional regulator CTCF and histone H2A.Z from the promoter resulting in heterochromatinization and silencing of

the gene. At the same time activation of a neighboring origin of replication takes place (**Figure 1.7**) (110).

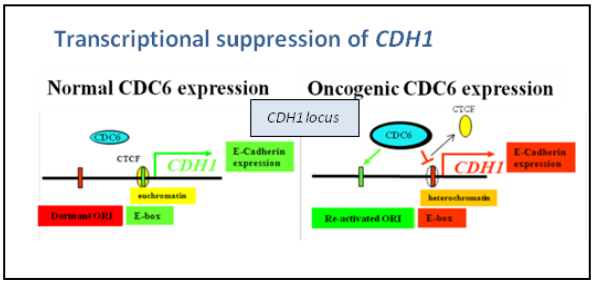


Figure 1.7: Proposed model for the involvement of CDC6 in transcriptional regulation of *CDH1* (110).

1.10 CDC6 AND PROSTATE CANCER

Cell Division Cycle 6 (CDC6) constitutes an AR transcriptional target gene. AR binds at specific regions in the promoter of Cdc6, named androgen-response element (ARE) sites, and this binding is necessary for androgen-dependent Cdc6 transcription. The peak of AR occupancy at the ARE sites happens during the G1/S phase of cell cycle similar with the peak of Cdc6 mRNA expression. Moreover, silencing of AR in prostate cancer cells leads to decreased Cdc6 expression as well as decreased androgen-dependent cellular proliferation. Conjointly, it is clear that Cdc6 is a crucial mediator of AR signaling that facilitates the comprehension of the mechanisms involved in prostate cancer cell proliferation (121).

Moreover, as it was mentioned again, prolonged CDC6 activation finally leads to “escape” from cellular senescence, and a bulk of senescent cells re-initiate proliferation through distinct genetic and epigenetic events, caused by the accumulated genomic instability (119, 122, 123). However, the role of CDC6 in prostate cancer as well as the implication of CDC6 in the regulation of senescence in the context of prostate cancer have not been thoroughly addressed and need further investigation.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL LINES

For this work we used the human prostate cancer cell lines LNCaP, C4-2B and PC3. The LNCaP and the enzalutamide-resistant C4-2B cell line were generously donated by MDAnderson Cancer Center in Houston, Texas of USA. Moreover, the enzalutamide-resistant PC-3 cell line was obtained by ATCC. The culture conditions of all cell lines included cell culture in RPMI medium (10% fetal bovine serum (FBS), 1% penicillin/streptomycin), maintenance at 37 °C and 5% CO_2 and growth to 70% confluency.

2.2 INHIBITION OF AR SIGNALING

The pharmacological inhibition of AR signaling node in all cell lines was carried out via 5-day treatment with Enzalutamide at a final concentration of 10 μM (stock concentration 10 mM). We replenished every 2 days enzalutamide in the cell culture medium and as control, we added DMSO in the cell culture medium at 0.1% final concentration. Enzalutamide (MDV3100) was purchased from Selleckchem (Catalog No. S1250).

2.3 RNA INTERFERENCE (RNAi) EXPERIMENTS

For siRNA-mediated silencing in LNCaP, C4-2B and PC-3 cell lines, we used Lipofectamine 2000 (Invitrogen) following the manufacturer instructions. The oligonucleotides that were used for these RNAi experiments are the following: non-targeting (CTRL): UAAGGUAUGAAGAGAUAC (Dharmacon) and siRNAs to GATA2 (GS2624, set of 4) that were obtained from Qiagen (stock concentration 10μM, used concentration 100 nM) and CDC6 Stealth siRNAs (HSS101647, HSS101648, HSS101649; set of 3) that were obtained from ThermoFischer Scientific (#1299001) (stock concentration 20μM, used concentration 150 nM).

2.4 PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

In order to extract total protein from cells and tissue samples, we washed them with cold PBS before their lysis with Laemmli Lysis Buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 50 mM NaF, 10 mM β-glycerophosphate, 0.5 mM Na_3VO_4 and 1 × EDTA free protease inhibitors) and then we normalized the cell lysates using the NanoDrop (Thermo Scientific). Western blotting was carried out as previously described by Papasyropoulos et al. (124). In brief, samples after their loading on 8-10% acrylamide/bis-acrylamide gels were transferred onto PVDF membrane (Millipore)and then were immunoblotted with the relevant primary antibodies overnight at 4°C. Primary antibodies that were used in this study are the following; AR (1:000 dilution, Cell Signaling #5153), PSA (1:1000 dilution, Cell Signaling #2475), CDC6 (1:200 dilution, Santa Cruz #9964), $p21^{WAF1/Cip1}$ (1:500 dilution, Cell Signaling #2947), E-Cadherin (CDH1; 1:500 dilution, Cell Signaling #3195) and GAPDH (:2000 dilution 1, Cell Signaling #5174). The HRP-linked secondary antibodies anti-mouse (Cell Signaling #7076) and anti-rabbit (Cell Signaling #7074) were used at a dilution of 1:1000.

2.5 CELL PROLIFERATION ASSAY

In order to investigate potential effects of Enzalutamide in the proliferation of LNCaP and C4-2B cells we performed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (CellTiter 96® Non-radioactive Cell Proliferation Assay, Promega). For this assay, we seeded 8000 cells from each cell line into a 96-well plate in octuplicate, and then cells were incubated at 37 °C and 5% CO_2. Then, we treated the cells either with Enzalutamide or DMSO as control. The process of the samples was achieved following the manufacturer's instructions. After we added the MTT solution in the culture medium, cells were maintained in the incubator for 2-4 h. Then we measured their absorbance at 570 nm using a 96-well plate reader (Anthos 2010 Microplate

Reader, Biochrom). We used as blanks of absorbance three control wells with only culture medium.

2.6 *IN VITRO* MIGRATION AND INVASION ASSAYS

For these assays, PC-3 cells were cultured in DMEM medium containing 10% FCS in 6-well dishes. When cells reached a 90% confluency, we changed the medium with fresh one containing or not enzalutamide at a final concentration of 10 μM. To evaluate cell migration, three days later we scraped the cell layer in a straight line using a 200 μl pipette tip. After removing the floating cells, we washed the remaining ones with PBS and then we added again fresh medium (with or without enzalutamide). We photographed the cell cultures under a Nikon Eclipse TS2 microscope immediately after scratching (0 hours) and after 24 hours. To evaluate the invasive potential of PC-3 cells (before and after enzalutamide treatment), we used a cell invasion assay kit (Chemicon), following the manufacturer's instructions.

2.7 RNA EXTRACTION, cDNA PREPARATION and qRT-PCR

In order to isolate total RNA from cells and tissue samples we used Nucleospin RNA extraction kit (Macherey-Nagel #740955) accordingly to the manufacturer's instructions. For the cDNA synthesis was we used the GoScript Reverse Transcriptase kit (Promega; A5000). Quantitative Real-Time PCR was carried out as previously described (124, 125) using the Power SYBR Green Cells-to-Ct kit protocol (Applied Biosystems). After we prepared the cDNA, we diluted it down 4 times in Nuclease-free water. Then, we produced the PCR Cocktail mix including the forward and reverse primers. PCR reactions were performed in 96-well plates (Life Technologies) and the PCR instrument (Applied Biosystems) was programmed as follows: Stage 1; Enzyme activation (hold), repeats 1, temperature 95 °C, time 10 min, Stage 2; PCR (cycle), repeats 40, Step 1; Temperature

95 °C, time 15 s, Step 2; Temperature 60 °C, time 1 min, Stage 3. Dissociation curve Step 1; Temperature 95 °C, time 15 s. Step 2; Temperature 60 °C, time 1 min, Step 3; Temperature 95 °C, time 30 s, Step 4; Temperature 60 °C, time 15 s. The primer sequences that we used are the following: E-CADHERIN (CDH1) forward: 5'-GCCTCCTGAAAAGAGAGTGGAAG-3' and reverse 5'-TGGCAGTGTCTCTCCAAATCCG-3', SNAIL (SNAI1) forward 5'- TGCCCTCAAGATGCACATCCGA-3' and reverse 5'- GGGACAGGAGAAGGGCTTCTC-3', ZEB1 forward 5'- GGCATACACCTACTCAACTACGG-3' and reverse 5'-TGGGCGGTGTAGAATCAGAGTC-3', GATA2 forward 5'-CAGCAAGGCTCGTTCCTGTTCA-3' and reverse 5'- ATGAGTGGTCGGTTCTGCCCAT-3', GAPDH forward 5'- TGCACCACCAACTGCTTAGC-3' and reverse 5'- GGCATGGACTGTGGTCATGAG-3'. We performed three independent experiments to average our results and we quantified them using the relative the 2^-ΔΔCt method.

2.8 IMMUNOFLUORESCENCE

For Immunofluorescence staining, we seeded cells at 70% confluency on coverslips, we fixed them applying 4% PFA in PBS for 10 min in 4^{o}C and then we permeabilized them applying Triton-X 0.3%/PBS for 15 min. Blocking of non-specific binding of antibodies was carried out by applying normal goat serum for 1 h at RT (dilution 1:40, Abcam ab138478). Then, in order to detect the lipofuscin, we stained the cells using SenTraGor^{TM} reagent as described in the SenTraGor^{TM} staining section below. The visualization of senescent cells was carried out using primary anti-biotin conjugated fluorescent antibody (dilution 1:100, Biotium BNC610400-100) for 1h at RT. Then, we washed the cells with PBS and we stained them with primary antibody anti-p21$^{WAF1/Cip1}$ (dilution 1:200, Cell Signaling #2947) for 1h at RT. After washing with PBS, we applied secondary antibody (Goat Anti-Rabbit: dilution 1:500, Abcam ab150077) for 1h at RT. We washed the cells with PBS and and we counterstained their nuclei with DAPI. Mounting of cells was performed

after washing them with dH_20 for 30 sec. In the end, we observed the cells under the Leica TCS-SP8 confocal microscope on the 10x Objective (100x magnification).

2.9 IMMUNOCYTOCHEMISTRY

Cells were seeded, fixed, permeabilized and blocked as previously reported in the Immunofluorescence section. Then we applied the SenTraGor™ reagent as will be described in the SenTraGor™ staining section below. We continued applying primary antibodies for 1h at RT (anti-biotin antibody (dilution 1:300, Abcam ab201341), anti-Ki67 (dilution 1:200, Abcam ab16667), anti-cleaved Caspase-3 (dilution 1:300, Cell Signaling #9664)). The development of positive signal was achieved using the Dako REAL EnVision Detection System, (Cat.no: K5007) following the manufacturer's instructions. Then, we stained the cells with Hematoxylin and we mounted them. In the end, we observed the cells under a ZEISS Axiolab5 microscope on the 10x, 20x or 40x Objectives (100x, 200x and 400x magnification, respectively).

2.10 IMMUNOHISTOCHEMISTRY

For Immunohistochemistry staining, we obtained 4 μm sections from formalin-fixed paraffin embedded (FFPE) mouse tumors. Tumor sections were deparaffinized, hydrated and antigen retrieval was performed by heating them in 10 mM citric acid (pH 6.0) for 10 minutes. Then we applied SenTraGor™ reagent as will be described in the SenTraGor™ staining section below. Blocking of non-specific binding of antibodies was carried out by applying normal goat serum for 1 h at RT (dilution 1:40, Abcam ab138478). Then we applied primary antibodies (anti-biotin antibody (dilution 1:300, Abcam ab201341), primary anti-Ki67 (dilution 1:200, Abcam ab16667)) and we incubated the sections overnight at 4 ˚C. Development of positive signal was achieved using the Dako REAL EnVision Detection System, (Cat.no: K5007) following the manufacturer's

instructions. In the end, we observed the tumor sections under a ZEISS Axiolab5 microscope on a 20x Objective (200x magnification).

2.11 SenTraGor™ (GL13) STAINING

SenTraGor™ staining was performed as previously described (126, 127). More specifically, we seeded cells on coverslips and we obtained tissue sections and after their blocking we applied in the beginning 50% ethanol for 5 min, 70% ethanol for 5 min and then SenTraGor™ for 10 min at 37^{o}C. Then we washed the cells/tissue sections with 50% ethanol for 2 min and then with 1X PBS. We removed excess amount of SenTraGor™ with a 3-min wash with Triton-X 0.3%/PBS. After washing cells/tissue sections with PBS we applied primary anti- biotin antibodies for 1 h at RT (dilution 1:300, Abcam ab201341 in the case of immunocytochemistry and immunohistochemistry; dilution 1:100, Biotium BNC610400-100 in the case of immunofluorescence).

2.12 TRANSMISSION ELECTRON MICROSCOPY

For this assay, we cultured LNCaP and C4-2B cells up to 80% confluence and then we fixed them applying 2.5% glutaraldehyde in 0.01M PBS for 30 min at room temperature (RT). Then, we harvested the cells using a scraper, collected them into a tube and centrifuged them at 800 x g for 5 min at RT. Then we aspirated the supernatant, while cells were resuspended in warmed 4% gelatin aquatic solution followed by centrifugation at 800 x g for 5 min at RT and cooled on ice. Under a stereoscope we extracted he solidified cell pellet with gelatin, we cut it into small fragments (1–2 mm^3) and then we transferred the fragments into PBS at 4 °C. The cell-gelatin fragments were then dehydrated in graded series of ethyl alcohol, followed by propylene oxide (PO) treatment, infiltrated gradually in a mixture of Epon/Araldite resins diluted in PO, and finally

embedded in fresh epoxy resin mixture. Then we cut ultrathin sections (70-90 nm thickness) using a Leica Ultracut R ultramicrotome, equipped with a Diatome diamond knife, and we mounted them onto 200-mesh copper grids. The sections were then counterstained with ethanolic uranyl acetate followed by lead citrate and observed on a Philips 420 transmission electron microscope equipped with an Olympus Megaview G2 CCD camera.

2.13 *IN VIVO* EXPERIMENTS

All animal studies were approved by the National Hellenic Research Foundation (NHRF) Animal Care and Use Committee. The study protocol was approved by the local ethics committee (Athens Prefecture Veterinarian Service; (315856/15-03-2023)). The *in vivo* study was conducted in the ISO 9001: 2015 operating (registration number I-030-02-100-01430) animal model unit of the Institute of Chemical Biology of the NHRF. Briefly, we subcutaneously inoculated male C.B-Igh-1^{b}/IcrTac-Prkdc^{scid} (SCID) mice (n=6 per condition) with $5x10^{6}$ LNCaP or C4-2B cells resuspended in BME (R&D Systems). When the size of tumor was around 50 mm^{3}, we treated the mice with 10 mg/kg enzalutamide diluted in DMSO and resuspended in corn oil/saline solution (final DMSO concentration at 0.001% w/v). Enzalutamide was administrated by oral gavage for 8 consecutive days. In order to avoid the adverse effect of enzalutamide, mice were monitored daily for any signs of illness or discomfort. At the end of the experiment, the mice were euthanized and the we surgically excised the tumors to procced with downstream molecular analysis and immunohistochemistry.

2.14 RNA SEQUENCING, BIOINFORMATICS AND STATISTICAL ANALYSIS

RNA sequencing, bioinformatics and statistical analysis were carried out as previously described (128).

CHAPTER 3

RESULTS

3.1 A GATA2-CDC6 AXIS MODULATES SENESCENCE IN RESPONSE TO AR SIGNALING INHIBITION.

To elucidate the molecular mechanisms underlying the sensitivity to AR inhibition, we initially implemented the LNCaP prostate cancer cell line, that represents androgen-sensitive localized cancer. Based on literature, AR was shown to directly promote the transcription of CDC6 (121). Furthermore, androgen deprivation was found to cause induction of senescence in LNCaP cells, however with temporary effects, driving finally CRPC development (93-95). For that reason, we initially wanted to ascertain whether inhibition of AR may induce senescence and if CDC6 is involved in this process. We found that treating LNCaP cells with the AR inhibitor enzalutamide led to complete abrogation of CDC6 expression. This result is in accordance with the previously reported role of AR in CDC6 transcription (**Figure 3.1**). Intriguingly, although senescence as a stress response was previously found to be provoked by CDC6 upregulation (79), CDC6 downregulation in LNCaP cells came along with $p21^{WAF1/Cip1}$ upregulation, a p53 downstream target that participates in senescence induction (66, 129) (**Figure 3.1**).

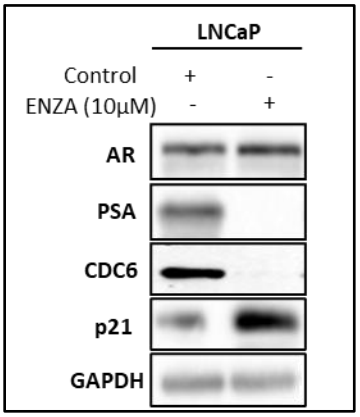


Figure 3.1: Western blotting of LNCaP control and LNCaP enzalutamide-treated cell lysates with the indicated antibodies. We verified the responsiveness of LNCaP cells to enzalutamide with the complete absence of PSA expression upon treatment. We used GAPDH as endogenous control (128).

Induction of senescence in LNCaP cells upon enzalutamide treatment was further confirmed by immunocytochemical GL13 staining that identifies the accumulation of lipofuscin in the cytoplasm of senescent cells. As previously reported, lipofuscin accumulation is one of the most distinguished hallmarks of senescence phenotype (**Figure 3.2**) (127).

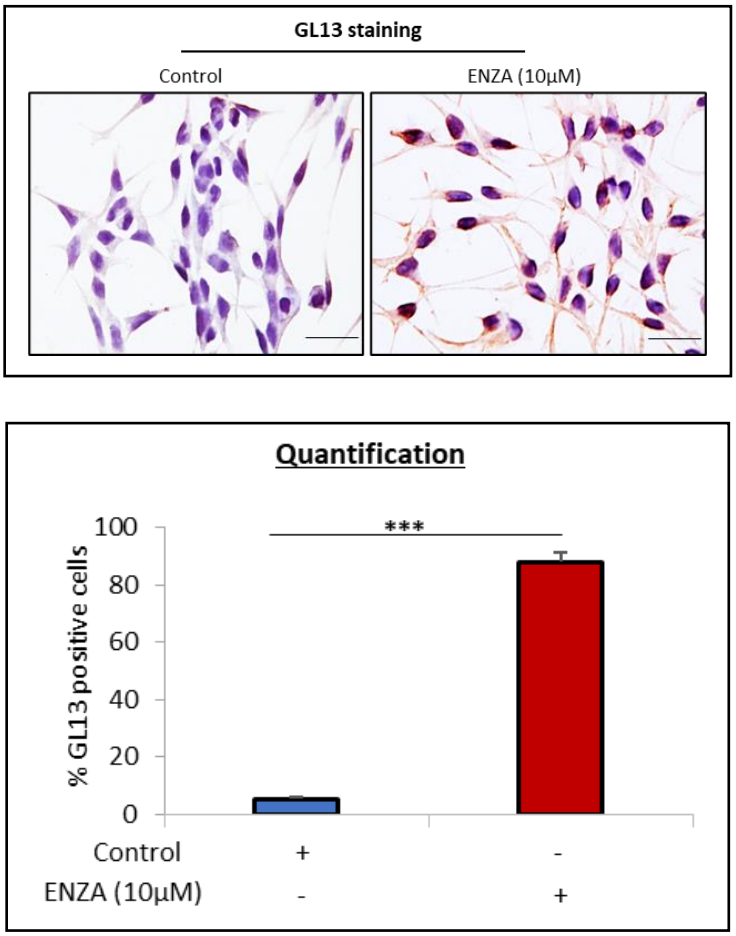


Figure 3.2: GL13 immunocytochemical staining of LNCaP control and LNCaP enzalutamide-treated cells (top) and quantification of GL13 positive cells (bottom). Lipofuscin mostly accumulates in the cytoplasm of LNCaP enzalutamide-treated cells. Magnification: 200x (Objective 20x), scale bars: 30 µm (128).

In agreement with the senescent phenotype, cellular proliferation was reduced in LNCaP cells receiving treatment (**Figure 3.3**).

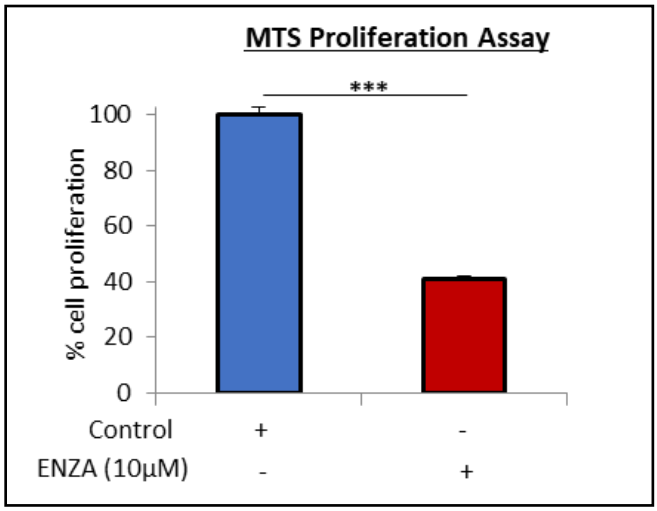


Figure 3.3: MTS proliferation assay confirmed that proliferation of LNCaP cells was decreased upon treatment with enzalutamide (128).

In order to investigate the molecular basis and the most important regulators of senescence induction in LNCaP cells upon CDC6 reduction, we performed RNA sequencing in LNCaP control and LNCaP enzalutamide-treated cells. Intriguingly, we found that the transcription factor GATA2 was one of the most significantly upregulated genes in response to therapy (**Figure 3.4**). GATA elements, according to previous studies, are located in the *CDC6* promoter, in specific regions named Androgen Response Element (ARE) sites (**Figure 3.4**) (121), implying that may GATA factors may directly modulate CDC6 transcription.

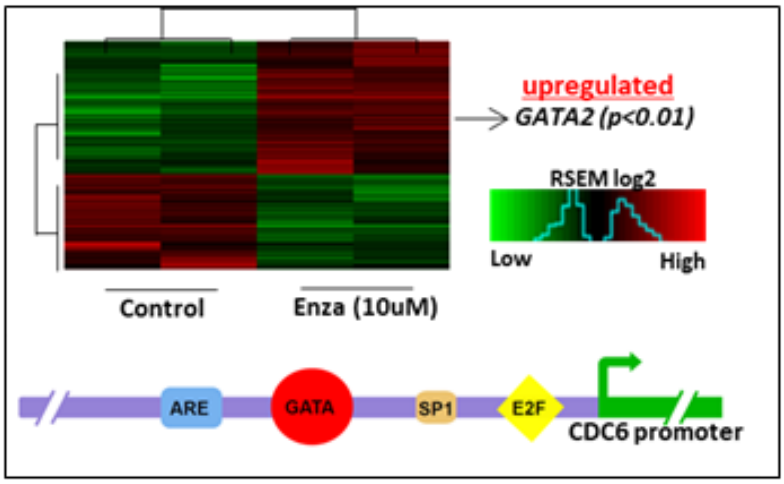


Figure 3.4: RNA sequencing analysis in LNCaP control and LNCaP enzalutamide-treated cells revealed GATA2 as one of the most predominantly upregulated factors (P<0.01). GATA elements have been found in the CDC6 promoter in specific regions named Androgen Response Element (ARE) sites (121, 128).

To investigate if CDC6 is regulated by GATA2, we silenced GATA2 in LNCaP control and LNCaP enzalutamide-treated cells, and we found that CDC6 was stabilized upon GATA2 loss regardless of treatment (**Figure 3.5, 3.6**). It is of note that, silencing of GATA2 didn't increase $p21^{WAF1/Cip1}$ expression even upon treatment with enzalutamide (**Figure 3.5, 3.6**). Interestingly, endogenous GATA2 levels were shown to be remarkably upregulated in response to enzalutamide, followed by CDC6 loss, $p21^{WAF1/Cip1}$ increase and induction of senescence phenotype (**Fig. 3.5-3.7**).

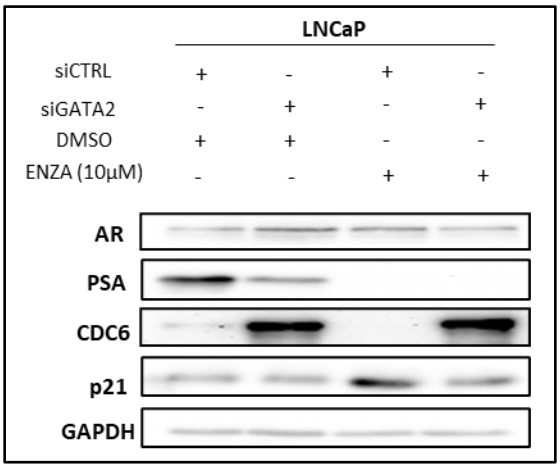


Figure 3.5: Western blotting of LNCaP cells transfected with the siCTRL (indicating non-targeting siRNA) or siGATA2 s and/or treated with DMSO or enzalutamide. CDC6 expression levels are shown to be increased upon GATA2 silencing regardless of treatment (128).

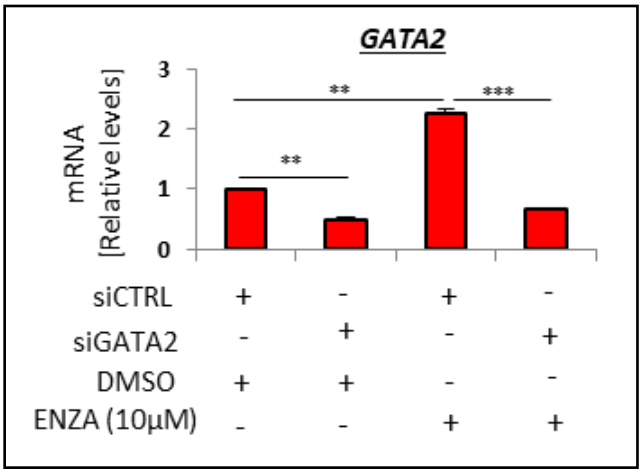


Figure 3.6: qPCR for GATA2 mRNA levels confirming successful GATA2 knock-down in Figure 3.5. Endogenous GATA2 mRNA levels are shown to be upregulated in response to enzalutamide (128).

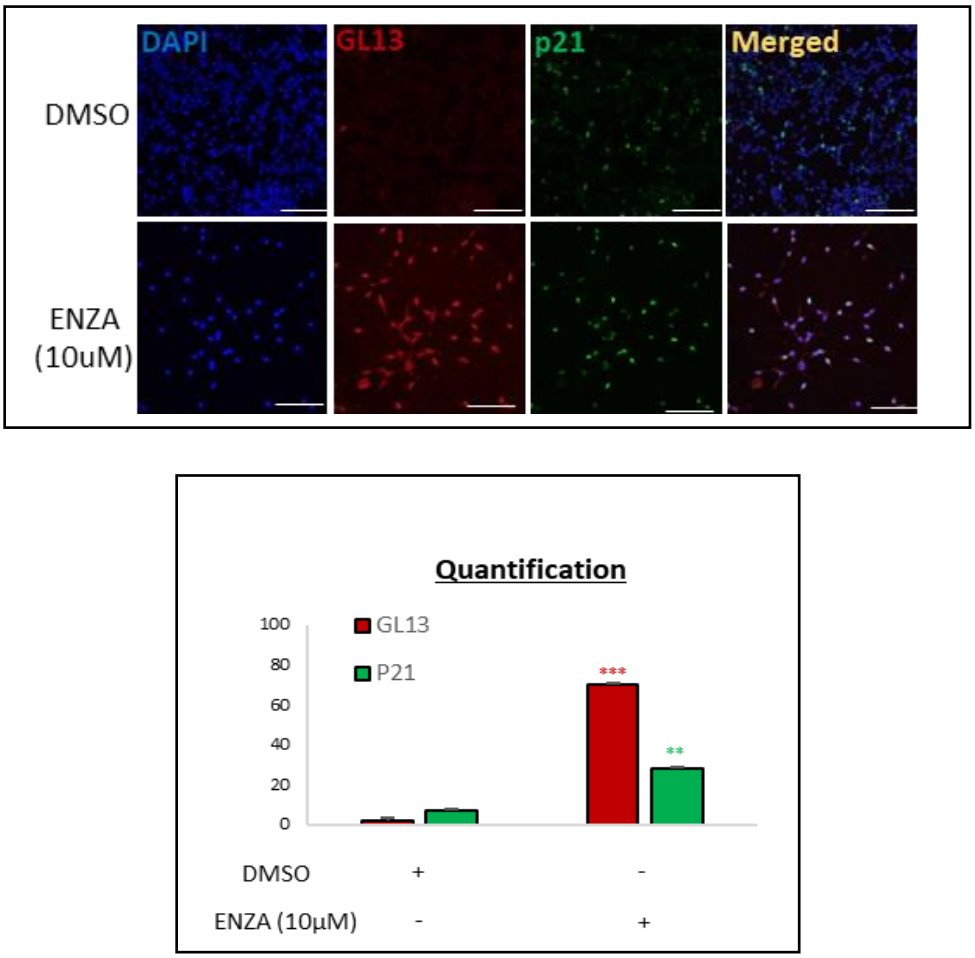


Figure 3.7: Double GL13/p21$^{WAF1/Cip1}$ Immunofluorescence staining (top) and quantification (bottom) of stained cells, validates senescence induction in LNCaP cells only upon enzalutamide treatment. Magnification: 100x (Objective 10x), scale bars: 60 µm (128)

These results indicate that AR signaling inhibition in androgen-dependent LNCaP cells triggers the natural activation of GATA2, which may directly repress CDC6, thereby establishing senescence phenotype.

We next wanted to elucidate the effect of the potential GATA2-CDC6 axis on LNCaP oncogenic characteristics. As expected, and in line with the senescence phenotype, cellular proliferation of LNCaP cells was significantly increased upon siGATA2-mediated CDC6 stabilization (**Figure 3.8**).

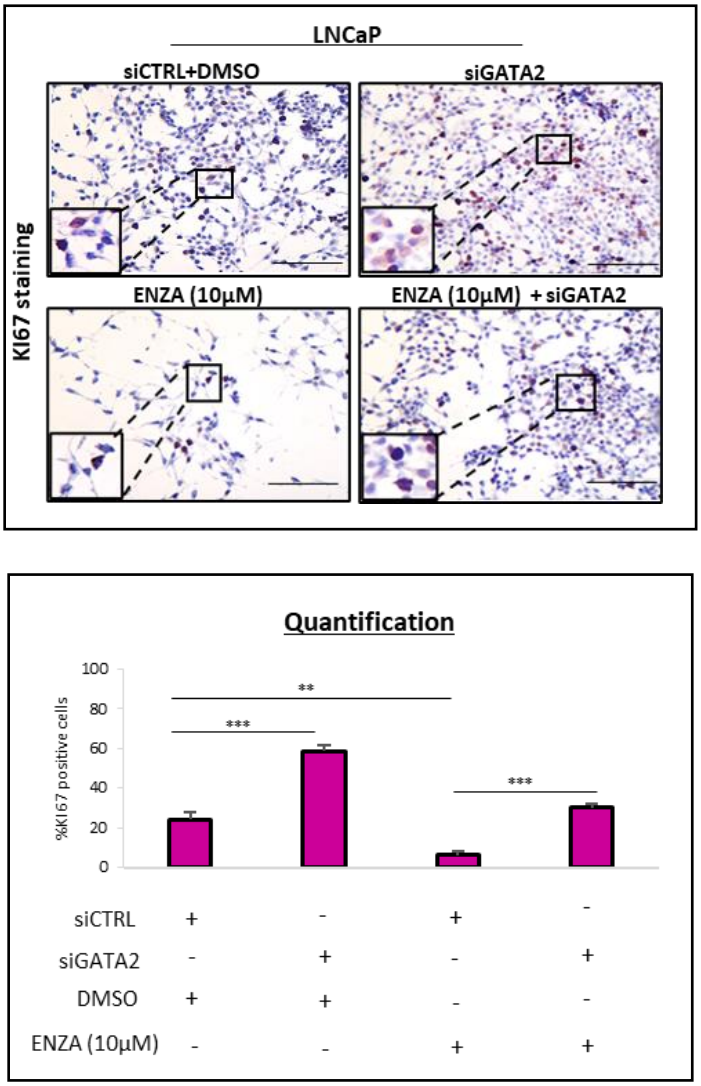


Figure 3.8: Ki67 immunocytochemical staining (top) and quantification (bottom) of LNCaP cells in Figure 3.5, reveal that LNCaP proliferation levels are in proportion with CDC6 expression levels. Magnification: 100x (Objective 10x), scale bars: 60 µm. Inset magnification: 400x (Objective 40x) (128).

Furthermore, silencing of GATA2 activated EMT, as shown by decreased levels of E-cadherin (CDH1) and increased levels of ZEB1 and SNAI1 (**Figure 3.9**).

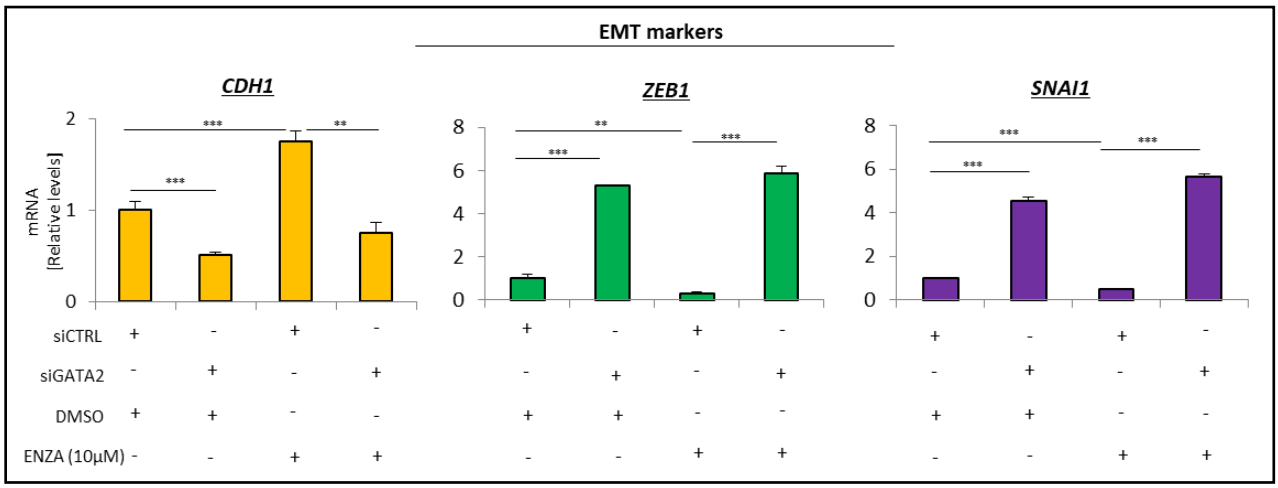


Figure 3.9: qPCR for EMT markers CDH1, ZEB1 and SNAI1 in the indicated conditions of LNCaP cells from Figure 3.5. GATA2 loss is followed by an EMT profile regardless of treatment with enzalutamide. Data are normalized to GAPDH expression (128).

These results point out that GATA2 loss promotes CDC6 upregulation that may be adequate to increase the invasiveness of localized prostate cancer cells, driving their metastatic transformation.

3.2 SILENCING OF CDC6 INDUCES SENESCENCE IN ENZALUTAMIDE-RESISTANT PROSTATE CANCER CELLS.

We next tried to investigate if AR inhibition in a CRPC setting may again depend on regulation of CDC6 expression, as shown in androgen-sensitive cells. For that aim, we implemented androgen-independent C4-2B and PC-3 cells. C4-2B cells are isolated from bone metastases that are formed in nude mice after their inoculation with LNCaP-derived, androgen-independent C4-2 cells (130). Since C4-2B are enzalutamide-resistant prostate cancer cells, in conjunction with LNCaP cells, they compose an exceptional preclinical model for the investigation of progression of localized androgen-dependent prostate cancer into metastatic and androgen-independent disease (131). PC-3 cells originate from bone metastatic, human prostate adenocarcinoma cells and are also shown to exhibit increased resistance to enzalutamide (132, 133).

Intriguingly, inhibition of AR in androgen-independent C4-2B cells via treatment with enzalutamide led to increased CDC6 levels, while no senescence induction was detected as indicated by no change in $p21^{WAF1/Cip1}$ levels as well as absence of GL13 staining (**Fig. 3.10, 3.11**).

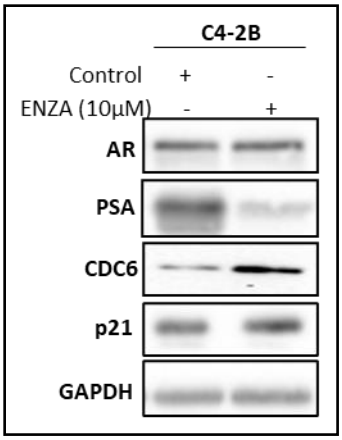


Figure 3.10: Western blotting of C4-2B cell lysates treated with DMSO or enzalutamide. CDC6 is shown to be stabilized upon enzalutamide treatment (128).

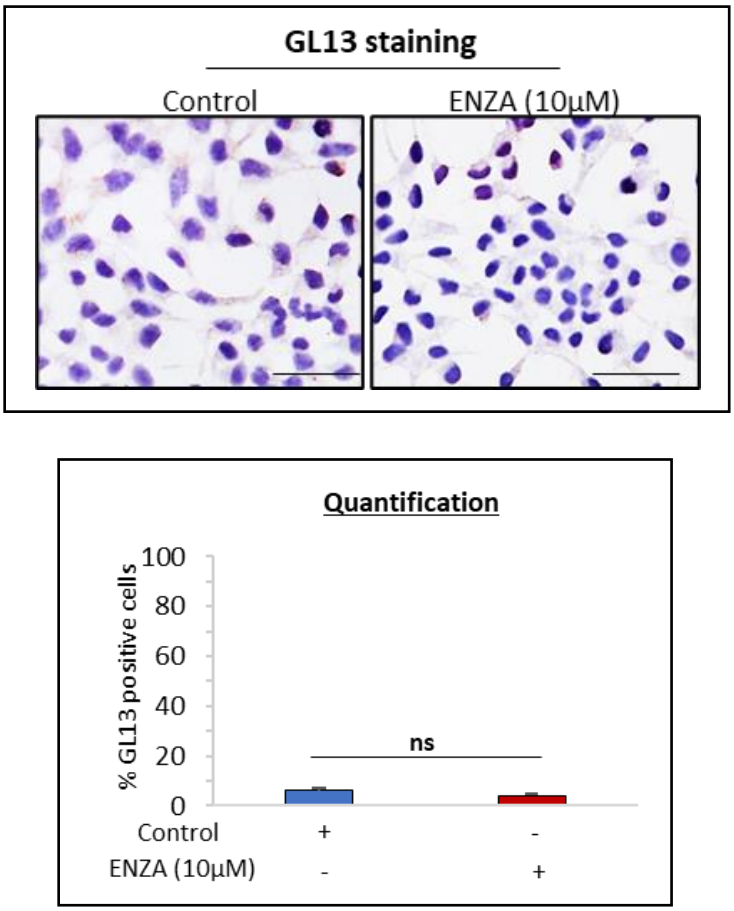


Figure 3.11: GL13 immunocytochemical staining of C4-2B control and C4-2B enzalutamide-treated cells (top) and quantification of GL13 positive cells (bottom) revealed non-significant differences in the accumulation of lipofuscin between the indicated conditions. Magnification: 200x (Objective 20x), scale bars: 30 µm (128).

Based on this result, we then checked if loss of CDC6 may lead to reversal of the C4-2B cell phenotype. To be sure that our results are cell line unbiased, we independently confirmed those results also in PC-3 cells. Indeed, silencing of CDC6 in C4-2B and PC-3 cells led to increased $p21^{WAF1/Cip1}$ levels as well as induction of senescence. Interestingly, CDC6 knockdown led to induction of senescence even upon enzalutamide treatment (**Figure 3.12, 3.13**).

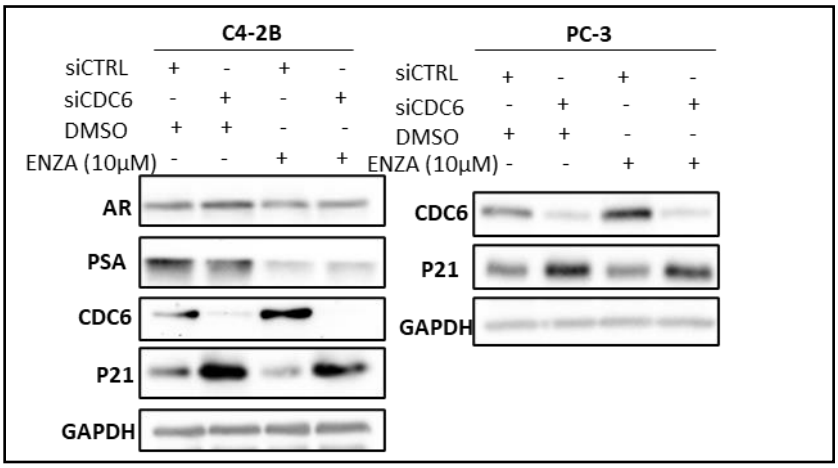
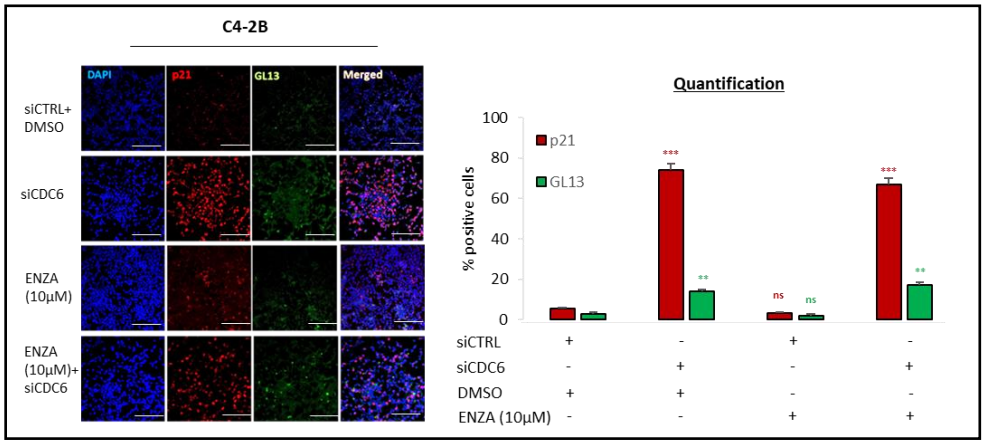


Figure 3.12: Western blotting of C4-2B and PC-3 cells transfected with the siCTRL (indicating non-targeting siRNA) or siCDC6 s and/or treated with DMSO or enzalutamide. CDC6 protein levels are stabilized upon enzalutamide at the expense of $p21^{WAF1/Cip1}$ (128).



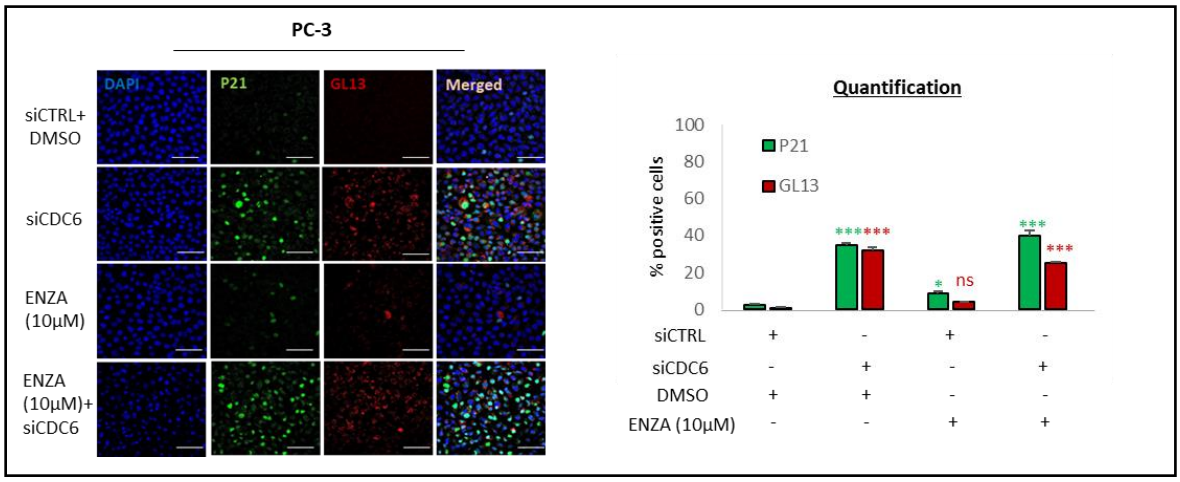


Figure 3.13: Double GL13/p21$^{WAF1/Cip1}$ Immunofluorescence staining and quantification of stained cells in C4-2B (top) and PC-3 (bottom) cells, validates senescence induction only in CDC6-depleted cells. Magnification: 100x (Objective 10x), scale bars: 60 µm (128).

In line with the senescent phenotype of C4-2B cells induced by CDC6 silencing, the proliferation of CDC6-depleted C4-2B cells was significantly reduced (**Figure 3.14, 3.15**).

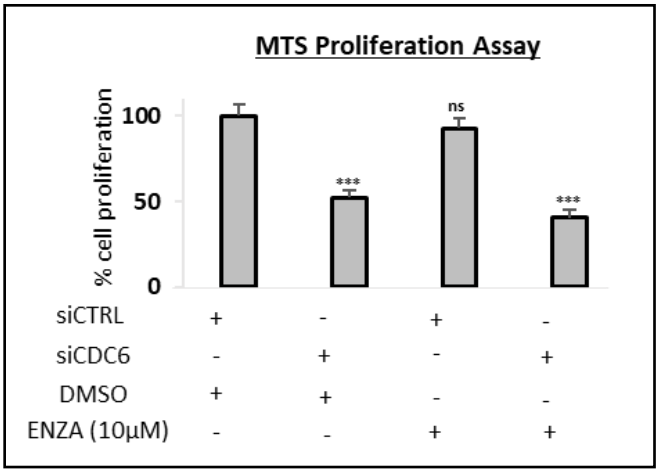


Figure 3.14: MTS proliferation assay in C4-2B cells identifies a significant proliferation decrease of C4-2B cells upon CDC6 depletion (128).

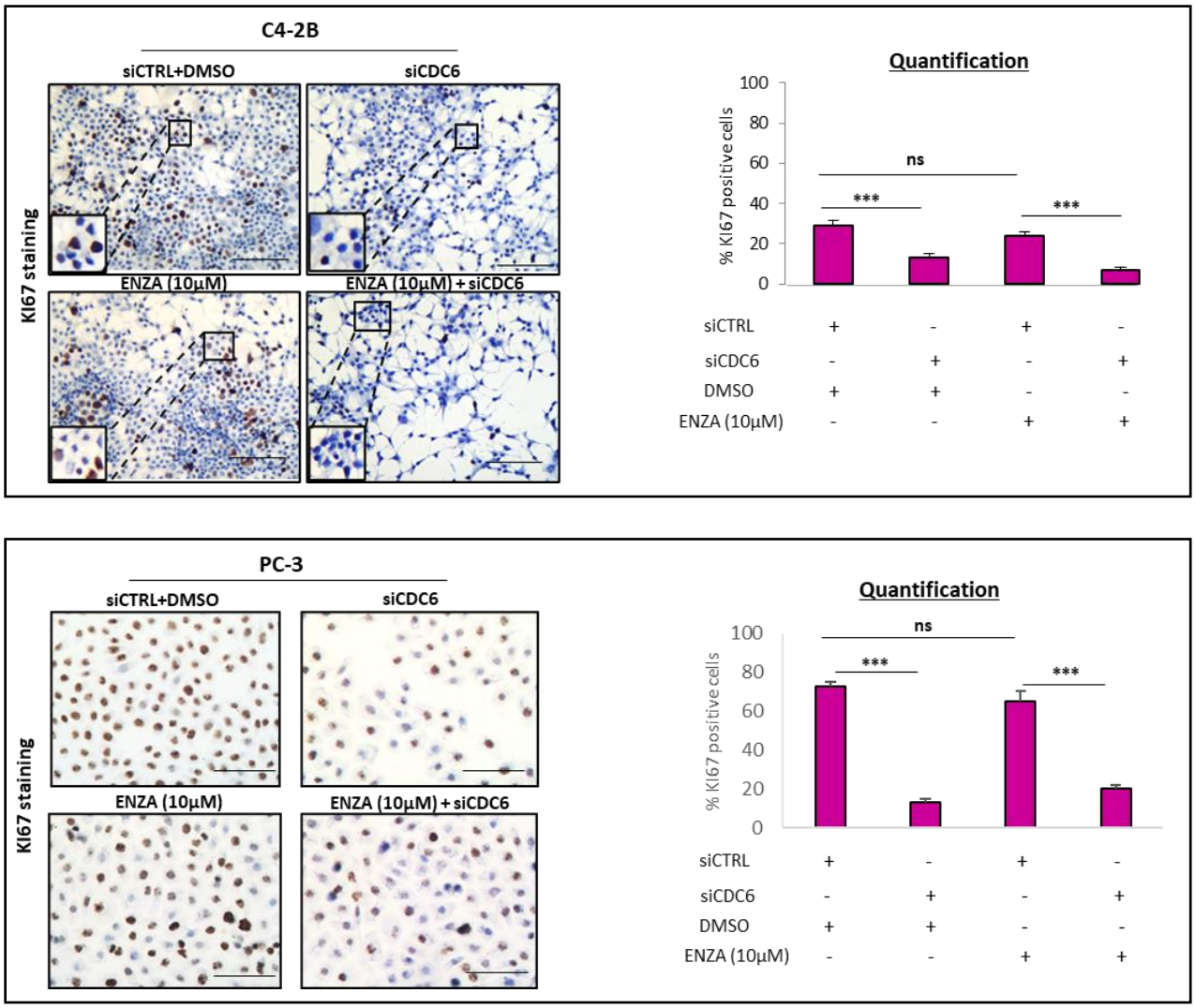


Figure 3.15: Ki67 immunocytochemical staining and quantification of stained C4-2B (top) and PC-3 (bottom) cells, reveal a proliferation decrease upon CDC6 loss, in both cell lines. Magnification (top): 100x (Objective 10x), scale bars: 60 μm. Inset magnification: 400x (Objective 40x). Magnification (bottom): 200x (Objective 20x), scale bars: 30 μm(128).

Moreover, silencing of CDC6 reduced EMT, as indicated by the remarkable changes in the mRNA levels of all EMT markers that we checked (**Figure 3.16**). Interestingly, enzalutamide treatment

alone caused a significant activation of EMT (**Figure 3.16**), signifying that CDC6 upregulation upon acquired resistance may exacerbate the invasiveness of prostate cancer cells.

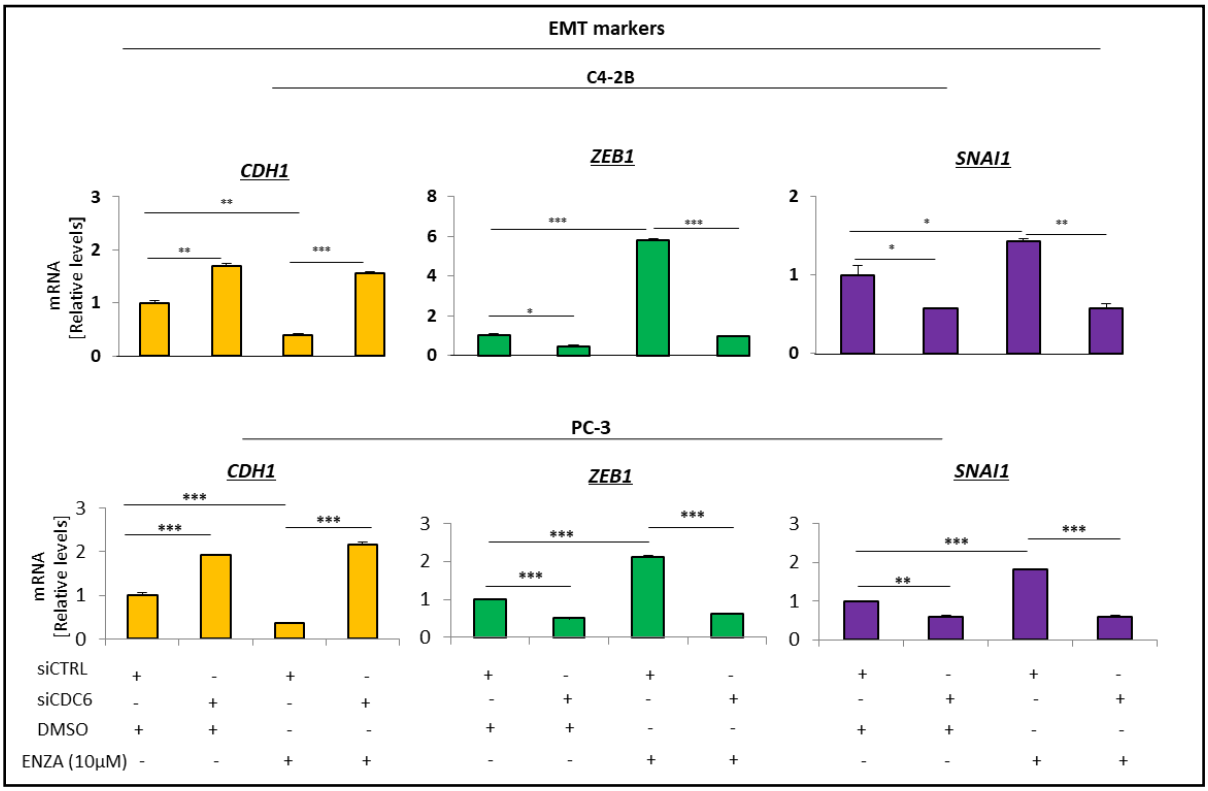


Figure 3.16: qPCR for EMT markers CDH1, ZEB1 and SNAI1 in the indicated conditions of C4-2B (top) d PC-3 (bottom) cells. CDC6 loss rescues EMT marker expression, while CDC6 stabilization upon enzalutamide treatment has the opposite effect. Data are normalized to GAPDH expression (128).

Combining our results, we conclude that there is a potentially opposite modulation pattern of CDC6 in androgen-dependent and –independent prostate cancer cells; however, the absence of CDC6 may induce senescence and rescue crucial oncogenic characteristics of androgen-independent cancer cells.

3.3 ENZALUTAMIDE MODULATES A GATA2-CDC6 AXIS TO WORSEN EMT PHENOTYPE UPON ACQUIRING RESISTANCE TO ANDROGEN BLOCKADE THERAPY.

In order to investigate if the upregulation of CDC6 that came up upon treatment with enzalutamide may again rely on GATA2 in a castration-resistant setting, we silenced GATA2 in C4-2B and PC-3 cells treated or not with enzalutamide. We observed that in both cell lines silencing of GATA2 resulted in CDC6 upregulation compared to control regardless of treatment (**Figure 3.17, 3.18**), confirming again the role of GATA2 as a CDC6 repressor. Intriguingly and conversely with our findings in LNCaP cells, enzalutamide treatment alone resulted in a significant decrease of endogenous GATA2 levels compared to control, followed by an increase of CDC6 protein levels (**Figure 3.17, 3.18**).

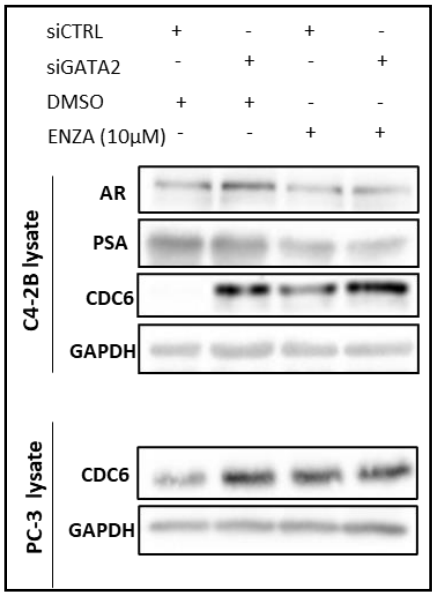


Figure 3.17: Western blotting of C4-2B (top) and PC-3 cells (bottom) transfected with the siCTRL (indicating non-targeting siRNA) or siGATA2 and/or treated with DMSO or enzalutamide. CDC6 protein levels are stabilized either upon GATA2 silencing or enzalutamide treatment (128).

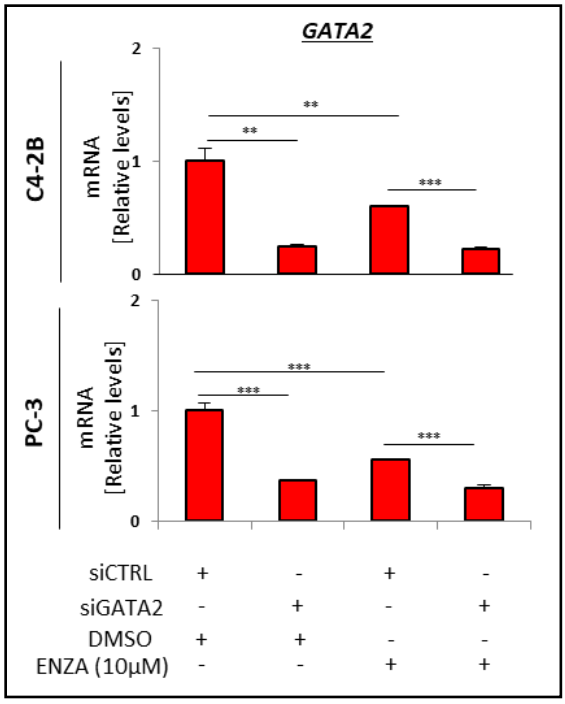


Figure 3.18: qPCR for GATA2 mRNA levels confirms successful GATA2 knock-down in Figure 3.17. Endogenous GATA2 mRNA levels are shown to be down regulated in response to enzalutamide in already enzalutamide-resistant cells (128).

In order to search for potential effects of GATA2-mediated CDC6 regulation in a castration-resistant cellular setting, we again evaluated various oncogenic characteristics of C4-2B and PC-3 cells upon depletion of GATA2, followed by treatment with DMSO or enzalutamide. No evidence of senescence phenotype was observed upon either GATA2 silencing or enzalutamide treatment (**Figure 3.19, 3.20**), coinciding with CDC6 upregulation.

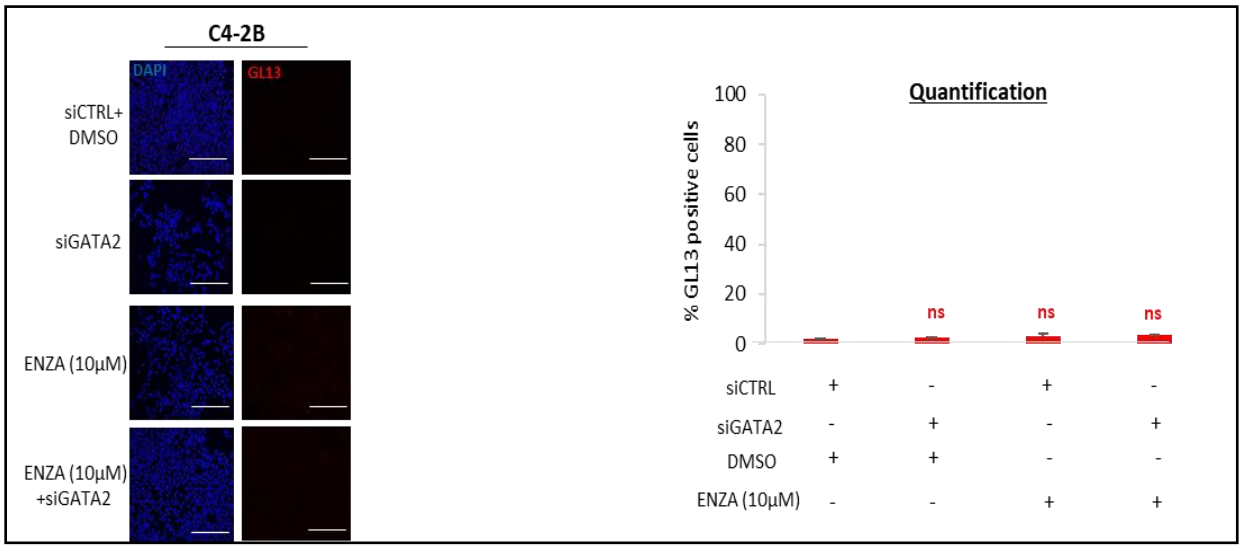


Figure 3.19: GL13 Immunofluorescence staining and quantification of stained C4-2B cells indicates no induction of senescence upon enzalutamide treatment. Magnification: 100x (Objective 10x), scale bars: 60 µm (128).

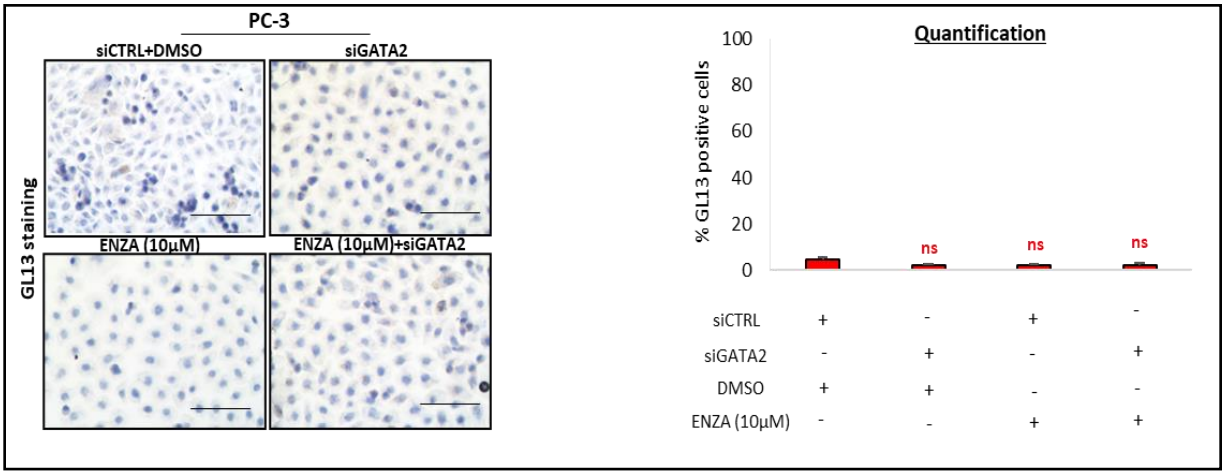


Figure 3.20: GL13 immunocytochemical and quantification of stained PC-3 cells indicates no induction of senescence upon enzalutamide treatment. Magnification: 200x (Objective 20x), scale bars: 30 µm (128).

Furthermore, although in both C4-2B and PC-3 cell lines no significant changes in their proliferation capacity upon silencing of GATA2 depletion or enzalutamide treatment were observed (**Figure 3.21**), we identified in both cell lines a significant activation of EMT markers, revealing that CDC6 stabilization via natural or RNAi-mediated GATA2 depletion is enough for already metastatic prostate cancer cells to obtain more aggressive characteristics (**Figure 3.22**).

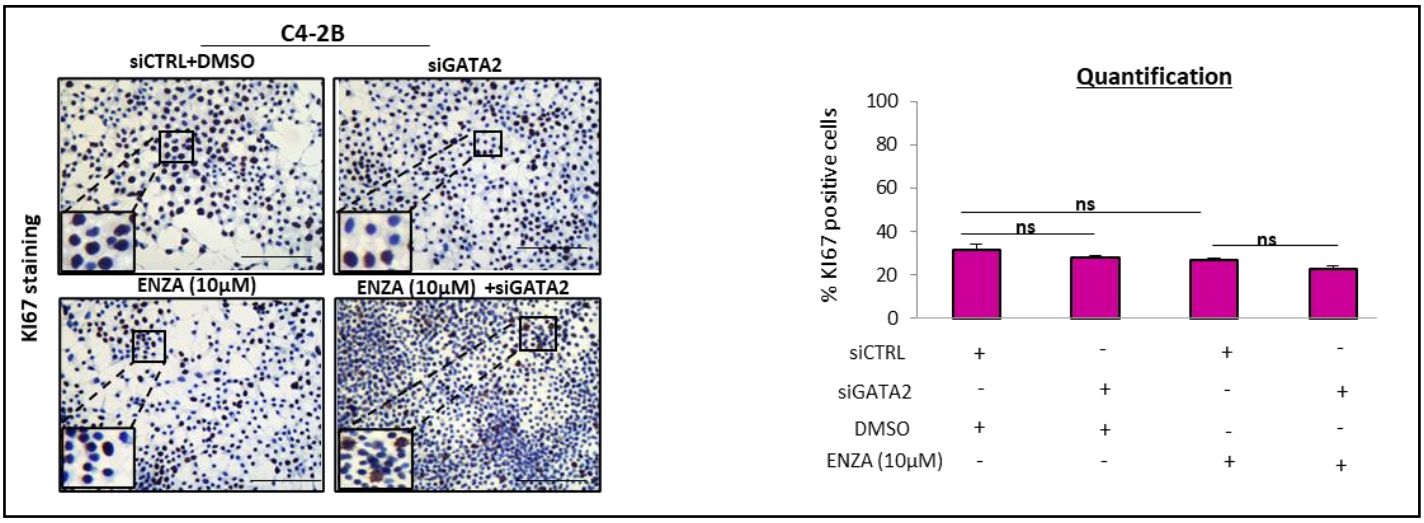


Figure 3.21: Ki67 immunocytochemical staining and quantification of stained C4-2B cells reveal a no significant change in their proliferation upon GATA2 loss. Magnification: 100x (Objective 10x), scale bars: 60 μm. Inset magnification: 400x (Objective 40x) (128).

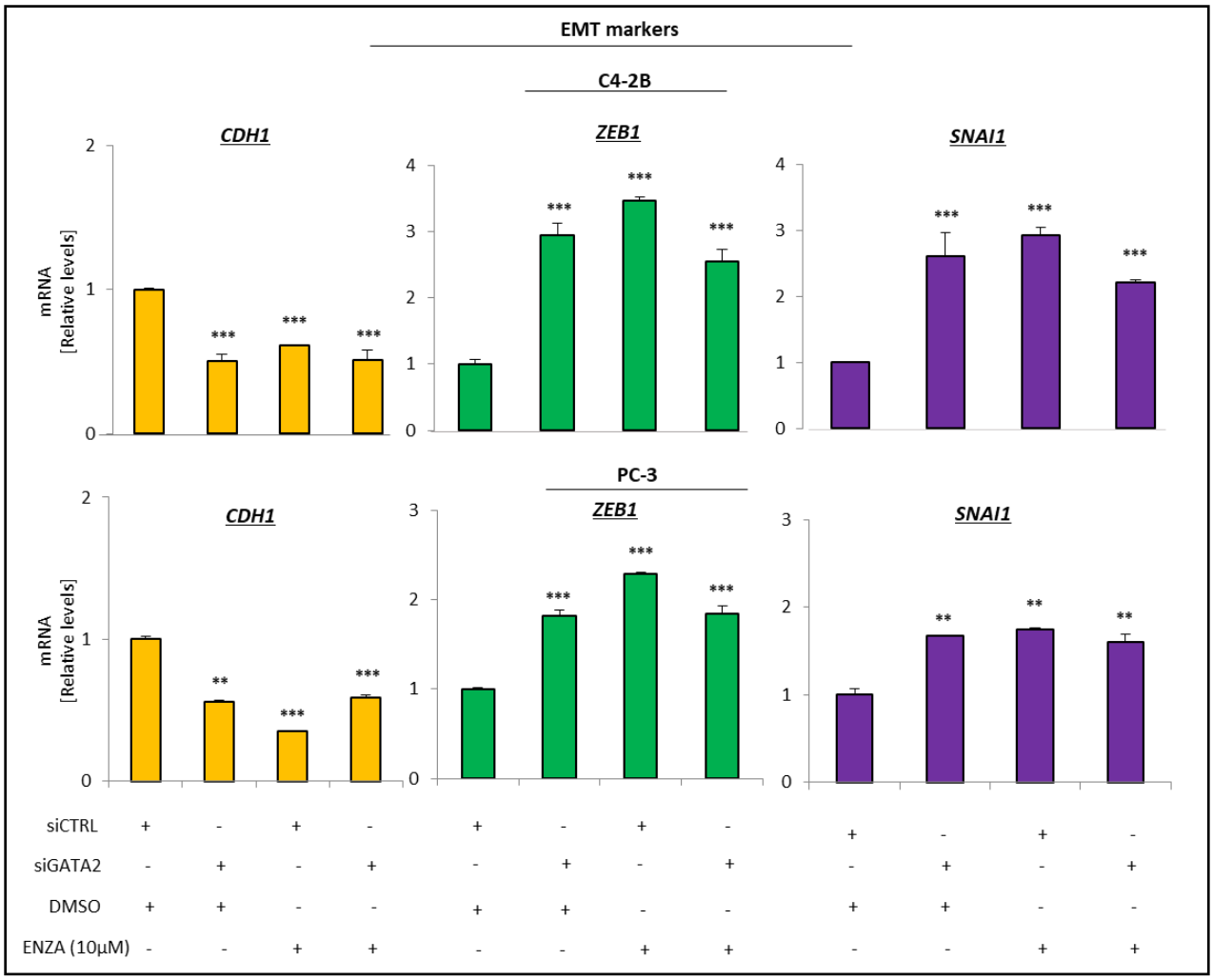


FIGURE 3.22: qPCR for EMT markers CDH1, ZEB1 and SNAI1 in the indicated conditions of C4-2B (top) d PC-3 (bottom) cells. GATA2 loss or enzalutamide treatment, promote EMT marker expression. Data are normalized to GAPDH expression (128).

This result was further validated with *in vitro* migration and invasion assays in PC-3 cells revealing that PC-3 cells treated with enzalutamide acquired an increased migratory and invasive potential (**Figure 3.23**).

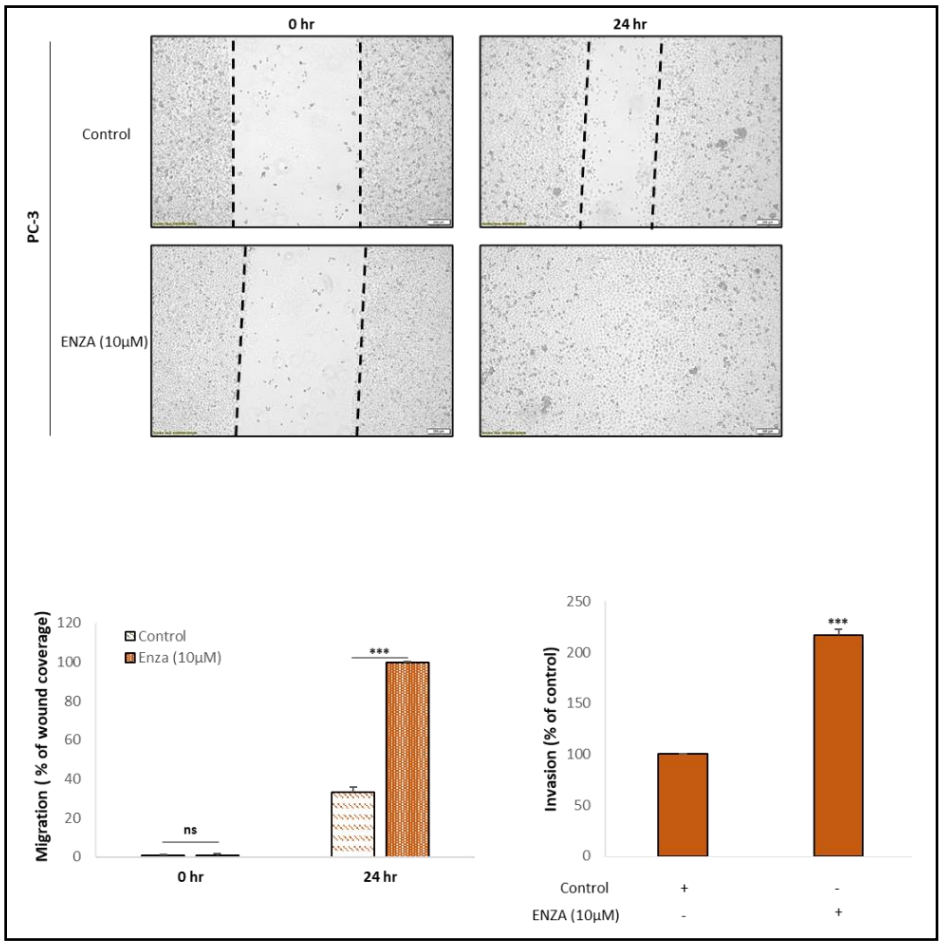


Figure 3.23: (Top): Wound healing assay of PC-3 control or PC-3 enzalutamide-treated cells, showing cell migration at the indicated time points after wound formation. Cells used to the assay have already been treated or not with enzalutamide for 72 h. Magnification: 50x (Objective 5x), scale bars: 200 µm. (Bottom left): Quantification of PC-3 control or PC-3 enzalutamide-treated cells migration potential and (Bottom right): Quantification of PC-3 control or PC-3 enzalutamide-treated cells invasion potential, using a colorimetric invasion assay (128).

Our findings so far show that the identified GATA2-CDC6 axis is modulated in a different way in enzalutamide-resistant C4-2B and PC-3 cells compared to enzalutamide-sensitive LNCaP cells. We observed that further treatment of CRPC cells with AR inhibitors may actually result in increased

invasiveness via GATA2-mediated CDC6 upregulation. This interesting result has to be taken into consideration in the treatment of CRPC patients, because therapy-induced oncogenic consequences may start immediately upon acquiring therapy-resistance.

With the aim of further validating our observations in a larger dataset, we recovered publicly available RNA-seq data from enzalutamide-resistant and enzalutamide-sensitive prostate cancer cell lines and tumor samples (134-139). Not surprisingly, a comparative pathway enrichment analysis between these two groups, revealed that in enzalutamide-resistant samples pathways linked to cell cycle progression, active cell division and proliferation were upregulated, whereas pathways related to cell adhesion and differentiation were downregulated (**Figure 3.24**).

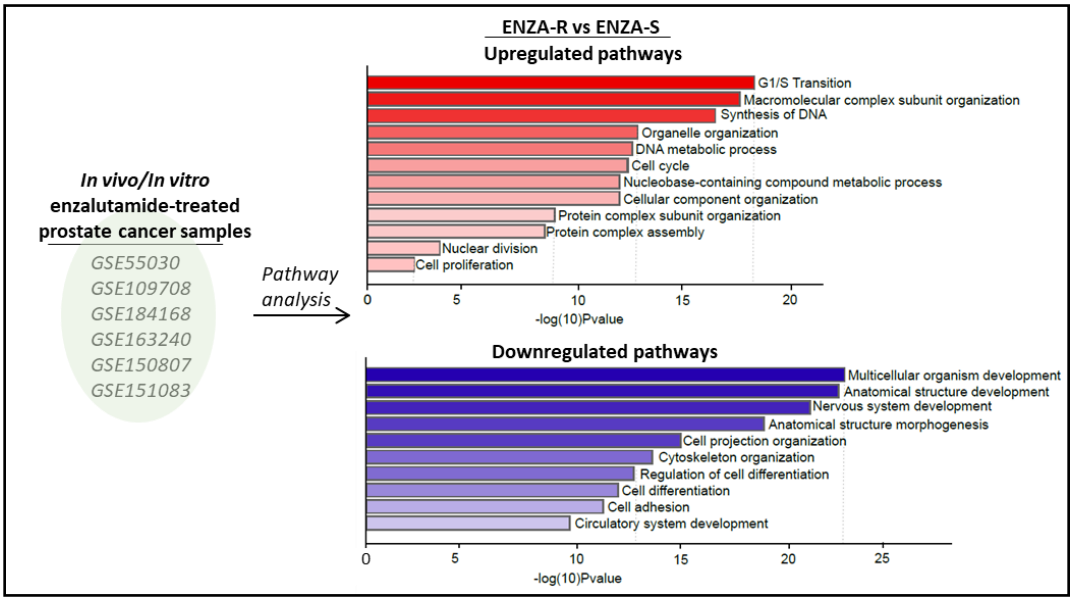


Figure 3.24: A comparative pathway enrichment analysis between enzalutamide-sensitive (ENZA-S) and enzalutamide–resistant (ENZA-R) prostate cancer cell lines or mouse xenografts, from publicly available RNA-seq datasets, showed that pathways linked to cell cycle progression, cell division and proliferation were upregulated in enzalutamide-resistant samples compared with pathways related to cell adhesion(128).

We next wanted to further explore the factors that participate in the predominant pathways that the comparative pathway enrichment analysis revealed. As expected, and in line with our *in vitro* findings, we observed that CDC6 was significantly upregulated in enzalutamide-resistant samples compared to enzalutamide-sensitive samples, while GATA2 was significantly downregulated, following the opposite pattern with CDC6 (**Figure 3.25**). Then, we tried to find a potential correlation between the GATA2-CDC6 pattern with senescence and/or EMT across the whole dataset. Indeed, we found that some established senescence markers (140, 141) were found significantly reduced in enzalutamide-resistant samples, while well-characterized EMT markers were significantly upregulated (**Figure 3.26**).

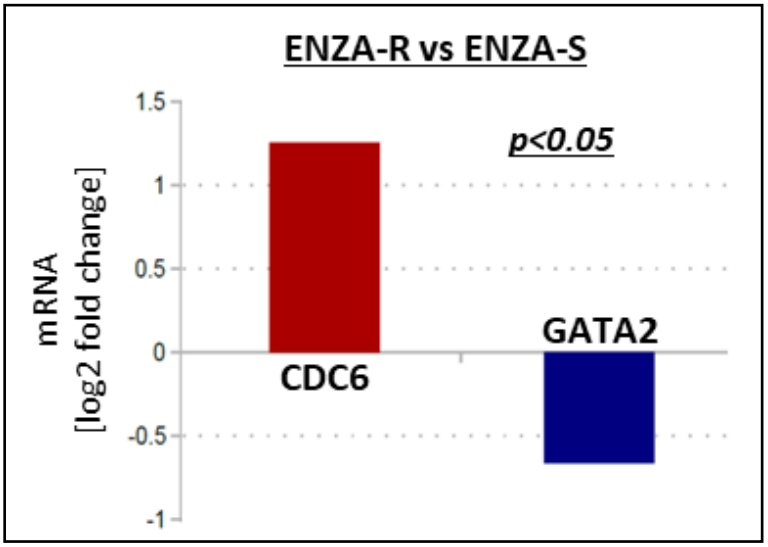


Figure 3.25: CDC6 mRNA levels were found significantly increased in enzalutamide-resistant samples, whereas GATA2 mRNA levels were decreased in enzalutamide-resistant samples compared to enzalutamide- sensitive counterparts (128).

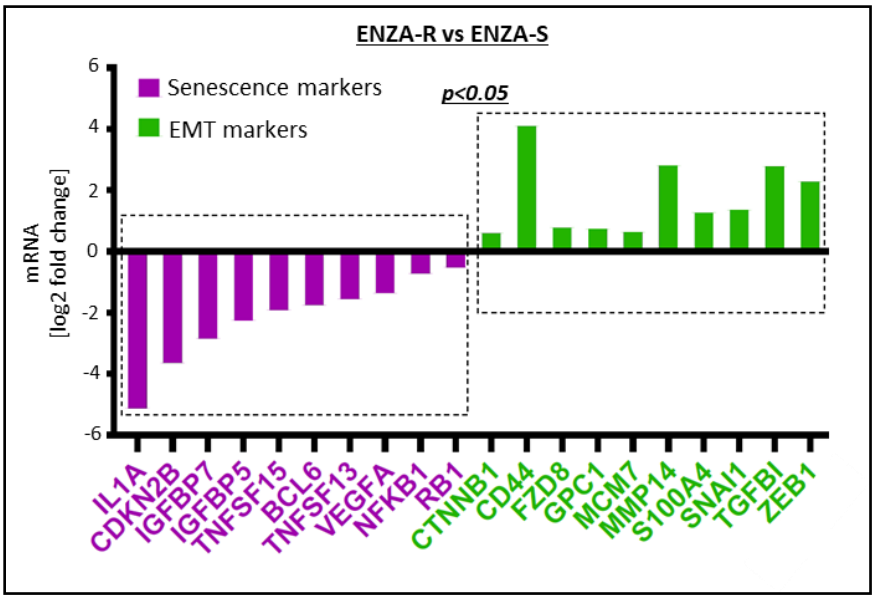


Figure 3.26: Known EMT markers (142), including ZEB1 and SNAI1, were upregulated in enzalutamide-resistant samples, while markers related to senescence and SASP followed the opposite pattern. Senescence and SASP markers were recovered from: https://www.reactome.org/content/detail/R-HSA-2559582 (128).

A heatmap of normalized RNA seq raw counts showed that CDC6 is clearly reversely correlated with GATA2 in both enzalutamide-resistant and enzalutamide–sensitive samples (**Figure 3.27**). Furthermore, $GATA2^{low}$ -$CDC6^{high}$ levels linked with EMT activation, whereas $GATA2^{high}$-$CDC6^{low}$ levels associated with the presence of senescence in all cases (**Figure 3.27**).

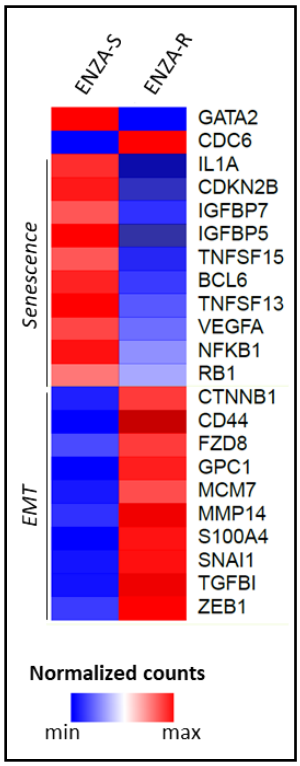


Figure 3.27: A heatmap of normalized RNA seq raw counts showed that CDC6 upregulation in enzalutamide-resistant samples links to increased EMT and reduced GATA2 and senescence, while the opposite pattern of all these counterparts was observed in enzalutamide-sensitive samples (128).

Of note, all these results confirm our *in vitro* findings, showing that if we experimentally regulate the GATA2-CDC6 axis we can drive the AR inhibition-resistant prostate cancer to bypass their harmful clinical phenotypes.

In order to be sure that the observed consequences of GATA2-CDC6 modulation were not due to activation of other cellular processes, such as autophagy or apoptosis, we subjected both LNCaP and C4-2B cells treated or not with enzalutamide, to transmission electron microscopy (TEM), and finally we excluded the possibility of autophagy induction, because we did not observe autophagic vacuoles in any condition (**Figure 3.28**).

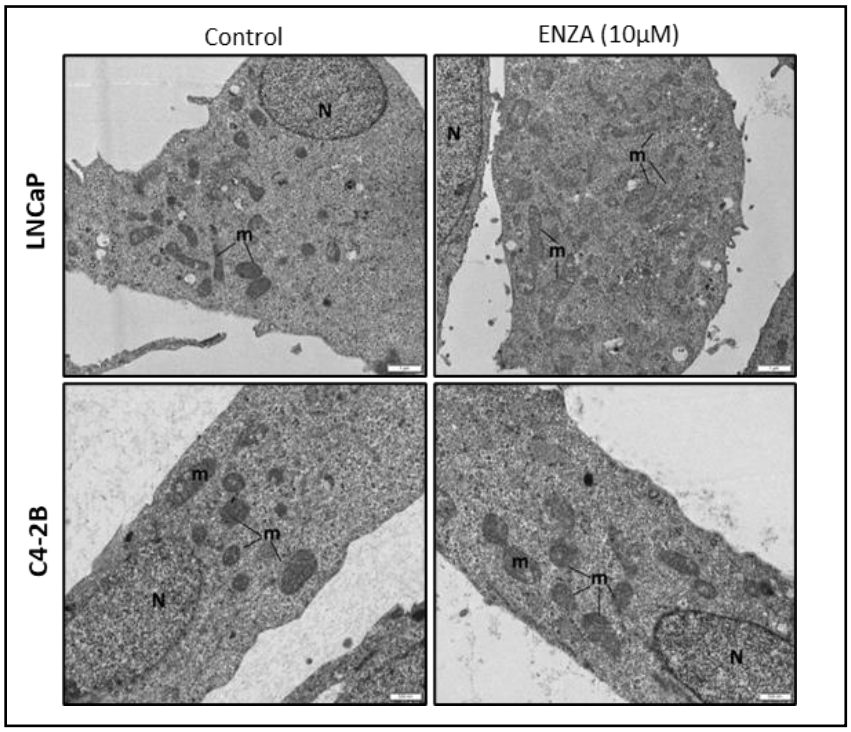


Figure 3.28: Electron micrographs of LNCaP and C4-2B control or enzalutamide-treated cells showed no autophagy induction upon treatment with enzalutamide. N: nucleus; m: mitochondrion. Scale bars for LNCaP cells: 1 μm; for C4-2B cells: 500 nm (128).

Furthermore, another observation that led us to the conclusion that enzalutamide did not promote induction of apoptosis in LNCaP, C4-2B and PC-3 cells is that all these cells were found negative for cleaved caspase-3 staining regardless of treatment (**Figure 3.29**).

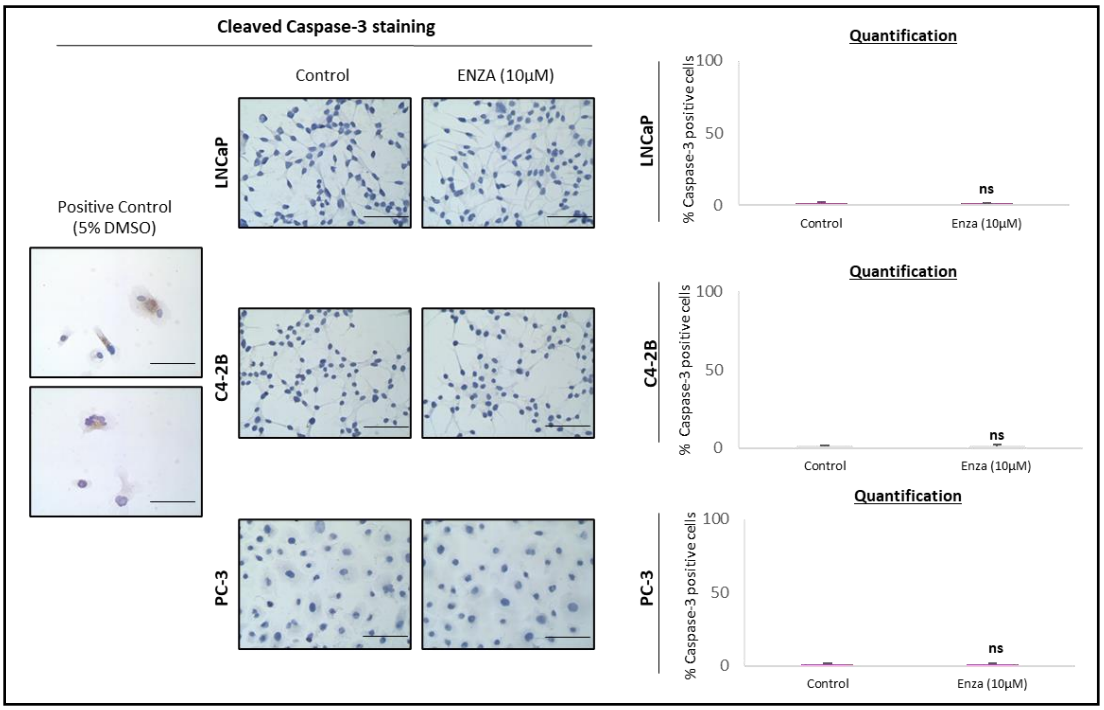


Figure 3.29: Cleaved caspase-3 Immunocytochemical staining of LNCaP-, C4-2B- and PC-3- control or enzalutamide-treated cells demonstrated no evidence of apoptosis induction. As positive control we used PC-3 cells treated with 5% DMSO for 24 h. Magnification: 200x (Objective 20x), scale bars: 30 µm (128).

In order to further validate our *in vitro* findings in an *in vivo* setting, we subcutaneously injected LNCaP and C4-2B cells in immunodeficient SCID mice. Then, when tumors were successfully formed, we treated mice with enzalutamide (10 mg/kg) for an overall period of 8 days. Not surprisingly and in line with our *in vitro* observations, enzalutamide-treated LNCaP xenografts appeared senescence and decreased proliferation compared to untreated LNCaP xenografts (**Figure 3.30**).

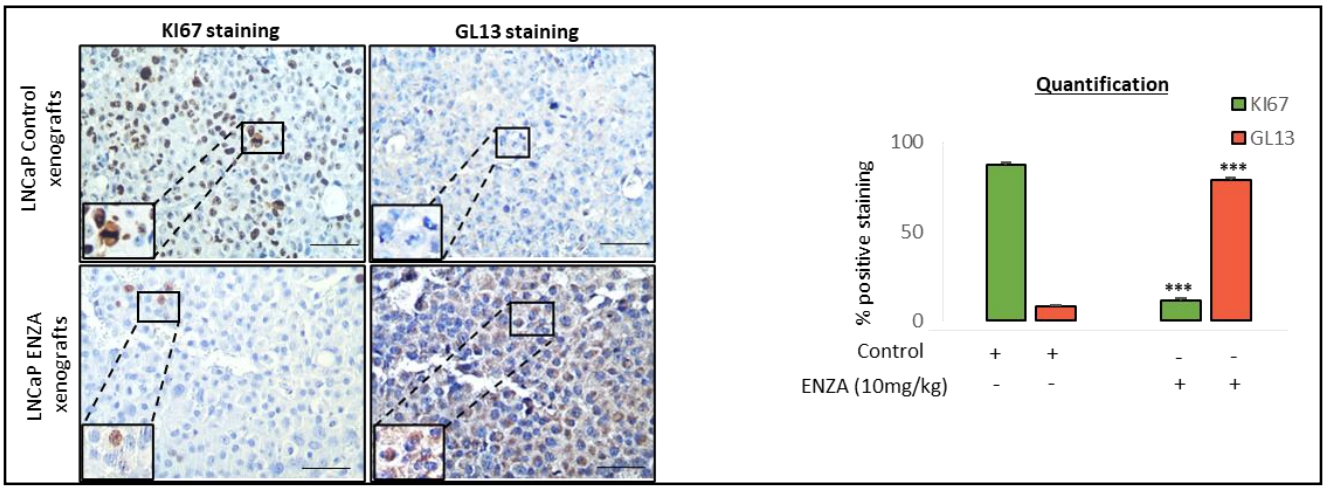


Figure 3.30: Ki67 and GL13 Immunohistochemical staining in serial sections of LNCaP- control and LNCaP -enzalutamide treated mouse xenografts (left) and their quantification (right) showed that senescence was induced and proliferation was decreased in LNCaP-enzalutamide treated mouse xenografts compared to control. Magnification: 200x (Objective 20x), scale bars: 30 µm. Inset magnification: 400x (Objective 40x) (128).

We then continued with further analysis of LNCaP cell lysates from extracted tumors. We found that CDC6 protein levels were decreased upon treatment with enzalutamide, followed by $p21^{WAF1/Cip1}$ increased levels (**Figure 3.31**). Moreover, we observed a significant increase in GATA2 levels (**Figure 3.32**), completely validating our *in vitro* observations.

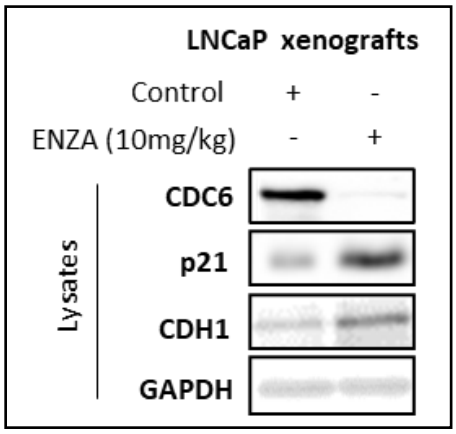


Figure 3.31: Western blotting of cell lysates from LNCaP mouse xenografts tumors, with or without treatment with enzalutamide, showed CDC6 downregulation accompanied by $p21^{WAF1/Cip1}$ and CDH1 upregulation, upon treatment with enzalutamide (128).

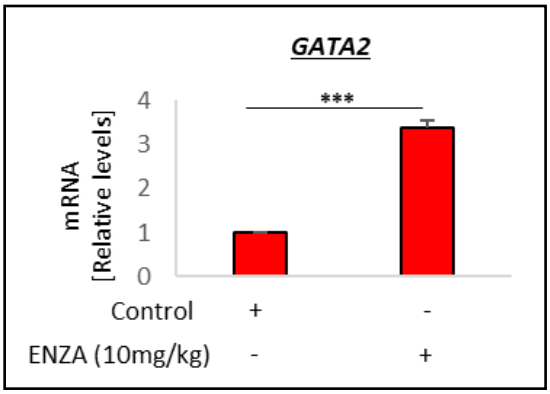


Figure 3.32: q-PCR for GATA2 mRNA levels of cell lysates from LNCaP mouse xenografts tumors, with or without treatment with enzalutamide demonstrated an increase of GATA2 levels upon treatment with enzalutamide (128).

Furthermore, we demonstrated that enzalutamide promoted a decrease of EMT in LNCaP mouse xenografts (**Figure 3.31, 3.33, 3.34)**.

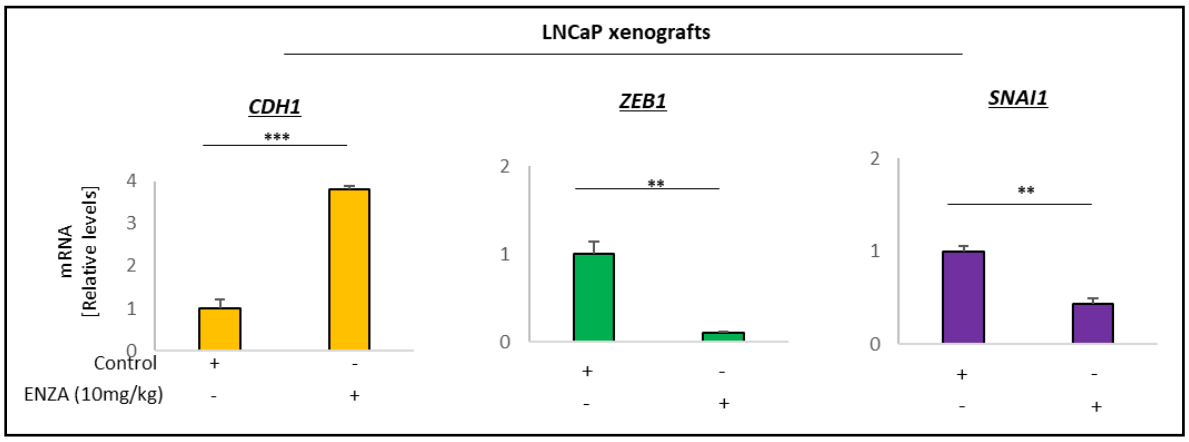


Figure 3.33: qPCR for EMT markers CDH1, ZEB1 and SNAI1 in LNCaP-control and LNCaP- enzalutamide treated (10 mg/kg) mouse xenografts revealed an EMT attenuation upon enzalutamide treatment. Data are normalized to GAPDH expression (128).

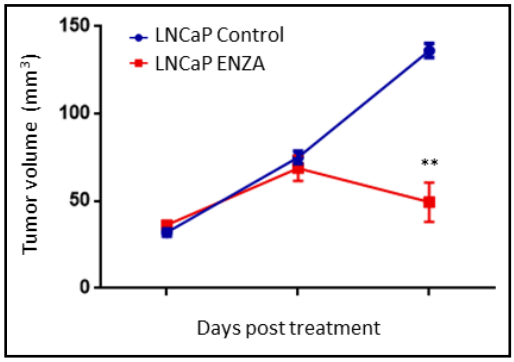


Figure 3.34: Curves showing tumor volume during treatment with enzalutamide in mice injected with LNCaP cells.

On the other hand, C4-2B tumors exhibited no proof of senescence irrespective of enzalutamide (**Figure 3.35, 3.36**), followed by CDC6 and EMT increase, enhanced invasiveness (**Figure 3.36, 3.38-3.40**), and GATA2 decrease (**Figure 3.37**).

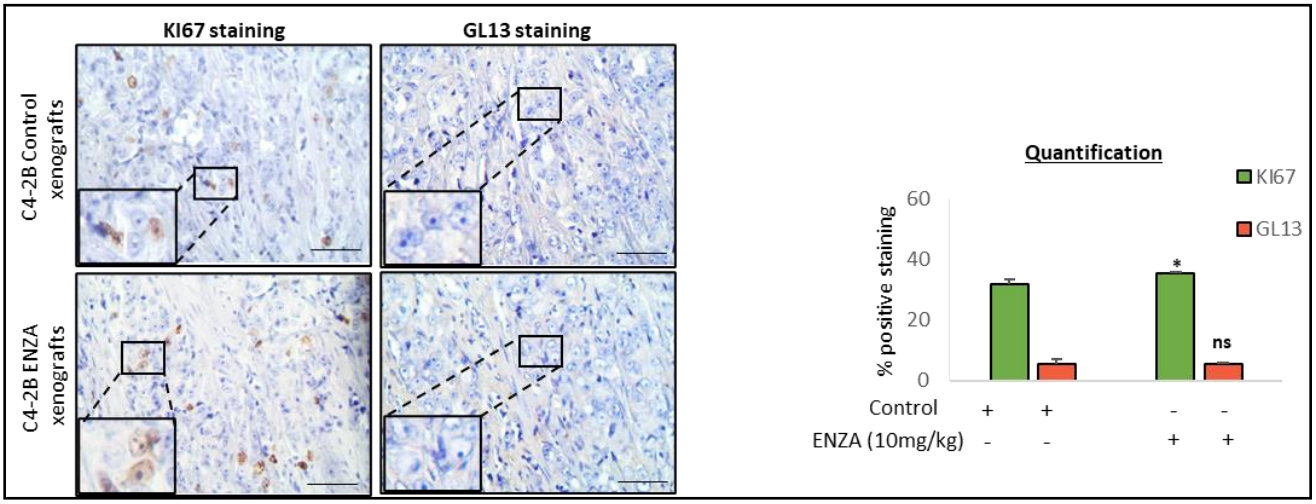


Figure 3.35: Ki67 and GL13 Immunohistochemical staining in serial sections of C4-2B - control and - enzalutamide treated mouse xenografts (left) and their quantification (right). Magnification: 200x (Objective 20x), scale bars: 30 µm. Inset magnification: 400x (Objective 40x)(128).

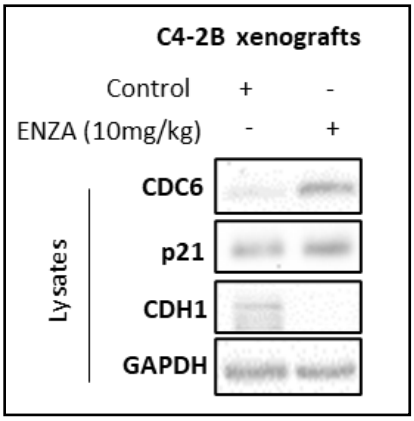


Figure 3.36: Western blotting of cell lysates from C4-2B mouse xenografts tumors, with or without treatment with enzalutamide, showed CDC6 stabilization and CDH1 decrease upon enzalutamide(128).

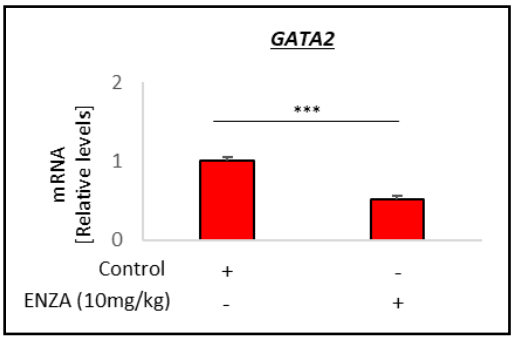


Figure 3.37: q-PCR for GATA2 mRNA levels of cell lysates from C4-2B mouse xenografts demonstrated a decrease of GATA2 levels upon treatment with enzalutamide (128).

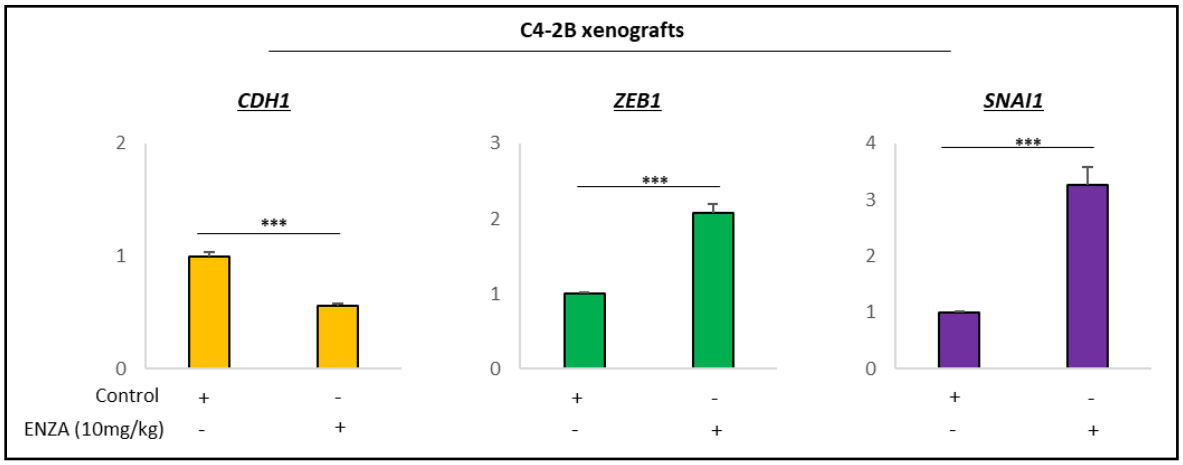


Figure 3.38: qPCR for EMT markers CDH1, ZEB1 and SNAI1 in C4-2B-control and C4-2B- enzalutamide treated (10 mg/kg) mouse xenografts revealed a clear EMT activation upon treatment with enzalutamide. Data are normalized to GAPDH expression (128).

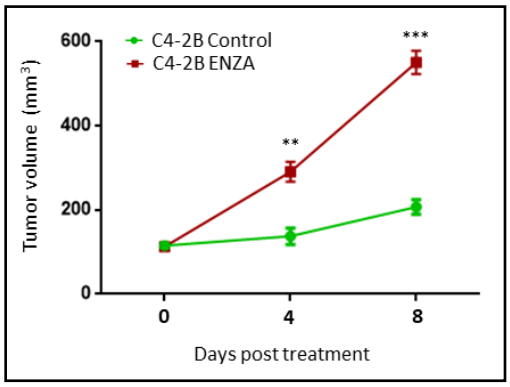


Figure 3.39: Curves showing tumor volume during treatment with enzalutamide in mice injected with C4-2B cells (128).

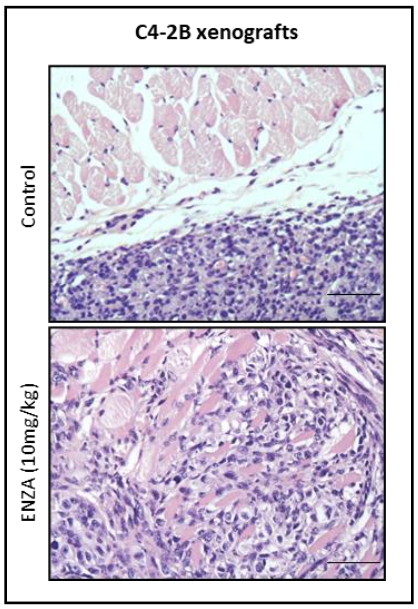


Figure 3.40: Immunohistochemistry of C4-2B mouse tumors revealed enhanced invasiveness upon treatment with enzalutamide, into the underlying muscle tissue. Magnification: 200x (Objective 20x), scale bars: 30 µm (128).

These results strengthen our previous observations revealing the role of enzalutamide as senescence-inducer in enzalutamide-responsive cells through GATA2^{high}-CDC6^{low} signaling axis. On the other hand, enzalutamide-resistant cells not only continue to exhibit no evidence of senescence upon treatment, but also display increased invasiveness followed by an opposite GATA2-CDC6 modulation pattern.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

In the present study we demonstrate a novel GATA2-CDC6 signaling axis that regulates senescence induction, with a different modulatory pattern in androgen-sensitive and androgen-resistant prostate cancer cells (**Figure 3.41**).

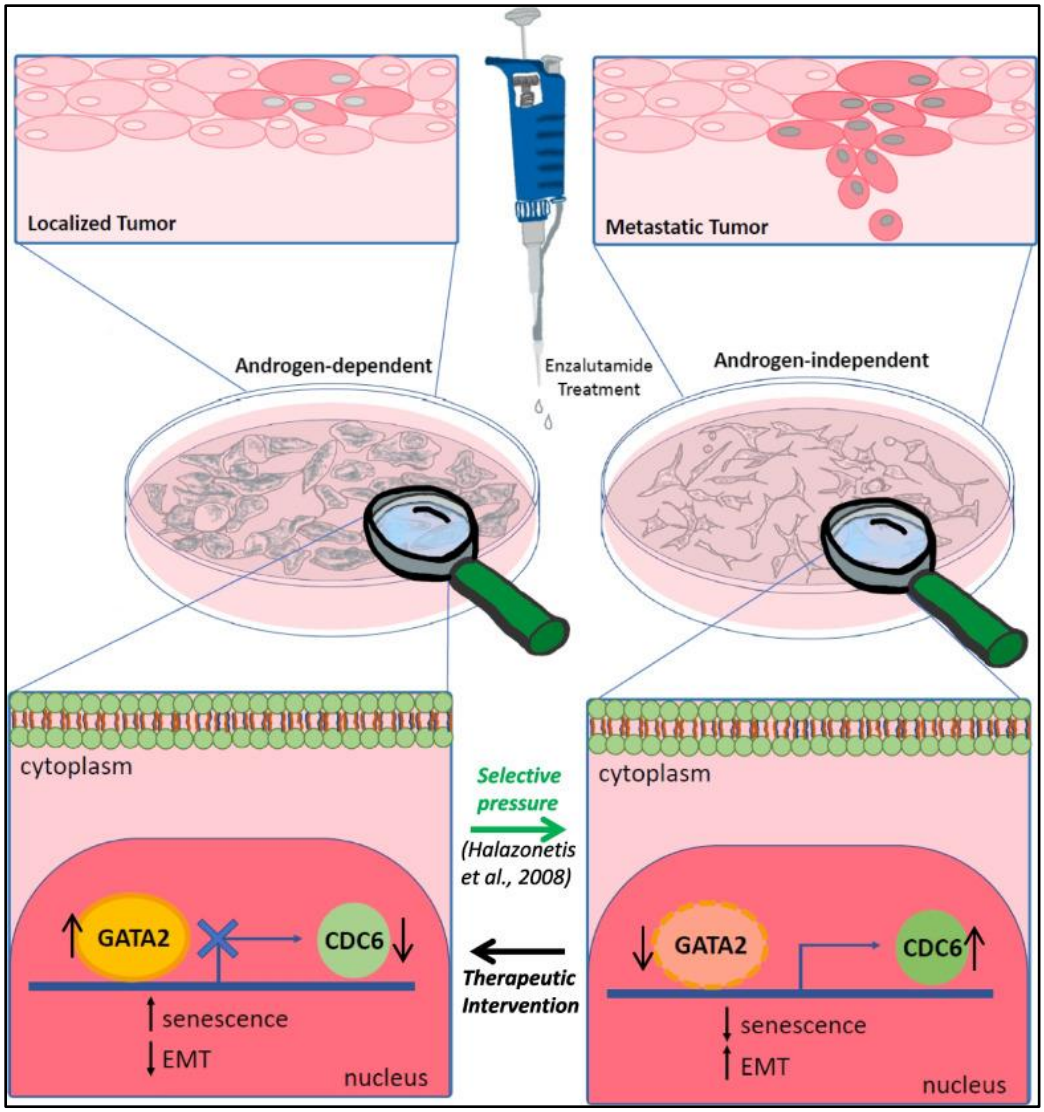


Figure 3.41: Schematic representation of the proposed GATA2-CDC6 signaling axis in androgen-dependent (LNCaP) and androgen-independent (C4-2B, PC-3) prostate cancer cells (128).

In this study we show that AR signaling inhibition via treatment with enzalutamide promotes GATA2 expression in androgen-sensitive prostate cancer cells, resulting in establishment of senescence and reduction of proliferation and EMT via direct CDC6 suppression. On the other hand, the GATA2-CDC6 axis exhibits a clearly converse modulatory pattern in androgen- resistant prostate cancer cells upon treatment with enzalutamide. In particular, AR signaling blockade in androgen-resistant prostate cancer cells reduces GATA2 expression, resulting in oncogenic growth, increased invasiveness but not senescence induction through CDC6 upregulation.

Remarkably, in this study we show that experimental depletion of CDC6 can bypass the oncogenic characteristics of metastatic and androgen-resistant cells and thus establishing senescence in this enzalutamide-resistant setting. This finding is really crucial as it outlines the molecular mechanisms that regulate Prostate Cancer progression and metastasis.

Additionally, in this work we indicate that previously unvalued harmful consequences in therapy of prostate cancer patients that acquired resistance to AR inhibitors may be due to GATA2-driven CDC6 expression. This finding has a very important impact in the therapy of Prostate Cancer as it delineates the clinical setting where therapy with AR inhibitors may represent a successful and productive therapeutic option.

It is well known that Androgen Deprivation Therapy and AR signaling inhibition represent the centerpiece in the field of Prostate Cancer therapy. However, it is of note that these therapeutic strategies are successful and effective only during early disease stages (28).

Enzalutamide is an oral second-generation nonsteroidal AR inhibitor that accordingly to clinical trials it improves the overall survival of patients with Castration Resistant Prostate Cancer (56, 143). However, it is of note that, not all the patients with Castration Resistant Prostate Cancer respond effectively to enzalutamide (144). Moreover, there are CRPC patients that initially respond to enzalutamide but gradually they acquire enzalutamide resistance (144). As a result, enzalutamide is currently not the first line treatment for patients with metastatic Castration Resistant Prostate Cancer.

Conversely, according to recent clinical data, enzalutamide may be remarkably more effective as first line therapeutic strategy in patients with localized and androgen-sensitive prostate cancer (145).

The results of the present work corroborate the clinical data, justifying mechanistically that enzalutamide is an effective drug for early-stage androgen-sensitive prostate patients but ineffective for many patients with metastatic Castration Resistant Prostate Cancer. Moreover, our work indicates that administration of enzalutamide in CRPC patients for long-time, results in acquiring therapy resistance and in enhanced oncogenic potential, through the GATA2-CDC6 axis that we propose in this study, explaining the restricted gain of enzalutamide in CRPC patients' overall survival rates (146).

The GATA family of transcription factors includes six proteins (GATA1-6) that actively regulate many physiological and pathological processes like the development and differentiation of various tissues (147). In the field of prostate carcinogenesis, GATA2 is a transcription factor of major importance, as it actively determines the development of the urogenital system (147). Moreover in the field of Prostate Cancer, according to recent literature, GATA2 induces AR signaling pathway (148) that in turn promotes the transcription of the replication licensing factor CDC6 (121, 149).

The model we propose in this study indicates that GATA2 functions as a CDC6 suppressor, where the regulation of CDC6 by GATA2 dominates over AR-mediated CDC6 regulation. Actually, our findings in androgen-sensitive LNCaP cells show that CDC6 downreguation due to AR inhibition can be reversed by GATA2 depletion (**Figure 3.5, 3.6**). However, the justification for the converse regulation of GATA2 levels in androgen-sensitive and –resistant cells still remains unknown.

Many factors that act upstream of GATA2 have been found in prostate cancer. For example, the E3 ubiquitin ligase COP1 promotes the ubiquitination of GATA2 at K419/K424 and its following degradation (148). Another upstream factor of GATA2 in prostate cancer is FOXA1. FOXA1 is a

transcription factor that recruits GATA2 to Forkhead DNA Binding Domain (FKHD)-containing genomic sites (150).

Moreover, in the CRPC setting, the GATA2 cistrome was shown to remarkably overlay with bromodomain and extraterminal (BET) proteins, whereas BET inhibitors were found to attenuate GATA2 activity (151). Although the differential response of androgen-sensitive and –resistant prostate cancer cells to AR blockade may be attributed to the above GATA2 regulators, the exact mechanism still needs to be further elucidated.

In the field of prostate cancer, there are molecular factors that bypass the AR signaling pathway and its importance for the survival of prostate cancer cells, modulating by their own the cellular growth. For example, *VAV3* and *TWIST1* oncogenes increased expression or *DKK3* tumor suppressor repression, have been associated with prostate cancer progression apart of AR signaling pathway (152). Additionally, kinase-dependent signaling pathways may also bypass AR signaling (153). In mice with *Pten* conditional prostate deletion, *Pten* loss that increases PI3K activity, may enhance the proliferation of prostate cancer cells even in the absence of androgens, rendering AR non-essential for their proliferation (154). Furthermore, AR inactivation has been shown to induce activation of the glucocorticoid receptor (GR), enhancing prostate cancer cells to progress into castration resistance (155, 156).

It is widely established the role of cellular senescence as a tumor barrier induced by a variety of stimuli, such as oncogene activation (79, 81, 120). In this context, CDC6 activation was shown to induce senescence through a process named oncogene-induced senescence (OIS) (79, 109). The findings of this work reveal a new side of CDC6-induced senescence, where CDC6 depletion rather than overexpression may induce senescence in prostate cancer. Our findings indicated that CDC6-mediated senescence was induced both upon AR inhibition in androgen-dependent cells and RNAi-mediated CDC6 silencing in CRPC cells, in line with observation from nasopharyngeal carcinoma cells where CDC6 loss resulted in induction of irradiation-mediated senescence (157).

In contrary, we indicate that CDC6 activation upon acquired enzalutamide resistance not only is ineffective for senescence induction, but also enhances EMT. One possible explanation for this result, is that prolonged activation of oncogenes such as CDC6, may promote senescence bypass and cell cycle re-entry, leading to the acquisition of even more malignant and aggressive oncogenic characteristics (66, 119, 158), caused by genetic and epigenetic modifications (122). Actually, ADT-induced senescence in androgen-dependent prostate cancer cells was found to allow their progression into CRPC, because of the existence of senescent cell subpopulations that regain their proliferative potential (93).

A very crucial point about acquired therapy resistance in prostate cancer hinges on the mechanism that regulates the progression of cells from a responsive to an irresponsive state. Based on our model, CDC6 downregulation that is promoted via treatment of androgen-sensitive cells with AR inhibitors, may cause DNA under-replication and consequently DNA damage, since cells may enter to mitosis without completing their DNA replication or having unresolved chromosomes (159, 160). DNA under-replication drives to gradual accumulation of genomic instability which finally represses tumor suppressive mechanisms due to selective pressure (79, 109, 119, 120, 161). So, the GATA2-CDC6 axis that acts as tumor suppressive mechanism in an androgen-responsive setting may be rewired to allow the progression to a more aggressive androgen- irresponsive state.

Many signaling pathways that have been shown to be involved in prostate cancer development and progression potentially regulate senescence induction (162-165). Our results indicate that CDC6 abrogation either via GATA2 or through direct CDC6 inhibition may induce senescence in both androgen-dependent and –resistant prostate cancer cells. As a result, the proposed GATA2-CDC6 axis with its senescence-modulating function offers a therapeutic window for the patients of prostate cancer, which could benefit by the rapid development of senolytics that selectively eliminate senescent cells in a tissue (166-168). Nowadays, several chemical compounds are being

tested as possible senolytic drugs (168-170), as well as numerous natural compounds with protective or anti-cancer effects are investigated as anti-senescence agents (171-173).

Our study proposes the GATA2-CDC6 signaling axis as a major regulator of cellular senescence and EMT in prostate cancer cells. Moreover, it sheds light on the clinical setting where AR signaling inhibition may be favorable for patients, or instead enhance their oncogenic features. Interestingly, our observations have direct therapeutic involvement, since direct modulation of the GATA2-CDC6 axis may greatly change prostate cancer cell fate and give rise to additional treatment options.

CHAPTER 5

LITERATURE

1. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin. 2023;73(1):17-48.
2. MacLean HE, Chu S, Warne GL, Zajac JD. Related individuals with different androgen receptor gene deletions. J Clin Invest. 1993;91(3):1123-8.
3. Saranyutanon S, Srivastava SK, Pai S, Singh S, Singh AP. Therapies Targeted to Androgen Receptor Signaling Axis in Prostate Cancer: Progress, Challenges, and Hope. Cancers (Basel). 2019;12(1).
4. Gelmann EP. Molecular biology of the androgen receptor. J Clin Oncol. 2002;20(13):3001-15.
5. Tan MH, Li J, Xu HE, Melcher K, Yong EL. Androgen receptor: structure, role in prostate cancer and drug discovery. Acta Pharmacol Sin. 2015;36(1):3-23.
6. Giovannucci E, Stampfer MJ, Krithivas K, Brown M, Dahl D, Brufsky A, et al. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. Proc Natl Acad Sci U S A. 1997;94(7):3320-3.
7. Schoenmakers E, Alen P, Verrijdt G, Peeters B, Verhoeven G, Rombauts W, et al. Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains. Biochem J. 1999;341 (Pt 3)(Pt 3):515-21.
8. Alen P, Claessens F, Verhoeven G, Rombauts W, Peeters B. The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. Mol Cell Biol. 1999;19(9):6085-97.
9. Brinkmann AO, Faber PW, van Rooij HC, Kuiper GG, Ris C, Klaassen P, et al. The human androgen receptor: domain structure, genomic organization and regulation of expression. J Steroid Biochem. 1989;34(1-6):307-10.
10. Haag P, Bektic J, Bartsch G, Klocker H, Eder IE. Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. J Steroid Biochem Mol Biol. 2005;96(3-4):251-8.
11. Henshall SM, Quinn DI, Lee CS, Head DR, Golovsky D, Brenner PC, et al. Altered expression of androgen receptor in the malignant epithelium and adjacent stroma is associated with early relapse in prostate cancer. Cancer Res. 2001;61(2):423-7.
12. Ruizeveld de Winter JA, Janssen PJ, Sleddens HM, Verleun-Mooijman MC, Trapman J, Brinkmann AO, et al. Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. Am J Pathol. 1994;144(4):735-46.
13. Chaturvedi AP, Dehm SM. Androgen Receptor Dependence. Adv Exp Med Biol. 2019;1210:333-50.
14. Denayer S, Helsen C, Thorrez L, Haelens A, Claessens F. The rules of DNA recognition by the androgen receptor. Mol Endocrinol. 2010;24(5):898-913.
15. Sahu B, Pihlajamaa P, Dubois V, Kerkhofs S, Claessens F, Janne OA. Androgen receptor uses relaxed response element stringency for selective chromatin binding and transcriptional regulation in vivo. Nucleic Acids Res. 2014;42(7):4230-40.
16. Traish AM, Morgentaler A. Epidermal growth factor receptor expression escapes androgen regulation in prostate cancer: a potential molecular switch for tumour growth. Br J Cancer. 2009;101(12):1949-56.
17. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, et al. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res. 1994;54(20):5474-8.
18. Edlind MP, Hsieh AC. PI3K-AKT-mTOR signaling in prostate cancer progression and androgen deprivation therapy resistance. Asian J Androl. 2014;16(3):378-86.

19. Ueda T, Bruchovsky N, Sadar MD. Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. J Biol Chem. 2002;277(9):7076-85.
20. Heinlein CA, Chang C. The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. Mol Endocrinol. 2002;16(10):2181-7.
21. Liao RS, Ma S, Miao L, Li R, Yin Y, Raj GV. Androgen receptor-mediated non-genomic regulation of prostate cancer cell proliferation. Transl Androl Urol. 2013;2(3):187-96.
22. Migliaccio A, Castoria G, Auricchio F. Analysis of androgen receptor rapid actions in cellular signaling pathways: receptor/Src association. Methods Mol Biol. 2011;776:361-70.
23. Kalyvianaki K, Panagiotopoulos AA, Malamos P, Moustou E, Tzardi M, Stathopoulos EN, et al. Membrane androgen receptors (OXER1, GPRC6A AND ZIP9) in prostate and breast cancer: A comparative study of their expression. Steroids. 2019;142:100-8.
24. Papakonstanti EA, Kampa M, Castanas E, Stournaras C. A rapid, nongenomic, signaling pathway regulates the actin reorganization induced by activation of membrane testosterone receptors. Mol Endocrinol. 2003;17(5):870-81.
25. Tan YG, Poon RJ, Pang LJ, Villanueva A, Huang HH, Chen K, et al. Comparative study of surgical orchidectomy and medical castration in treatment efficacy, adverse effects and cost based on a large prospective metastatic prostate cancer registry. Urol Oncol. 2020;38(8):682 e1- e9.
26. Huggins C, Hodges CV. Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. CA Cancer J Clin. 1972;22(4):232-40.
27. Roach M, 3rd, DeSilvio M, Lawton C, Uhl V, Machtay M, Seider MJ, et al. Phase III trial comparing whole-pelvic versus prostate-only radiotherapy and neoadjuvant versus adjuvant combined androgen suppression: Radiation Therapy Oncology Group 9413. J Clin Oncol. 2003;21(10):1904-11.
28. Karantanos T, Corn PG, Thompson TC. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. Oncogene. 2013;32(49):5501-11.
29. Harris WP, Mostaghel EA, Nelson PS, Montgomery B. Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. Nat Clin Pract Urol. 2009;6(2):76-85.
30. Crawford ED. Hormonal therapy in prostate cancer: historical approaches. Rev Urol. 2004;6 Suppl 7(Suppl 7):S3-S11.
31. Xu Y, Jiang YF, Wu B. New agonist- and antagonist-based treatment approaches for advanced prostate cancer. J Int Med Res. 2012;40(4):1217-26.
32. Rhee H, Gunter JH, Heathcote P, Ho K, Stricker P, Corcoran NM, et al. Adverse effects of androgen-deprivation therapy in prostate cancer and their management. BJU Int. 2015;115 Suppl 5:3-13.
33. Thompson IM. Flare Associated with LHRH-Agonist Therapy. Rev Urol. 2001;3 Suppl 3(Suppl 3):S10-4.
34. Heidegger I, Massoner P, Eder IE, Pircher A, Pichler R, Aigner F, et al. Novel therapeutic approaches for the treatment of castration-resistant prostate cancer. J Steroid Biochem Mol Biol. 2013;138:248-56.
35. Klotz L, Boccon-Gibod L, Shore ND, Andreou C, Persson BE, Cantor P, et al. The efficacy and safety of degarelix: a 12-month, comparative, randomized, open-label, parallel-group phase III study in patients with prostate cancer. BJU Int. 2008;102(11):1531-8.
36. Crawford ED, Hou AH. The role of LHRH antagonists in the treatment of prostate cancer. Oncology (Williston Park). 2009;23(7):626-30.

37. Gomella LG. Effective testosterone suppression for prostate cancer: is there a best castration therapy? Rev Urol. 2009;11(2):52-60.
38. Boccon-Gibod L, van der Meulen E, Persson BE. An update on the use of gonadotropin-releasing hormone antagonists in prostate cancer. Ther Adv Urol. 2011;3(3):127-40.
39. Rick FG, Block NL, Schally AV. An update on the use of degarelix in the treatment of advanced hormone-dependent prostate cancer. Onco Targets Ther. 2013;6:391-402.
40. Damber JE, Tammela TL, Iversen P, Abrahamsson PA, Boccon-Gibod L, Olesen TK, et al. The effect of baseline testosterone on the efficacy of degarelix and leuprolide: further insights from a 12-month, comparative, phase III study in prostate cancer patients. Urology. 2012;80(1):174-80.
41. Van Poppel H, Tombal B, de la Rosette JJ, Persson BE, Jensen JK, Kold Olesen T. Degarelix: a novel gonadotropin-releasing hormone (GnRH) receptor blocker--results from a 1-yr, multicentre, randomised, phase 2 dosage-finding study in the treatment of prostate cancer. Eur Urol. 2008;54(4):805-13.
42. Attard G, Reid AH, Auchus RJ, Hughes BA, Cassidy AM, Thompson E, et al. Clinical and biochemical consequences of CYP17A1 inhibition with abiraterone given with and without exogenous glucocorticoids in castrate men with advanced prostate cancer. J Clin Endocrinol Metab. 2012;97(2):507-16.
43. Chandrasekar T, Yang JC, Gao AC, Evans CP. Mechanisms of resistance in castration-resistant prostate cancer (CRPC). Transl Androl Urol. 2015;4(3):365-80.
44. Trachtenberg J, Pont A. Ketoconazole therapy for advanced prostate cancer. Lancet. 1984;2(8400):433-5.
45. Pont A, Williams PL, Azhar S, Reitz RE, Bochra C, Smith ER, et al. Ketoconazole blocks testosterone synthesis. Arch Intern Med. 1982;142(12):2137-40.
46. Pont A, Williams PL, Loose DS, Feldman D, Reitz RE, Bochra C, et al. Ketoconazole blocks adrenal steroid synthesis. Ann Intern Med. 1982;97(3):370-2.
47. Attard G, Reid AH, Yap TA, Raynaud F, Dowsett M, Settatree S, et al. Phase I clinical trial of a selective inhibitor of CYP17, abiraterone acetate, confirms that castration-resistant prostate cancer commonly remains hormone driven. J Clin Oncol. 2008;26(28):4563-71.
48. Yamaoka M, Hara T, Hitaka T, Kaku T, Takeuchi T, Takahashi J, et al. Orteronel (TAK-700), a novel non-steroidal 17,20-lyase inhibitor: effects on steroid synthesis in human and monkey adrenal cells and serum steroid levels in cynomolgus monkeys. J Steroid Biochem Mol Biol. 2012;129(3-5):115-28.
49. Vaughan ED. Long-Term Experience With 5-alpha-Reductase Inhibitors. Rev Urol. 2003;5 Suppl 5(Suppl 5):S22-7.
50. Juniewicz PE, McCarthy M, Lemp BM, Barbolt TA, Shaw C, Hollenbaugh DM, et al. The effect of the steroidal androgen receptor antagonist, Win 49,596, on the prostate and testis of beagle dogs. Endocrinology. 1990;126(5):2625-34.
51. Winneker RC, Wagner MM, Batzold FH. Studies on the mechanism of action of Win 49596: a steroidal androgen receptor antagonist. J Steroid Biochem. 1989;33(6):1133-8.
52. d'Ancona FC, Debruyne FM. Endocrine approaches in the therapy of prostate carcinoma. Hum Reprod Update. 2005;11(3):309-17.
53. Osguthorpe DJ, Hagler AT. Mechanism of androgen receptor antagonism by bicalutamide in the treatment of prostate cancer. Biochemistry. 2011;50(19):4105-13.
54. Goldspiel BR, Kohler DR. Flutamide: an antiandrogen for advanced prostate cancer. DICP. 1990;24(6):616-23.
55. Davis NB, Ryan CW, Stadler WM, Vogelzang NJ. A phase II study of nilutamide in men with prostate cancer after the failure of flutamide or bicalutamide therapy. BJU Int. 2005;96(6):787-90.

56. Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. N Engl J Med. 2012;367(13):1187-97.
57. Clegg NJ, Wongvipat J, Joseph JD, Tran C, Ouk S, Dilhas A, et al. ARN-509: a novel antiandrogen for prostate cancer treatment. Cancer Res. 2012;72(6):1494-503.
58. Rathkopf DE, Morris MJ, Fox JJ, Danila DC, Slovin SF, Hager JH, et al. Phase I study of ARN-509, a novel antiandrogen, in the treatment of castration-resistant prostate cancer. J Clin Oncol. 2013;31(28):3525-30.
59. Tetel MJ. Nuclear receptor coactivators in neuroendocrine function. J Neuroendocrinol. 2000;12(9):927-32.
60. Tetel MJ. Nuclear receptor coactivators: essential players for steroid hormone action in the brain and in behaviour. J Neuroendocrinol. 2009;21(4):229-37.
61. Fizazi K, Albiges L, Loriot Y, Massard C. ODM-201: a new-generation androgen receptor inhibitor in castration-resistant prostate cancer. Expert Rev Anticancer Ther. 2015;15(9):1007-17.
62. Echeverria PC, Picard D. Molecular chaperones, essential partners of steroid hormone receptors for activity and mobility. Biochim Biophys Acta. 2010;1803(6):641-9.
63. Pratt WB, Morishima Y, Osawa Y. The Hsp90 chaperone machinery regulates signaling by modulating ligand binding clefts. J Biol Chem. 2008;283(34):22885-9.
64. Haase M, Fitze G. HSP90AB1: Helping the good and the bad. Gene. 2016;575(2 Pt 1):171-86.
65. De Leon JT, Iwai A, Feau C, Garcia Y, Balsiger HA, Storer CL, et al. Targeting the regulation of androgen receptor signaling by the heat shock protein 90 cochaperone FKBP52 in prostate cancer cells. Proc Natl Acad Sci U S A. 2011;108(29):11878-83.
66. Gorgoulis V, Adams PD, Alimonti A, Bennett DC, Bischof O, Bishop C, et al. Cellular Senescence: Defining a Path Forward. Cell. 2019;179(4):813-27.
67. d'Adda di Fagagna F, Teo SH, Jackson SP. Functional links between telomeres and proteins of the DNA-damage response. Genes Dev. 2004;18(15):1781-99.
68. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature. 1990;345(6274):458-60.
69. Takai H, Smogorzewska A, de Lange T. DNA damage foci at dysfunctional telomeres. Curr Biol. 2003;13(17):1549-56.
70. Herbig U, Jobling WA, Chen BP, Chen DJ, Sedivy JM. Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). Mol Cell. 2004;14(4):501-13.
71. Chen QM, Prowse KR, Tu VC, Purdom S, Linskens MH. Uncoupling the senescent phenotype from telomere shortening in hydrogen peroxide-treated fibroblasts. Exp Cell Res. 2001;265(2):294-303.
72. Di Leonardo A, Linke SP, Clarkin K, Wahl GM. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. Genes Dev. 1994;8(21):2540-51.
73. Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P, et al. Reversal of human cellular senescence: roles of the p53 and p16 pathways. EMBO J. 2003;22(16):4212-22.
74. Jacobs JJ, de Lange T. Significant role for p16INK4a in p53-independent telomere-directed senescence. Curr Biol. 2004;14(24):2302-8.
75. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell. 1997;88(5):593-602.

76. Lin AW, Barradas M, Stone JC, van Aelst L, Serrano M, Lowe SW. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes Dev. 1998;12(19):3008-19.
77. Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature. 2005;436(7051):720-4.
78. Dimri GP, Itahana K, Acosta M, Campisi J. Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14(ARF) tumor suppressor. Mol Cell Biol. 2000;20(1):273-85.
79. Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature. 2006;444(7119):633-7.
80. Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 2005;434(7035):864-70.
81. Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, et al. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature. 2006;444(7119):638-42.
82. Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B, et al. Oncogene-induced senescence as an initial barrier in lymphoma development. Nature. 2005;436(7051):660-5.
83. Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, et al. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature. 2005;436(7051):725-30.
84. Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, et al. Tumour biology: senescence in premalignant tumours. Nature. 2005;436(7051):642.
85. Lazzerini Denchi E, Attwooll C, Pasini D, Helin K. Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. Mol Cell Biol. 2005;25(7):2660-72.
86. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol. 2007;8(9):729-40.
87. Campisi J, Robert L. Cell senescence: role in aging and age-related diseases. Interdiscip Top Gerontol. 2014;39:45-61.
88. Salama R, Sadaie M, Hoare M, Narita M. Cellular senescence and its effector programs. Genes Dev. 2014;28(2):99-114.
89. Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature. 1998;396(6706):84-8.
90. Dickson MA, Hahn WC, Ino Y, Ronfard V, Wu JY, Weinberg RA, et al. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. Mol Cell Biol. 2000;20(4):1436-47.
91. Ramirez RD, Morales CP, Herbert BS, Rohde JM, Passons C, Shay JW, et al. Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. Genes Dev. 2001;15(4):398-403.
92. Haugstetter AM, Loddenkemper C, Lenze D, Grone J, Standfuss C, Petersen I, et al. Cellular senescence predicts treatment outcome in metastasised colorectal cancer. Br J Cancer. 2010;103(4):505-9.
93. Carpenter V, Saleh T, Min Lee S, Murray G, Reed J, Souers A, et al. Androgen-deprivation induced senescence in prostate cancer cells is permissive for the development of castration-resistance but susceptible to senolytic therapy. Biochem Pharmacol. 2021;193:114765.

94. Ewald JA, Desotelle JA, Church DR, Yang B, Huang W, Laurila TA, et al. Androgen deprivation induces senescence characteristics in prostate cancer cells in vitro and in vivo. Prostate. 2013;73(4):337-45.
95. Burton DG, Giribaldi MG, Munoz A, Halvorsen K, Patel A, Jorda M, et al. Androgen deprivation-induced senescence promotes outgrowth of androgen-refractory prostate cancer cells. PLoS One. 2013;8(6):e68003.
96. Blow JJ, Dutta A. Preventing re-replication of chromosomal DNA. Nat Rev Mol Cell Biol. 2005;6(6):476-86.
97. Baker TA, Bell SP. Polymerases and the replisome: machines within machines. Cell. 1998;92(3):295-305.
98. Petrakis TG, Komseli ES, Papaioannou M, Vougas K, Polyzos A, Myrianthopoulos V, et al. Exploring and exploiting the systemic effects of deregulated replication licensing. Semin Cancer Biol. 2016;37-38:3-15.
99. DePamphilis ML, Blow JJ, Ghosh S, Saha T, Noguchi K, Vassilev A. Regulating the licensing of DNA replication origins in metazoa. Curr Opin Cell Biol. 2006;18(3):231-9.
100. Neuwald AF, Aravind L, Spouge JL, Koonin EV. AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res. 1999;9(1):27-43.
101. Cook JG, Park CH, Burke TW, Leone G, DeGregori J, Engel A, et al. Analysis of Cdc6 function in the assembly of mammalian prereplication complexes. Proc Natl Acad Sci U S A. 2002;99(3):1347-52.
102. Mailand N, Diffley JF. CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. Cell. 2005;122(6):915-26.
103. Clijsters L, Wolthuis R. PIP-box-mediated degradation prohibits re-accumulation of Cdc6 during S phase. J Cell Sci. 2014;127(Pt 6):1336-45.
104. Walter D, Hoffmann S, Komseli ES, Rappsilber J, Gorgoulis V, Sorensen CS. SCF(Cyclin F)-dependent degradation of CDC6 suppresses DNA re-replication. Nat Commun. 2016;7:10530.
105. Herbig U, Marlar CA, Fanning E. The Cdc6 nucleotide-binding site regulates its activity in DNA replication in human cells. Mol Biol Cell. 1999;10(8):2631-45.
106. Jeruzalmi D, O'Donnell M, Kuriyan J. Crystal structure of the processivity clamp loader gamma (gamma) complex of E. coli DNA polymerase III. Cell. 2001;106(4):429-41.
107. Borlado LR, Mendez J. CDC6: from DNA replication to cell cycle checkpoints and oncogenesis. Carcinogenesis. 2008;29(2):237-43.
108. Karakaidos P, Taraviras S, Vassiliou LV, Zacharatos P, Kastrinakis NG, Kougiou D, et al. Overexpression of the replication licensing regulators hCdt1 and hCdc6 characterizes a subset of non-small-cell lung carcinomas: synergistic effect with mutant p53 on tumor growth and chromosomal instability--evidence of E2F-1 transcriptional control over hCdt1. Am J Pathol. 2004;165(4):1351-65.
109. Liontos M, Koutsami M, Sideridou M, Evangelou K, Kletsas D, Levy B, et al. Deregulated overexpression of hCdt1 and hCdc6 promotes malignant behavior. Cancer Res. 2007;67(22):10899-909.
110. Sideridou M, Zakopoulou R, Evangelou K, Liontos M, Kotsinas A, Rampakakis E, et al. Cdc6 expression represses E-cadherin transcription and activates adjacent replication origins. J Cell Biol. 2011;195(7):1123-40.
111. Gorgoulis VG, Zacharatos P, Mariatos G, Kotsinas A, Bouda M, Kletsas D, et al. Transcription factor E2F-1 acts as a growth-promoting factor and is associated with adverse prognosis in non-small cell lung carcinomas. J Pathol. 2002;198(2):142-56.

112. Zacharatos P, Kotsinas A, Evangelou K, Karakaidos P, Vassiliou LV, Rezaei N, et al. Distinct expression patterns of the transcription factor E2F-1 in relation to tumour growth parameters in common human carcinomas. J Pathol. 2004;203(3):744-53.
113. Tsantoulis PK, Gorgoulis VG. Involvement of E2F transcription factor family in cancer. Eur J Cancer. 2005;41(16):2403-14.
114. Evangelou K, Kotsinas A, Mariolis-Sapsakos T, Giannopoulos A, Tsantoulis PK, Constantinides C, et al. E2F-1 overexpression correlates with decreased proliferation and better prognosis in adenocarcinomas of Barrett oesophagus. J Clin Pathol. 2008;61(5):601-5.
115. Jacot W, Fiche M, Zaman K, Wolfer A, Lamy PJ. The HER2 amplicon in breast cancer: Topoisomerase IIA and beyond. Biochim Biophys Acta. 2013;1836(1):146-57.
116. Krishnamurti U, Silverman JF. HER2 in breast cancer: a review and update. Adv Anat Pathol. 2014;21(2):100-7.
117. Martin V, Cappuzzo F, Mazzucchelli L, Frattini M. HER2 in solid tumors: more than 10 years under the microscope; where are we now? Future Oncol. 2014;10(8):1469-86.
118. Akman BH, Can T, Erson-Bensan AE. Estrogen-induced upregulation and 3'-UTR shortening of CDC6. Nucleic Acids Res. 2012;40(21):10679-88.
119. Galanos P, Vougas K, Walter D, Polyzos A, Maya-Mendoza A, Haagensen EJ, et al. Chronic p53-independent p21 expression causes genomic instability by deregulating replication licensing. Nat Cell Biol. 2016;18(7):777-89.
120. Halazonetis TD, Gorgoulis VG, Bartek J. An oncogene-induced DNA damage model for cancer development. Science. 2008;319(5868):1352-5.
121. Jin F, Fondell JD. A novel androgen receptor-binding element modulates Cdc6 transcription in prostate cancer cells during cell-cycle progression. Nucleic Acids Res. 2009;37(14):4826-38.
122. Zampetidis CP, Galanos P, Angelopoulou A, Zhu Y, Polyzou A, Karamitros T, et al. A recurrent chromosomal inversion suffices for driving escape from oncogene-induced senescence via subTAD reorganization. Mol Cell. 2021;81(23):4907-23 e8.
123. Angelopoulou A, Papaspyropoulos A, Papantonis A, Gorgoulis VG. CRISPR-Cas9-mediated induction of large chromosomal inversions in human bronchial epithelial cells. STAR Protoc. 2022;3(2):101257.
124. Papaspyropoulos A, Angelopoulou A, Mourkioti I, Polyzou A, Pankova D, Toskas K, et al. RASSF1A disrupts the NOTCH signaling axis via SNURF/RNF4-mediated ubiquitination of HES1. EMBO Rep. 2022;23(2):e51287.
125. Papaspyropoulos A, Bradley L, Thapa A, Leung CY, Toskas K, Koennig D, et al. RASSF1A uncouples Wnt from Hippo signalling and promotes YAP mediated differentiation via p73. Nat Commun. 2018;9(1):424.
126. Evangelou K, Veroutis D, Paschalaki K, Foukas PG, Lagopati N, Dimitriou M, et al. Pulmonary infection by SARS-CoV-2 induces senescence accompanied by an inflammatory phenotype in severe COVID-19: possible implications for viral mutagenesis. Eur Respir J. 2022;60(2).
127. Kohli J, Wang B, Brandenburg SM, Basisty N, Evangelou K, Varela-Eirin M, et al. Algorithmic assessment of cellular senescence in experimental and clinical specimens. Nat Protoc. 2021;16(5):2471-98.
128. Mourkioti I, Polyzou A, Veroutis D, Theocharous G, Lagopati N, Gentile E, et al. A GATA2-CDC6 axis modulates androgen receptor blockade-induced senescence in prostate cancer. J Exp Clin Cancer Res. 2023;42(1):187.

129. McConnell BB, Starborg M, Brookes S, Peters G. Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. Curr Biol. 1998;8(6):351-4.
130. Thalmann GN, Anezinis PE, Chang SM, Zhau HE, Kim EE, Hopwood VL, et al. Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. Cancer Res. 1994;54(10):2577-81.
131. Lin HM, Mak B, Yeung N, Huynh K, Meikle TG, Mellett NA, et al. Overcoming enzalutamide resistance in metastatic prostate cancer by targeting sphingosine kinase. EBioMedicine. 2021;72:103625.
132. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol. 1979;17(1):16-23.
133. Smith R, Liu M, Liby T, Bayani N, Bucher E, Chiotti K, et al. Enzalutamide response in a panel of prostate cancer cell lines reveals a role for glucocorticoid receptor in enzalutamide resistant disease. Sci Rep. 2020;10(1):21750.
134. Zhao J, Zhao Y, Wang L, Zhang J, Karnes RJ, Kohli M, et al. Alterations of androgen receptor-regulated enhancer RNAs (eRNAs) contribute to enzalutamide resistance in castration-resistant prostate cancer. Oncotarget. 2016;7(25):38551-65.
135. Russo JW, Gao C, Bhasin SS, Voznesensky OS, Calagua C, Arai S, et al. Downregulation of Dipeptidyl Peptidase 4 Accelerates Progression to Castration-Resistant Prostate Cancer. Cancer Res. 2018;78(22):6354-62.
136. Farah E, Zhang Z, Utturkar SM, Liu J, Ratliff TL, Liu X. Targeting DNMTs to Overcome Enzalutamide Resistance in Prostate Cancer. Mol Cancer Ther. 2022;21(1):193-205.
137. Furlan T, Kirchmair A, Sampson N, Puhr M, Gruber M, Trajanoski Z, et al. MYC-Mediated Ribosomal Gene Expression Sensitizes Enzalutamide-resistant Prostate Cancer Cells to EP300/CREBBP Inhibitors. Am J Pathol. 2021;191(6):1094-107.
138. Verma S, Shankar E, Kalayci FNC, Mukunda A, Alassfar M, Singh V, et al. Androgen Deprivation Induces Transcriptional Reprogramming in Prostate Cancer Cells to Develop Stem Cell-Like Characteristics. Int J Mol Sci. 2020;21(24).
139. Verma S, Shankar E, Chan ER, Gupta S. Metabolic Reprogramming and Predominance of Solute Carrier Genes during Acquired Enzalutamide Resistance in Prostate Cancer. Cells. 2020;9(12).
140. Hernandez-Segura A, Nehme J, Demaria M. Hallmarks of Cellular Senescence. Trends Cell Biol. 2018;28(6):436-53.
141. Young AR, Narita M. SASP reflects senescence. EMBO Rep. 2009;10(3):228-30.
142. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. 2015;1(6):417-25.
143. Beer TM, Armstrong AJ, Rathkopf DE, Loriot Y, Sternberg CN, Higano CS, et al. Enzalutamide in metastatic prostate cancer before chemotherapy. N Engl J Med. 2014;371(5):424-33.
144. Tucci M, Zichi C, Buttigliero C, Vignani F, Scagliotti GV, Di Maio M. Enzalutamide-resistant castration-resistant prostate cancer: challenges and solutions. Onco Targets Ther. 2018;11:7353-68.
145. Armstrong AJ, Azad AA, Iguchi T, Szmulewitz RZ, Petrylak DP, Holzbeierlein J, et al. Improved Survival With Enzalutamide in Patients With Metastatic Hormone-Sensitive Prostate Cancer. J Clin Oncol. 2022;40(15):1616-22.
146. Linder S, van der Poel HG, Bergman AM, Zwart W, Prekovic S. Enzalutamide therapy for advanced prostate cancer: efficacy, resistance and beyond. Endocr Relat Cancer. 2018;26(1):R31-R52.
147. Rodriguez-Bravo V, Carceles-Cordon M, Hoshida Y, Cordon-Cardo C, Galsky MD, Domingo-Domenech J. The role of GATA2 in lethal prostate cancer aggressiveness. Nat Rev Urol. 2017;14(1):38-48.

148. Shen T, Dong B, Meng Y, Moore DD, Yang F. A COP1-GATA2 axis suppresses AR signaling and prostate cancer. Proc Natl Acad Sci U S A. 2022;119(43):e2205350119.
149. Karanika S, Karantanos T, Li L, Wang J, Park S, Yang G, et al. Targeting DNA Damage Response in Prostate Cancer by Inhibiting Androgen Receptor-CDC6-ATR-Chk1 Signaling. Cell Rep. 2017;18(8):1970-81.
150. Zhao JC, Fong KW, Jin HJ, Yang YA, Kim J, Yu J. FOXA1 acts upstream of GATA2 and AR in hormonal regulation of gene expression. Oncogene. 2016;35(33):4335-44.
151. Chaytor L, Simcock M, Nakjang S, Heath R, Walker L, Robson C, et al. The Pioneering Role of GATA2 in Androgen Receptor Variant Regulation Is Controlled by Bromodomain and Extraterminal Proteins in Castrate-Resistant Prostate Cancer. Mol Cancer Res. 2019;17(6):1264-78.
152. Marques RB, Dits NF, Erkens-Schulze S, van Weerden WM, Jenster G. Bypass mechanisms of the androgen receptor pathway in therapy-resistant prostate cancer cell models. PLoS One. 2010;5(10):e13500.
153. Wyatt AW, Gleave ME. Targeting the adaptive molecular landscape of castration-resistant prostate cancer. EMBO Mol Med. 2015;7(7):878-94.
154. Wang S, Gao J, Lei Q, Rozengurt N, Pritchard C, Jiao J, et al. Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. Cancer Cell. 2003;4(3):209-21.
155. Santer FR, Erb HH, McNeill RV. Therapy escape mechanisms in the malignant prostate. Semin Cancer Biol. 2015;35:133-44.
156. Xie N, Cheng H, Lin D, Liu L, Yang O, Jia L, et al. The expression of glucocorticoid receptor is negatively regulated by active androgen receptor signaling in prostate tumors. Int J Cancer. 2015;136(4):E27-38.
157. Yu X, Liu Y, Yin L, Peng Y, Peng Y, Gao Y, et al. Radiation-promoted CDC6 protein stability contributes to radioresistance by regulating senescence and epithelial to mesenchymal transition. Oncogene. 2019;38(4):549-63.
158. Komseli ES, Pateras IS, Krejsgaard T, Stawiski K, Rizou SV, Polyzos A, et al. A prototypical non-malignant epithelial model to study genome dynamics and concurrently monitor micro-RNAs and proteins in situ during oncogene-induced senescence. BMC Genomics. 2018;19(1):37.
159. Bertolin AP, Hoffmann JS, Gottifredi V. Under-Replicated DNA: The Byproduct of Large Genomes? Cancers (Basel). 2020;12(10).
160. Spies J, Lukas C, Somyajit K, Rask MB, Lukas J, Neelsen KJ. 53BP1 nuclear bodies enforce replication timing at under-replicated DNA to limit heritable DNA damage. Nat Cell Biol. 2019;21(4):487-97.
161. Georgakilas AG, Gorgoulis VG. Systemic dna damage: Mechanisms, effects and mitigation strategies. Semin Cancer Biol. 2016;37-38:1-2.
162. Mourkioti I, Angelopoulou A, Belogiannis K, Lagopati N, Potamianos S, Kyrodimos E, et al. Interplay of Developmental Hippo-Notch Signaling Pathways with the DNA Damage Response in Prostate Cancer. Cells. 2022;11(15).
163. Papaspyropoulos A, Hazapis O, Lagopati N, Polyzou A, Papanastasiou AD, Liontos M, et al. The Role of Circular RNAs in DNA Damage Response and Repair. Cancers (Basel). 2021;13(21).
164. Lagopati N, Belogiannis K, Angelopoulou A, Papaspyropoulos A, Gorgoulis V. Non-Canonical Functions of the ARF Tumor Suppressor in Development and Tumorigenesis. Biomolecules. 2021;11(1).
165. Papaspyropoulos A, Lagopati N, Mourkioti I, Angelopoulou A, Kyriazis S, Liontos M, et al. Regulatory and Functional Involvement of Long Non-Coding RNAs in DNA Double-Strand Break Repair Mechanisms. Cells. 2021;10(6).

166. Raffaele M, Vinciguerra M. The costs and benefits of senotherapeutics for human health. Lancet Healthy Longev. 2022;3(1):e67-e77.
167. Kirkland JL, Tchkonia T. Senolytic drugs: from discovery to translation. J Intern Med. 2020;288(5):518-36.
168. Wang L, Lankhorst L, Bernards R. Exploiting senescence for the treatment of cancer. Nat Rev Cancer. 2022;22(6):340-55.
169. Baker DJ, Childs BG, Durik M, Wijers ME, Sieben CJ, Zhong J, et al. Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. Nature. 2016;530(7589):184-9.
170. Baker DJ, Wijshake T, Tchkonia T, LeBrasseur NK, Childs BG, van de Sluis B, et al. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature. 2011;479(7372):232-6.
171. Tzekaki EE, Geromichalos G, Lavrentiadou SN, Tsantarliotou MP, Pantazaki AA, Papaspyropoulos A. Oleuropein is a natural inhibitor of PAI-1-mediated proliferation in human ER-/PR- breast cancer cells. Breast Cancer Res Treat. 2021;186(2):305-16.
172. Tzekaki EE, Papaspyropoulos A, Tsolaki M, Lazarou E, Kozori M, Pantazaki Alpha A. Restoration of BMI1 levels after the administration of early harvest extra virgin olive oil as a therapeutic strategy against Alzheimer's disease. Exp Gerontol. 2021;144:111178.
173. Varela-Eirin M, Carpintero-Fernandez P, Sanchez-Temprano A, Varela-Vazquez A, Paino CL, Casado-Diaz A, et al. Senolytic activity of small molecular polyphenols from olive restores chondrocyte redifferentiation and promotes a pro-regenerative environment in osteoarthritis. Aging (Albany NY). 2020;12(16):15882-905.