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The effects of *DDX4*-deletion on PC3 tumor formation, growth and transcriptome

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Cancer-germline (CG) antigen-genes are a set of genes normally expressed in germ cells, trophoblasts and a few somatic tissues but also aberrantly expressed in a wide range of human malignancies. Cancer cells share multiple functional characteristics with germ cells, such as hyperproliferation and extensive migration, and it has long been proposed that cancer cells harness their reservoir of normally silent germline genes to adapt and face challenges in their new environment. Germ cells are characterized by germline specific ribonucleoprotein granules, also known as germ granules. The germ granules appear in the cytoplasm of germ cells during haploid differentiation and serve as an important platform for coordinating different RNA-regulatory pathways. Interestingly, components of the germ granules are also cancer-germline antigens, suggesting a role in cancer formation and progression.

One of the main germ granule components, DEAD-box helicase 4 (DDX4), appears in the cytoplasm of several human cancers. This thesis aims to elucidate the effects of *DDX4* deletion on tumor transcriptome and xenograft tumor formation in immunodeficient mice.

Our data revealed that *DDX4*-deletion does in fact hinder tumor formation, growth, progression and likely metastasis in xenograft tumor models. In addition, DDX4-deletion has major effects on tumor transcriptome. Therefore, research on DDX4 and its role in cancer could provide valuable information regarding its utility as a marker for certain cancers or even as a therapeutic target. In addition, the results stress the need to validate RNA-sequencing results.

Key words: cancer germline antigen gene, DDX4, germ granule.

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1 Introduction

1.1 Germ cells

Germ cells are central components in the biology of sexually reproducing organisms and the route to transfer genetic information from one generation to the next (Alberts et al., 2015). Germ cells are unique in their capacity to, after a period of mitotic proliferation, undergo meiotic division and differentiate into haploid gametes; oocytes in the female and spermatozoa in the male (Alberts et al., 2015; Mayr, 2001). Later, the fusion of these two types of gametes produces an embryo, where a new set of primordial germ cells (PGCs) begin the cycle of sexual reproduction all over again (Alberts et al., 2015). Germ cells are the only type of cells capable of creating a new organism (Mayr, 2001; Teletin et al., 2017).

Early in their development, primordial germ cells migrate into the forming gonad, which will differentiate to ovaries in females and testes in males. During their migration, the primordial germ cells receive signals from the adjacent somatic cells promoting survival, proliferation and migration (Alberts et al., 2015). After reaching the gonadal ridge and proliferating for a couple of days, the PGCs will commit to their respective developmental pathway to become either an egg or sperm (Figure 1). This commitment to a certain developmental pathway depends not only on the sex chromosome of the primordial cell, but also by the sex chromosome and signals of the somatic cells of the surrounding gonad (Alberts et al., 2015; Mayr, 2001). Determining the differentiation of the gonad, i.e. sex determination, is a complex and dynamic process, largely affected by multiple genetic and epigenetic factors (Gunes et al., 2016; Koopman & Wilhelm, 2011). However, in the formation of gametes, oogenesis and spermatogenesis, the key events of meiosis and cellular differentiation are closely intertwined (Alberts et al., 2015).

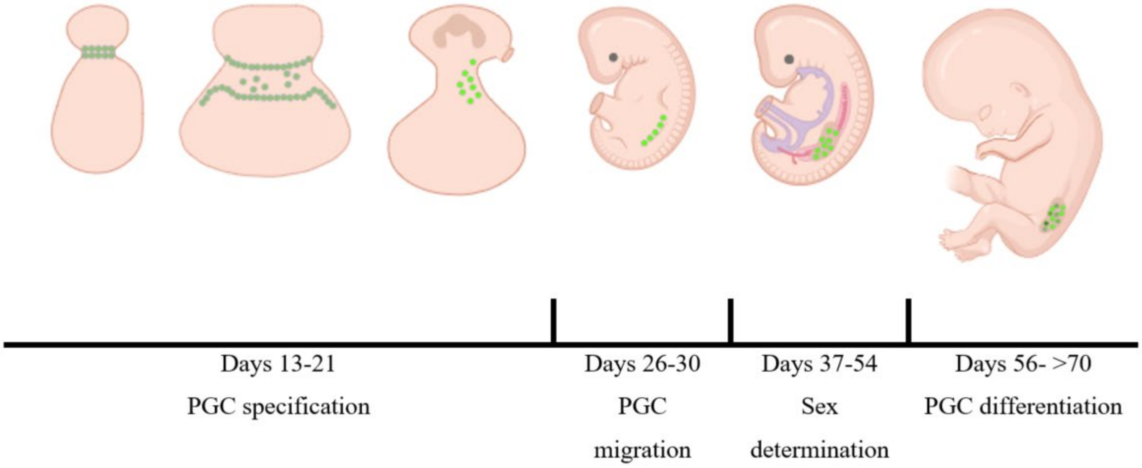

Figure 1. Primordial germ cell (PGC) development. Human primordial germ cells (PGCs) are specified around weeks 2-3 after ovulation and subsequently migrate to the genital ridges. There, they interact with somatic cells to form the indifferent gonads. Sex determination begins approximately at weeks 5-6 post-ovulation. The embryonic stage concludes at week 8, followed by the fetal stages. During fetal development, PGCs undergo heterogeneous differentiation, developing into oocytes in females and pro-spermatogonia in males. Germ cells depicted in green, days post ovulation. Modified, with permission, from "*Mitochondrial DNA selection in human germ cells"* by Chen & Clark 2018, Nature Cell Biology 20, p. 118-120. Created with BioRender.com

1.1.1 Testis development

Testis development is a complex process that occurs during embryonic development in mammals, including humans (Mäkelä et al., 2019). The development of both gonads, testes and ovaries, originate from the gonadal ridge, a bipotential precursor of gonads. The gonadal primordia of XY and XX individuals are indistinguishable until a single gene, Sex-determining region Y (*Sry*), located in the Y chromosome is activated. The *Sry* gene acts as a genetic switch that turns the undifferentiated gonadal ridge into the male pathway and is therefore considered the master regulator of male sex determination (Koopman & Wilhelm, 2011; She & Yang, 2017). In case *Sry* is not expressed, or dysfunctional due to a mutation or deletion, a default genetic program is initiated and an ovary forms (Koopman & Wilhelm, 2011). The first morphological outcome of Sry expression is a rapid increase in proliferation leading to a noticeable change in size of the developing testis compared to a developing ovary of the same age (Mäkelä et al., 2019; Schmahl et al., 2000). However, even though deemed as the master regulator of testis determination pathway, the activation of *Sry* expression does very little itself. Only after the Sry protein reaches a certain threshold level, it upregulates Sox9 leading to a complex cascade of downstream effects, further stimulating the expression of other male-specific genes (Mäkelä et al., 2019; She & Yang, 2017). Sex differentiation does not conclude during embryonic development; instead, it continues throughout adulthood to guarantee maintenance of the gonads and accurate sex development (She & Yang, 2017).

1.1.1.1 Sertoli cells

During early embryonic development, the unipotent PGCs, progenitors of gametes, migrate into the gonadal ridge. This migration of PGCs and activation of *Sry* is accompanied by the formation of various somatic cell types within the coelomic epithelium (Mäkelä et al., 2019). The first somatic cells to differentiate in the XY gonad are the Sox9 expressing pre-Sertoli cells (Karl & Capel, 1998). The number of Sox9 expressing cells is crucial in order to secure normal testis development. Once the pre-Sertoli cells have fully differentiated, they are unable to proliferate making their number finite (Sharpe et al., 2003). Among the mechanisms helping to maximize the number of Sox9-positive cells are the expression of FGF9 and prostaglandin D2 (PGD2), along with cell proliferation. FGF9 plays a dual role; primarily to repress pro-ovary genes like Wnt4, through which its secondary effect is to upregulate Sox9. The role of PGD2 is to signal neighbouring XX gonadal cells to induce Sox9 expression, high jacking these cells towards Sertoli fate. Both Sry and Sox9 play a pivotal role in testis development, however the hallmark of Sertoli cell differentiation is Sox9 (Sekido et al., 2004; Svingen & Koopman, 2013). Furthermore, Sox9 coordinates the maintenance of Sertoli cell identity and regression of Müllerian ducts (Mäkelä et al., 2019).

1.1.1.2 Therapeutic potential of immune privileged Sertoli cells

Sertoli cells are a structural component of the seminiferous tubules in human testes. As large amorphous cells their main function is to nurture the developing spermatozoa (Shah et al., 2021). Each Sertoli cell is responsible for supporting a certain number of developing germ cells, therefore their number determines the capacity of spermatogenesis (Sharpe et al., 2003). In order for Sertoli cells to protect the maturing germ cells from immune destruction, they reside in an immune privileged environment. This immune protection is created by one of the tightest tissue barriers in mammals, the blood-testis barrier, which comprises of Sertoli-Sertoli cell junctions. (Luaces et al., 2023; Mruk & Cheng, 2015). In addition to a physical barrier, Sertoli cells partake in creating a tolerogenic microenvironment by expressing several immunoregulatory factors (Kaur et al., 2014). Furthermore, when Sertoli cells are transplanted ectopically they are able to produce a number of complement inhibitors as well as proteins that regulate both B-cell proliferation and NK-cell activity, showing that the immunoregulatory function of Sertoli cells is not limited to the testis (Washburn, Hibler, Thompson, et al., 2022).

Due to the capabilities in maintaining an immunoregulatory environment, Sertoli cells have become attractive candidates in the treatment of various different diseases. They have shown potential in treating various diseases like diabetes, Parkinson's, Alzheimer's, amyotrophic lateral sclerosis, male infertility, infections, and spinal cord injuries, improving symptoms and reversing disease progression in animal models. Sertoli cells can also enhance the viability and function of co-transplanted cells, act as a drug delivery platform, and produce therapeutic molecules, making them a valuable area for further research and exploration in alternative therapies (Kaur et al., 2015; Washburn, Hibler, Kaur, et al., 2022).

1.1.2 Spermatogenesis

Spermatogenesis is the complex process involving several stages by which spermatozoa are produced from male primordial germ cells within the seminiferous tubules of the testes. During early embryonic development, the unipotent PGCs, progenitors of gametes, migrate into the gonadal ridge. In males, the subsequent differentiation produces spermatogonial stem cells, ultimately giving rise to spermatozoa in puberty (Cheng et al., 2022a).

In humans, the multifaceted process of spermatogenesis takes 2.5 months to complete and occurs in three phases: mitosis, meiosis and spermiogenesis (Gilbert, 2000; Mäkelä & Toppari, 2017). When male PGCs enter the seminiferous cords they become gonocytes and remain in cell cycle arrest until birth. After birth, the gonocytes resume to divide, and differentiate into spermatogonial stem cells. Spermatogenesis starts when these spermatogonial stem cells commit to further differentiate and take the first steps towards germ cell maturation. (Cheng et al., 2022b; Mäkelä & Toppari, 2017).

The road from spermatogonia to spermatozoa can be divided into three phases. During the first phase, the early stage cells undergo mitotic division allowing spermatogonia to multiply and produce primary spermatocytes. In the second phase, the diploid cells form haploid cells: primary spermatocytes undergo two sequential meiotic divisions producing round spermatids. (Mäkelä & Toppari, 2017; Suede et al., 2023). In the third and final stage, the round spermatids mature and become mobile spermatozoa through a process called spermiogenesis (Suede et al., 2023). The phases of spermatogenesis are depicted in Figure 2.

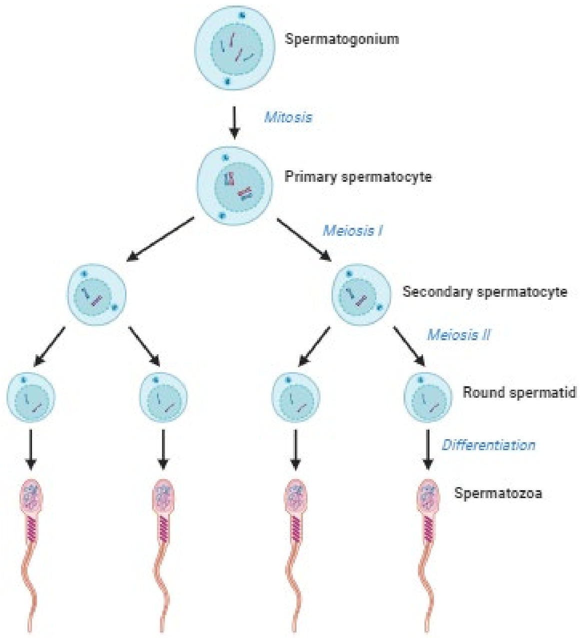

Figure 2. Spermatogenesis. Spermatogenesis begins with spermatogonia undergoing mitosis to produce primary spermatocytes, which then undergo two rounds of meiosis to form haploid spermatids. These spermatids mature through a process called spermiogenesis, transforming into fully developed spermatozoa. Adapted from *"Autophagy: A Double-Edged Sword in Male Reproduction"*, by Yan et al. 2022, Int. J. Mol. Sci. 23, 15273. Used under CC BY 4.0. Created with BioRender.com

Sertoli cells play a crucial role in spermatogenesis by providing structural and nutritional support to the developing spermatozoa. They secrete necessary growth factors and hormones assist in the phagocytosis of residual cytoplasm from spermatids, and regulate the process of sperm maturation through paracrine signalling. (Griswold, 1998; Ruthig & Lamb, 2022) Additionally, Sertoli cells create the blood-testis barrier, allowing the developing spermatozoa to reside in an immune-privileged environment. This barrier separates the developing germ cells from the bloodstream, preventing immune cells from accessing and potentially attacking the genetically distinct sperm cells. Moreover, Sertoli cells produce immunosuppressive factors that further protect the spermatozoa from immune responses. (Luaces et al., 2023b; Zhao et al., 2014).

1.2 Germline-specific RNP granules

Cytoplasmic germ cell-specific ribonucleoprotein (RNP) granules, also knowns as germ granules, appear in the cytoplasm of male germ cells throughout their differentiation. These non-membrane-bound organelles comprise of RNA molecules and RNA-binding proteins. Germ granules orchestrate critical post-transcriptional events such as messenger-RNA (mRNA) stabilization, translational repression, and RNA localization within germ cells. In addition, they play a crucial role in germ cell development and reproductive health as abnormalities in RNP granule components is associated with infertility across diverse model organisms. (Lehtiniemi & Kotaja, 2018; Mukherjee & Mukherjee, 2021). Unravelling the intricate regulatory mechanisms leading to the formation and function of these granules enhances our understanding of germ cell biology but also holds promise for insights into addressing reproductive challenges (M. Gao & Arkov, 2013; Meikar et al., 2011).

The two most notable germ granules are intermitochondrial cement (IMC) in meiotic spermatocytes and the chromatoid body (CB) in haploid round spermatids. The largest RNP granule, the CB, appears in the cytoplasm of male germ cells during spermatogenesis when gene expression is at its highest level, indicating a role in engineering the complex transcriptome. Under electron microscopy, the CB presents as a single, large, and electron-dense granule, approximately 0.5-1 µm in diameter. CB precursors initially develop in late pachytene spermatocytes, briefly co-existing with the IMC, before aggregating into a singular large granule in the perinuclear region of haploid round spermatids (Lehtiniemi & Kotaja, 2018; Olotu et al., 2023). Presence of the CB in haploid round spermatids aligns with a phase of intense genome-wide transcription as meiotic spermatocytes transition into haploid round spermatids (Da Ros et al., 2015, 2017). At the end of round spermatid differentiation, the CB decreases in size and migrates towards the base of the flagellum, forming a ring-like structure around the axoneme during mitochondrial sheath formation before ultimately disappearing (Fawcett et al., 1970; Shang et al., 2010). The CB is thought to take part in RNA regulation, as it is rich in various RNA species and RNA-binding proteins, including those involved in the piwi-interacting RNA pathway and non-sense-mediated RNA decay pathway (Kotaja & Sassone-Corsi, 2007; Parvinen, 2005). Moreover, the CB is closely associated with the endomembrane system, facilitating material exchange crucial for acrosome biogenesis (Da Ros et al., 2015).

1.2.1 Germ granule component DDX4

One of the components consistently found in all germ granules is the DEAD-box helicase 4 (DDX4) protein. DDX4, also known as VASA, is a member of the DEAD-box RNA helicase family, one of the largest human RNA helicase gene families. RNA helicases are enzymes that have multiple roles in the biochemical processes involving alteration of RNA, including transcription, transport, splicing, translation and decay (Lehtiniemi & Kotaja, 2018; Nagamori et al., 2011). Multiple studies have provided novel insights to the regulatory complexity underlying gene expression control during germ cell differentiation containing DDX4. DDX4 along with DDX6 and DDX3X has been shown to play a role in RNA granule formation without relying on adenosine triphosphate (ATP), and to also contribute to the breakdown of these granules when ATP turnover occurs. (Bourgeois et al., 2016). Another study suggest a developmental regulation mechanism within the CB where DDX4-mediated RNA binding and release are modulated by acetylation, affecting mRNA translation dynamics during spermatogenesis. The study proposes a temporally regulated process involving DDX4, where mRNA storage, release, and translation occur in a coordinated manner. The same study found specific intracellular signalling pathways, possibly related to stress responses and metabolic regulation, in controlling the acetylation of DDX4 and, consequently, the physiological function of the CB in germ cell differentiation. (Nagamori et al., 2011).

Many germ granule protein components, including DDX4, are highly conserved in a variety of invertebrate and vertebrate species. Originally, *Vasa* was identified in *Drosophila melanogaster* but has been since found in species such as Xenopus, Zebrafish, echinoderms, molluscs, marsupials and monotremes. This extensive conservation throughout different species underscores its crucial role in germ granules and germ line development in general. (Castrillon et al., 2000; Gustafson & Wessel, 2010). In fact, the disordered sequences in both tails of Ddx4 enables it to drive germ granule formation (Nott et al., 2015; C. Xu et al., 2022). While DDX4 is expressed in both male and female germlines, the loss-of-function mutation or knockout in mice results in fertility defects only in males. In contrast, in female *Vasa*-null Drosophila both nurse cells and oocytes face series of defects. (Lehtiniemi & Kotaja, 2018; C. Xu et al., 2022). Despite extensive research and comprehensive reviews detailing the versatile roles of Vasa/Ddx4 in the germline and other tissues across various species, the exact functions of Vasa/Ddx4 remain unclear (C. Xu et al., 2022).

1.3 Cancer

Hippocrates coined the term cancer circa year 400 BC, but the earliest description of human cancer dates back over 3000 years to an old Egyptian Papyrus. The word cancer comes from the Latin word for crab, describing the hard center and spiny projections of tumors. (Koeffler et al., 1991) These first descriptions of cancer have been from a rather small collection of solid masses in the breast but over time, it became apparent that cancer comes in multiple different shapes and forms. Essentially, cancer is a genetic disease where cells start to uncontrollably divide and multiply, eventually causing damage to different organs and tissues. Furthermore, cancerous cells may become able to move and spread to other locations in the body becoming even more dangerous to the patient, and if left untreated ultimately lead to death. (Koeffler et al., 1991; M. E. Miller, 2016). However, cancer research and subsequently its treatment have made great advances during the past few decades improving outcomes of these patients (K. D. Miller et al., 2022)

Living organisms are complex entities with multi-dimensional and interconnected causes and effects but their common denominator is the cell, a fundamental living unit. (Kellenberger, 2004; M. E. Miller, 2016) In multicellular organisms, cells sustain a homeostasis by constant interaction with one another and the adjacent tissue architecture. Their state of resting, growing and dividing, differentiating or dying is dependent on extracellular signals that work to achieve a common goal of normal cell number and tissue function. Disturbances in these extracellular signals can disrupt and overthrow the homeostasis of the intricate multicellular entity. (King & Cidlowski, 1995).

1.3.1 Characteristics of cancer

Cancer develops when normal cells gain and accumulate genetic and epigenetic mutations that disrupt their key cell cycle mechanisms (M. E. Miller, 2016). In the year 2000, Hanahan described a set of six essential alterations in cell physiology that most cancers have in common. These six physiological changes were termed as the hallmarks of cancer and described to help unravel the complexity of cancer as a disease and create a holistic view of its underlying mechanisms. With this knowledge, complexities would become a set of understandable underlying principles and ultimately aid in cancer diagnosis, prognosis and treatment based on rational science. The first six hallmarks included sustaining proliferative signaling, evading growth-inhibitory signals, resisting cell death, unlimited replicative potential, sustained angiogenesis and enabling invasion and metastasis. (Hanahan & Weinberg, 2000).

Arguably, the most fundamental trait that characterizes a malignant cell is its ability to sustain proliferative signaling that leads to unrestrained growth (Bast et al., 2017). This characteristic is acquired largely by self-generating growth signals ensuring an active proliferative state and reducing the dependence of extracellular signaling from normal tissue microenvironment. Alternatively, cancer cells can stimulate the surrounding normal cells to provide growth factors in return. Growth factor independence can also derive from deregulating the levels of receptor proteins or their signaling pathways, resulting in overexpression of cell-surface receptors and cells hyperresponsive to growth factors. Many of these dysregulated receptors carry tyrosine kinase activities and are overexpressed in cancers. (Hanahan & Weinberg, 2000). The role of tyrosine kinases in cancer, alongside other hallmarks, have led to novel and meaningful therapeutic discoveries against cancer. Some of these paradigm changing therapeutics include human epidermal growth factor receptor 2 (HER2), vascular endothelial growth factor (VEGF), cyclin-dependent kinase (CDK) 4/6, and Kirsten rat sarcoma virus (KRAS) inhibitors. (Bailón-Moscoso et al., 2014). However, to increase response rates and overcome inevitable resistance, targeting multiple hallmarks is likely the path forward (Hanahan, 2023).

1.3.2 Tumor transcriptome

Transcriptomics involves studying RNA molecules on a large scale using advanced techniques. It reveals the abundance and composition of RNA in a cell, which varies greatly among different cell types and states. Unlike deoxyribonucleic acid (DNA), which remains largely constant, RNA reflects the dynamic nature of cellular activity and regulatory processes. By analyzing transcriptome profiles, it is possible to gain valuable insights into the unique biology of specimens, as RNA serves as a signature of their environmental conditions and underlying cellular states. (Cieślik & Chinnaiyan, 2018). Transcriptomics covers multiple features like identification of noncoding RNAs, assessments of pre-messenger RNA processing including alternative splicing and polyadenylation, identification of fusion transcripts, transcript annotation, and detection of novel transcripts, all of which make it a valuable tool for characterizing tumor mechanisms and identifying biomarkers. Advances in these novel technologies as well as bioinformatics approaches have significantly enhanced our understanding of cancer development and progression and led to new clinical applications. (Tsimberidou et al., 2022).

Heterogeneity is a prevalent feature across various cancer types with diverse phenotypic expressions. The diversity stems from different genetic, epigenetic, and transcriptomic variations, as well as the interaction of cells with their complex microenvironments. (Fan et al., 2020). This heterogeneity contributes to multiple aspects of tumor characteristics like growth, survival, metastasis, immune evasion and therapeutic resistance, which, in turn, affect clinical outcomes (Ottaiano et al., 2023). One of the primary applications of transcriptomics has been to utilize its unique capacity in describing the high-dimensional molecular state of cancer. This gene expression data remains a powerful tool in, for example, predicting drug sensitivity in various diseases, including cancer. (Cieślik & Chinnaiyan, 2018; Y. Xu et al., 2021; J. Zhang et al., 2020). As mentioned, cancer cells exhibit altered protein function and abnormal transcriptional patterns due to somatic mutations and epigenetic changes, leading to a growth advantage (Cieślik & Chinnaiyan, 2018). Genetically engineered mouse models have offered strategies to study how activated oncogenes or deactivated tumor suppressors contribute to cancer heterogeneity, at least in a controlled manner (Ottaiano et al., 2023).

Cell lines derived from human cancers are extensively utilised as preclinical models in cancer research and drug discovery and development. Projects like the Cancer Cell Line Encyclopedia (CCLE) have generated comprehensive sequencing data across various molecular levels, including genomics, transcriptomics, epigenetics, proteomics, and metabolomics (Barretina et al., 2012; Basu et al., 2013). Additionally, initiatives such as the Genomics of Drug Sensitivity in Cancer (GDSC) project and the Cancer Therapeutics Response Portal (CTRP) offer invaluable resources on drug sensitivity across numerous cancer cell lines (Basu et al., 2013; Cieślik & Chinnaiyan, 2018; Yang et al., 2013).

Despite the utilization of these tools and advances in cancer treatments, a large proportion of patients fail to achieve durable responses, experience relapse or do not respond to treatment in the first place. These shortcomings could be tackled with combinatorial approaches like epigenetics drugs and immunotherapy. Synergy of different treatment approaches could also reshape the tumor microenvironment (TME) to restore anticancer responses thus overcoming some of the limitations in current cancer care. (Villanueva et al., 2020). Integration of transcriptomics along with genomics, proteomics and epigenetics, to future drug development offers great opportunities to develop biomarkers and personalized treatments (Tsimberidou et al., 2022; Villanueva et al., 2020).

1.3.3 Metastasis

Metastasis, one of the hallmarks of cancer, has intrigued scientist for over a hundred years. Despite extensive research efforts, advances in technology and novel therapeutic strategies, metastasis remains poorly understood. (Q. Liu et al., 2017). The invasion of nearby tissues and dissemination to more distant sites indicates an advanced disease and contributes to resistance to currently available treatment options. As a large proportion of patients are ultimately faced with metastatic disease, it constitutes a major cause of cancer-related deaths. (Dillekås et al., 2019; Fares et al., 2020; Q. Liu et al., 2017). The formation of metastasis is a complex cascade of events, often regarded as something happening in the later, advanced stages of cancer.

However, the order in which cancers acquire their characteristic capabilities differ vastly. (Hanahan & Weinberg, 2000; Riaz et al., 2024).

Factors contributing to and influencing metastasis include genetic mutations, cell adhesion molecules, epithelial-mesenchymal transition, and the TME, among others (Riaz et al., 2024). To date, dozens of mutated oncogenes driving cancer formation have been identified in human malignancies. Some of the most well-known oncogenes include HER2, epidermal growth factor receptor (EGFR), BRAF and KRAS. The identification of oncogenes has been widely utilized in developing anticancer drugs and there are currently tens of different oncogene-targeting drugs. (Croce, 2008). The role of oncogenes has been extensively studied and characterized, in both primary tumors and metastases. Over the years, studies have shown that compared to primary tumors, metastases are generally more prone to chromosomal instability and, unsurprisingly, carry a higher frequency of mutations in TP53 in several tumor types. In addition, certain metastasis patterns and genomic alterations have been identified in a cancer-specific manner. (Fares et al., 2020; Nguyen et al., 2022). However, metastatic cells are genetically heterogeneous, and can differ in the genes that they express. This can lead to treatment complications where some cells respond and others do not. Therefore, further studies are needed to better understand intra- and intertumoral heterogeneity, and their impact on treatment resistance. (Fares et al., 2020; Vitale et al., 2021).

1.3.4 Cancer germline antigen genes and their implications in cancer

Possible similarities between germ cell development and tumor development were hypothesised for over a hundred years ago. The production of chorionic gonadotropin and other trophoblastic hormones by human cancers drew increasing attention to the association between tumorigenesis and germ cell development. (Simpson et al., 2005). Cancer and germ line cells share multiple functional characteristics, such as hyperproliferation periods and extensive migration, among others (Figure 3). One of the most fundamental characteristics of cancer cells is their ability to sustain constant proliferation (Hanahan & Weinberg, 2000). Likewise, the gonial stem cells undergo a period of rapid proliferation within the presumptive gonad (Rooij, 2001). Furthermore, another hallmark of cancer, capability to metastasize, shares similar traits with PGCs, which migrate to the gonadal ridge where they initiate germ cell differentiation. It has long been proposed that cancer cells harness their reservoir of normally silent germline genes to adapt and face challenges in their new environment. (Schudrowitz et al., 2017). Majority of deaths from solid tumors are caused by metastases. Therefore, understanding the molecular mechanisms of cancer metastasis can help in developing cancer therapeutics. (Dillekås et al., 2019).

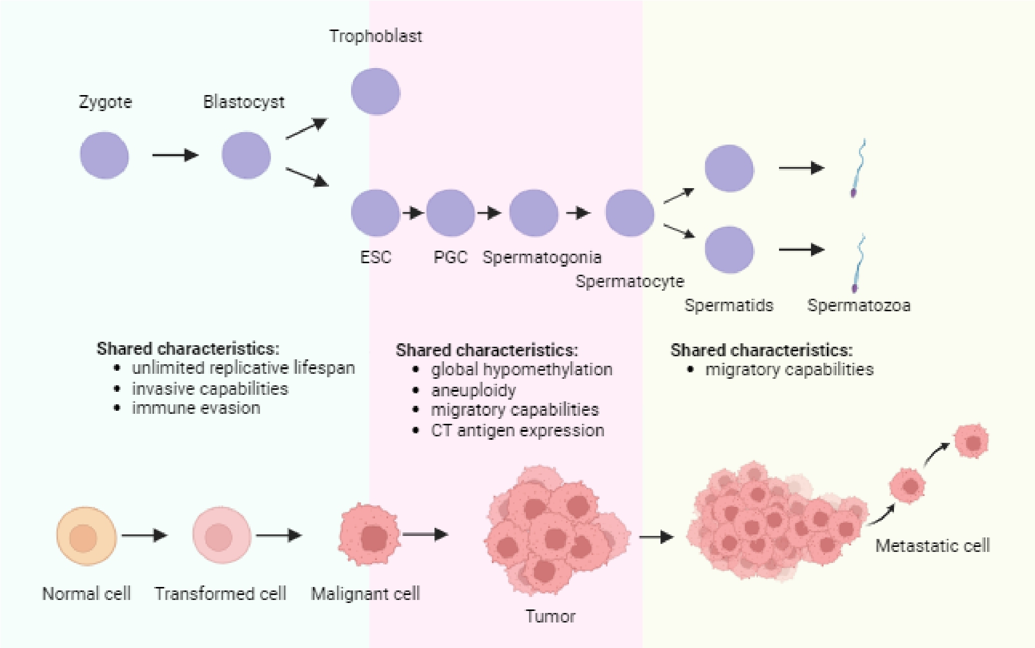

Figure 3. Embryo/spermatogenesis (upper panel) and tumorigenesis (lower panel) share multiple characteristics. Modified under CC BY 4.0 and with permission from *"Biology of Cancer-Testis Antigens and Their Therapeutic Implications in Cancer"* by Nin & Deng 2023, Cells 12(6): 926 and *"Cancer/testis antigens, gametogenesis and cancer"* by Simpson et al. 2005, Nature Reviews Cancer 5, 615-625. Created with BioRender.com.

Cancer germline antigen genes are a set of genes primarily expressed in germ cells and trophoblast tissues, but aberrantly expressed in multiple human malignancies of somatic origin (Karpf, 2008). The first cancer antigen recognized by autologous cytolytic T lymphocytes was identified from a human melanoma cell line, and initially named as MZ2-E (van der Bruggen et al., 1991). This cancer antigen was later denominated as melanoma antigen-1 and further renamed as melanoma associated antigen 1 (MAGE-A1) (Traversari et al., 1992). MAGE-A1 was found to be a part of a larger family of MAGE genes, followed by the identification of related BAGE and GAGE gene families, among others (Boih et al., 1995; De Smet et al., 1994; Gaugler et al., 1994; Van Den Eynde et al., 1995). These antigens were found to be expressed in various types of tumors but silent in normal tissues, with the exception of the testis (De Smet et al., 1994; Gaugler et al., 1994). Based on their expression pattern, this growing set of antigens was described as cancer-testis (CT) antigens (Old & Chen, 1998). However, a proportion of CT antigens were detected in one or two gametogenic tissues: testis, ovary and/or placenta (Scanlan et al., 2004). Further work showed that the expression of CT antigens was restricted to germ cells within the testis as well as fetal ovary and trophoblasts. Therefore, the terminology varies between cancer-testis and cancer-germline (CG) antigen [gene] (Scanlan et al., 2004; Simpson et al., 2005). The CG antigens share some general characteristics; they are typically members of large multigene families that are mostly located on the X chromosome and have a heterogeneous expression in tumor tissues (Akers et al., 2010).

The discovery of CT genes raised a theory that the abnormal activation of CT antigens contribute to tumorigenic processes as one of the driving forces (Akers et al., 2010; Simpson et al., 2005). In fact, several studies have found that individual CT genes affect a great number of cellular pathways, suggesting that CT antigens have diverse functions in human malignancies and are involved in a multitude of cancer hallmarks (Table 1).

Table 1. CT antigens have been associated with almost every hallmark from the original to the emerging ones.

Cancer hallmark	**Cancer/testis antigen**	
Sustained proliferative signalling	TFDP3, ZNF165	Original hallmarks
Evading growth suppressors	CAGE, CASG, MAGE, PRAME	
Inducing angiogenesis	CAGE	
Activating invasion and metastasis	CAGE, CTAG, CT45, GAGE, HORMAD1, MAGE, PRAME, SPANX, SSX	
Resisting cell death	FATE1, GAGE, MAGE, PAGE	
Enabling replicative immortality	MAGE, SSX	
Tumor-promoting inflammation		Emerging hallmarks
Genome instability & mutation	ACRBP, CEP55, MPHOSPH1, HORMAD1/2, SPO11, SYCP1/3, TEX12/15, TTK	
Avoiding immune destruction	MAGE, PRAME	Enabling factors
Deregulating energetics	BORIS, CAGE, COXB2, CT55, FSIP1, LDH-C, MAGE, SEMG1/2, TFDP3	

Multiple CT/CG genes have been found to be involved in the regulation of transcription, one of these being the first discovered CT antigen gene family MAGE (Nin & Deng, 2023). MAGEA1 interacts with the Ski-interacting protein (SKIP) to suppress transcription by recruiting histone deacetylase 1 (HDAC1) to SKIP target genes, potentially promoting tumor growth due to altered HDAC activity in cancer cells (Monte et al., 2006). MAGEA2, another MAGE family member, inhibits the potent tumor suppressor p53 by recruiting HDAC3 and creating an inhibitory loop. This association prevents p53 from binding DNA leading to the failed activation of genes like p21, MDM2, and PUMA, ultimately favouring tumor progression (Marcar et al., 2010).

Transforming growth factor beta (TGFβ) signaling has both oncogenic and tumor-suppressive roles depending on the context. In breast cancer, TGFβ seems to be tumor-suppressive in luminal A/B subtypes but oncogenic in triple-negative breast cancer (TNBC). (Bruna et al., 2012; Massagué, 2008). A loss-of-function screen identified the zinc finger transcription factor (ZNF) 165 as crucial for TGFβ's oncogenic effects in TNBC. ZNF165 interacts with TGFβ loci, promoting an oncogenic transcriptional program (Maxfield et al., 2015). It also modulates TGFβ-dependent gene transcription through its association with SMAD3, driving oncogenic pathways (Gibbs et al., 2020).

These studies, among others, suggest that CG/CT antigens influence transcriptional networks involved in cancer progression. Further mechanistic studies are needed to understand how CTAs control transcription, which could enhance our knowledge of their roles in cancer progression, development, and gametogenesis. (Nin & Deng, 2023).

1.3.4.1 Cancer germline antigens as potential immunotherapeutic targets

Traditional cancer therapies like surgery, radiotherapy, and chemotherapy extend patient lifespans but still face issues with resistance, metastasis, and relapse (Nin & Deng, 2023). Advances in molecularly targeted therapies, such as antibodies against EGFR, HER2, and cluster of differentiation (CD) 20, have improved treatment outcomes but often lead to resistance over time (Coiffier et al., 2002; Slamon et al., 2011; Vermorken et al., 2008). The emergence of immune-based therapies, particularly immune checkpoint inhibitors targeting the programmed cell death pathway (PD-1, PD-L1), and cytotoxic T-lymphocyte associated protein 4 (CTLA-4), were a breakthrough in cancer treatment and have shown success in eliciting prolonged responses and improving outcomes in various cancers (H. Zhang & Chen, 2018). However, a substantial number of patients do not respond to treatment, or ultimately face relapse. Therefore, the need for new, well-tolerated treatments that provide lasting clinical responses remains. (Chamoto et al., 2020).

The discovery of CG antigens raised questions about the relation of gametogenesis and tumorigenesis and opened up possibilities in finding tumour-specific antigens that could serve as cancer immunotherapy targets, especially since they reside in immune privileged sites protected by the blood-testis and Sertoli cell barriers (Boon et al., 1997; Kaur et al., 2014; Old, 2001). Thus far, over 1000 CG genes and their expression patterns in 19 different tumour types have been identified through systematic multiplatform analyses (C. Wang et al., 2016).

Several clinical trials are exploring vaccine-based immunotherapy using antigenic peptides against MAGE and New York esophageal squamous cell carcinoma 1 (NY-ESO-1) to elicit immune responses against various cancers (Kothandan et al., 2020; Wei et al., 2019). Although vaccines have been the popular choice in targeting CG/CT antigens T cell -based therapies, like targeting intracellular antigens through chimeric antigen or T- cell receptors, have recently gained popularity (Nin & Deng, 2023). For example, genetically engineered T-lymphocytes targeting NY-ESO-1 have shown tumor regression in patients with metastatic synovial cell sarcoma and melanoma (Robbins et al., 2011). Despite the promise of CG/CT antigen targeting cancer therapies, challenges remain. Therefore, careful and systematic analysis of CG/CT antigen expression and their role in cancer is imperative. (Naik et al., 2024). In the future, targeting CG/CT antigens along with multiple other cancer hallmarks is likely the path forward (Hanahan, 2023).

1.3.4.2 DDX4 in cancer

DDX4 is not only one of the most conserved germline factors among all multicellular organisms and a well characterized germ granule component, but also among the CG antigens reported to be aberrantly expressed in multiple human cancers (Noyes et al., 2023; C. Xu et al., 2022). Expression of DDX4 has been reported in at least ovarian cancer, multiple myeloma and leukemia (D'oronzo et al., 2020; Hashimoto et al., 2008; Schudrowitz et al., 2017). In epithelial ovarian cancer, expression of DDX4 was found in around 30% of cases and its expression correlates with older age and serous histology. DDX4 was shown to downregulate the expression of 14-3-3σ leading to disruptions in the G2 checkpoint and subsequent accumulation of chromosomal abnormalities. This might indicate that DDX4 directly contributes to the progression of epithelial ovarian cancer and could serve as a tumor marker. (Hashimoto et al., 2008). Another study looked at the relationship between DDX4 and ovarian cancer stem cells. It was shown that DDX4 and CD133, a cancer stem cell marker, share similar expression patterns and colocalize in multiple ovarian cancer tissues. This suggests that DDX4 could serve as one cancer stem cell marker in ovarian cancers. (K. H. Kim et al., 2014).

Aside from ovarian cancer, the expression of DDX4 has been reported in small cell lung cancer (SCLC) and blood-derived cancer cell lines, mainly in multiple myeloma-derived cell line IM9 and acute monocytic leukemia-derived cell line THP-1 (Noyes et al., 2023; Schudrowitz et al., 2017). In blood-derived cell lines, DDX4 is shown to have an essential role in cell proliferation and migration, in line with its function in the germ line (Schudrowitz et al., 2017). In SCLC DDX4 has been associated with increased cell survival and resistance to cisplatin-mediated chemoresistance (Noyes et al., 2023).

Germline-specific RNP granules have been a long-term research topic of the group, and we have previously shown that many of the components orchestrating RNA regulation in those germ granules are also CG antigens. One of the CG antigens consistently found in all germ granules is DDX4. Our group has demonstrated DDX4-positive germ granules appear in the cytoplasm of human cancer cells but not in non-cancerous somatic cells. It has also been noted by our group that there is no significant difference in cell proliferation of WT vs. *DDX4*-null PC3 cells under 2D culture conditions, whereas in 3D cultures the spheroid formation of *DDX4*-null is compromised. Aberrant expression of DDX4 has been found in several human malignancies, but its effects on tumor transcriptome have been less studied. Hereby, we propose that DDX4, as a prominent germ granule component, has a role in cancer formation, growth and migration, and is greatly involved in the RNA regulation of cancer cells.

2 Results

2.1 Deletion of *DDX4* delays the formation of PC3 tumors

2.1.1 Subcutaneous PC3 xenograft tumors

Previous data by the group suggested that the deletion of *DDX4* does not significantly hinder the proliferative capability of PC3 tumor cells compared to wild type (WT) PC3 cells in monolayer cell cultures (data not shown). However, in three-dimensional cultures spheroid formation was compromised in *DDX4*-null compared to WT PC3 cells. To understand how the deletion of *DDX4* affects PC3 xenograft tumor formation and growth *in vivo*, we performed subcutaneous inoculation of *DDX4*-null PC3 tumor cells into immunodeficient male mice. The targeted deletion in exon 11 of *DDX4* in PC3 cells was done previously in the group by using CRISPR/Cas9 technology (data not shown). Tumor formation and growth was followed for 4 weeks with weekly measurements of mouse weight and tumor volume by ultrasound. During weekly measurements, it was clear the formed *DDX4*-null tumors were visibly smaller, firm to touch and disc-like in shape when palpated.

Aside from itching, revealed by scratching marks on the skin above tumors, the tumors did not seem to affect the welfare of the mice. The mice from three cages had to be separated due to fighting, but the reasons behind this behavior could not be determined. There were no significant differences between body weights of mice carrying subcutaneous PC3 WT tumors or PC3 *DDX4*-null tumors (Figure 4).

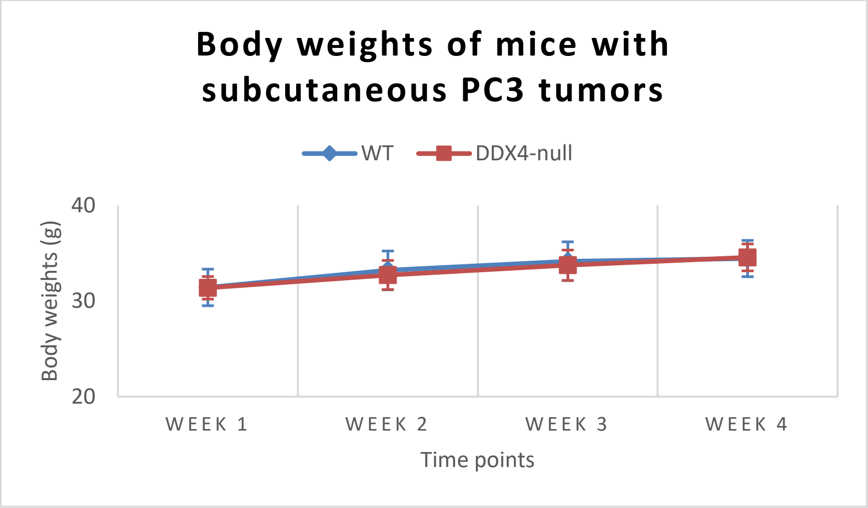

Figure 4. The 4-week progression of body weight (g) of mice inoculated with subcutaneous WT (blue, n=5) and DDX4-null (red, n=5) PC3 cells depicted in a line graph. Error bars represent a 95% confidence interval.

To evaluate tumor burden and growth, we conducted weekly ultrasound measurements (Figure 5). Measuring the subcutaneous tumors was straightforward due to their prominent location. *DDX4*-null tumors exhibited more consistent in shape, resulting in easier and more accurate measurements. In contrast, WT tumors were less symmetrical in shape, making it slightly more challenging to determine their widest and longest points.

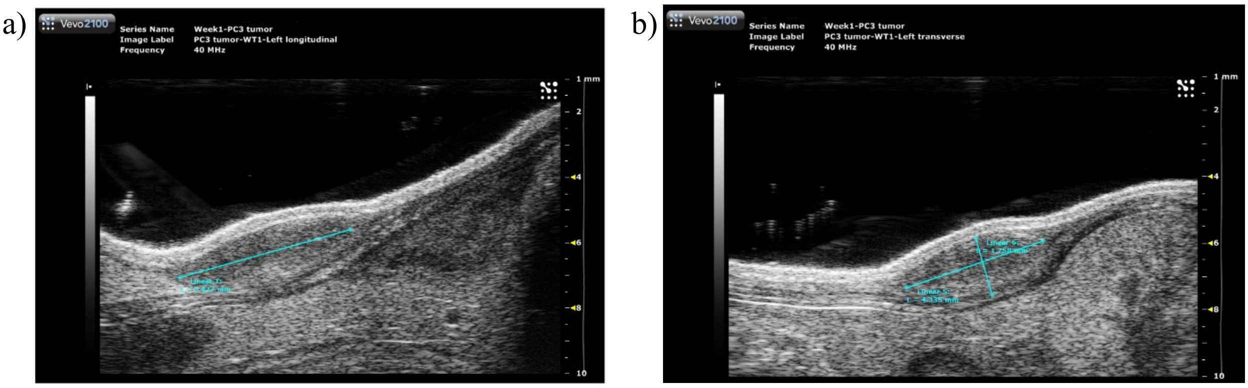

Figure 5. Selected images of ultrasound measurements for illustrative purposes. a) Longitudinal measurement taken from the greatest length of the tumor. b) Transverse measurement taken from the broadest section of the tumor.

The weekly ultrasound measurements showed that the volume of *DDX4*-null PC3 tumors was significantly lower than WT PC3 tumors, indicating that the deletion of *DDX4* hinders the growth of PC3 cells and delays tumor formation *in vivo* (Figure 6).

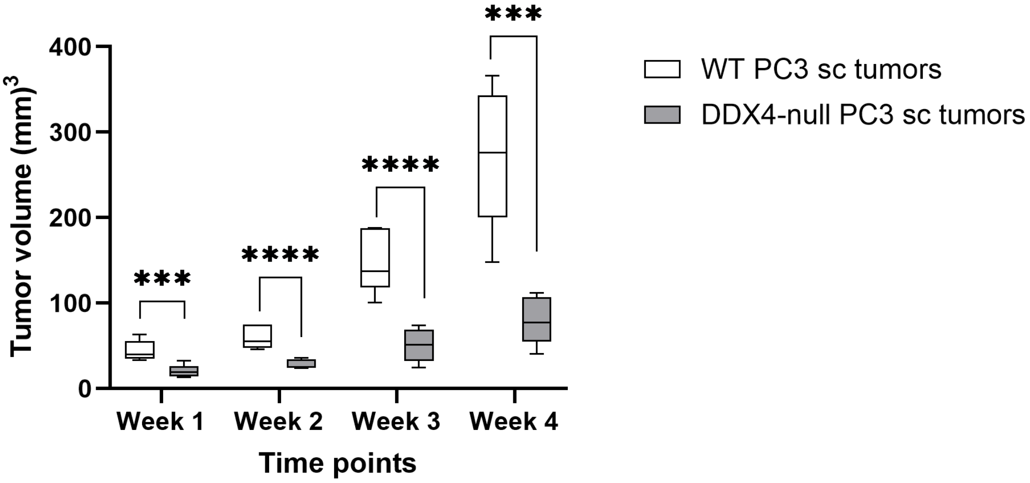

Figure 6. Box plot showing the difference in tumor volume of DDX4-null (gray, n=10) versus WT (white, n=10) PC3 tumors (p-value <0.0001 on week 1, p-value <0.0001 on week 2, p-value <0.0001 on week 3, p-value 0.0002 on week 4; Mann-Whitney U-test).

The effect of DDX4-deletion on tumor formation and growth was further confirmed when the dissected tumors were weighed (Figure 7). Each mouse (*DDX4*-null, n=5; WT, n=5) carried two tumors, one on each flank. The dissected *DDX4*-null PC3 tumors were significantly smaller (p-value 0.0232) also in weight compared to WT PC3 tumors.

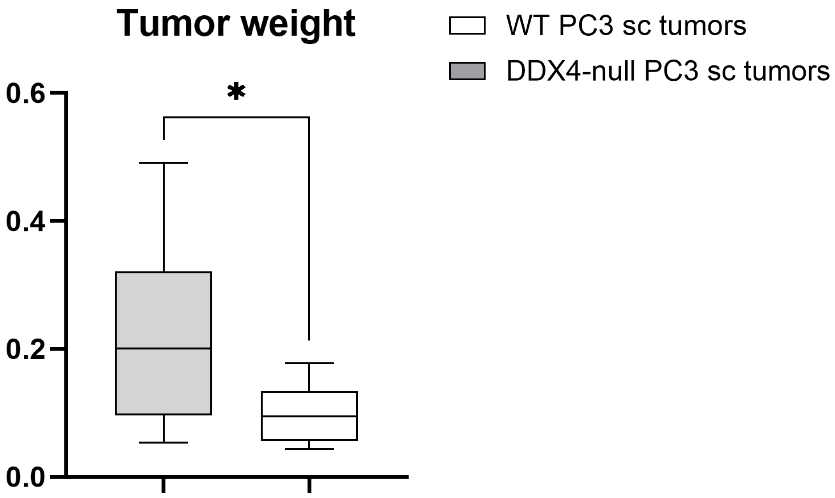

Figure 7. Weights of subcutaneous WT (gray, n=10) and DDX4-null (white, n=10) PC3 tumors after dissecting (p-value 0.0232; Mann-Whitney U test).

The subcutaneous *DDX4*-null and WT PC3 xenograft tumors revealed that *DDX4*-deletion causes a delay in tumor formation and hinders growth at least in subcutaneously inoculated PC3 cells.

2.1.2 Orthotopic PC3 xenograft tumors

To understand how the deletion of *DDX4* affects PC3 tumor formation, growth and invasive properties in a more authentic TME, we performed orthotopic inoculations of *DDX4*-null PC3 (n=5) and WT PC3 (n=5) cells into the prostate of immunodeficient male mice. Tumor formation and growth was followed for 8 weeks with weekly measurements of mouse weight and tumor volume by ultrasound.

There were no significant differences between body weights of mice carrying orthotopic PC3 WT tumors or PC3 *DDX4*-null tumors (Figure 8).

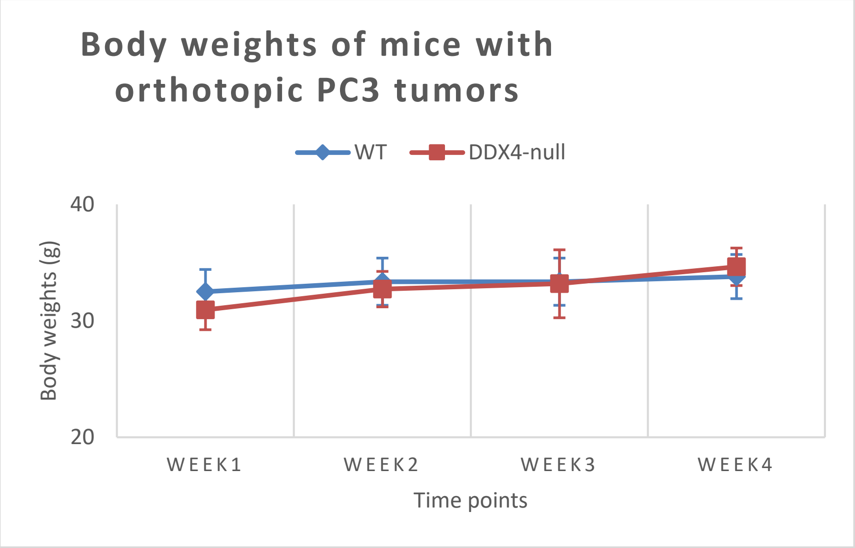

Figure 8. The 4-week progression of body weight (g) of mice inoculated with orthotopic WT (blue, n=5) and *DDX4*-null (red, n=5) PC3 tumors depicted in a line graph. Error bars represent a 95% confidence interval.

Similarly to the subcutaneous tumors, we did weekly ultrasound measurements to assess tumor volume. Unfortunately, this proved to be far more difficult than with the subcutaneous tumors and the measurements were either lacking or not accurate enough for conducting calculations on differences in size and/or growth rate during the experiment.

The experiment was terminated after 8 weeks, and both primary tumors and metastases were dissected. We found four orthotopic WT PC3 primary tumors and two *DDX4*-null PC3 primary tumros. Secondary, metastasised tumors were also found in lymph nodes of the abdominal area. In total, there were four secondary tumors in three mice inoculated with WT PC3 cells and three secondary tumors in two mice inoculated with DDX4-null PC3 cells. As with the subcutaneous tumors, the orthotopic *DDX4-null* tumor formation was delayed and the tumors were smaller in size compared to the orthotopic WT PC3 tumors (Figure 9). Similarly to the primary orthotopic tumors, also the secondary *DDX4*-null tumors were smaller than the secondary WT PC3 tumors.

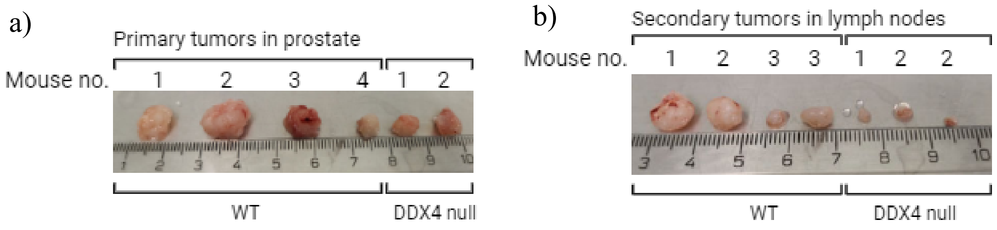

Figure 9. a) Primary orthotopic WT PC3 (n=4) and *DDX4*-null (n=2) tumors pictured after dissecting. b) Secondary WT PC3 (n=4) and *DDX4*-null (n=3) tumors found in lymph nodes of orthotopically inoculated mice pictured after dissecting.

After dissecting both the primary and secondary tumors were weighed for RNA isolation. The weights of the primary tumors are presented in Figure 10. Due to the small sample size and large variation in weight, no significant difference was observed. Only the primary tumors (*DDX4*-null n=2, WT n=4) were selected for subsequent RNA isolation.

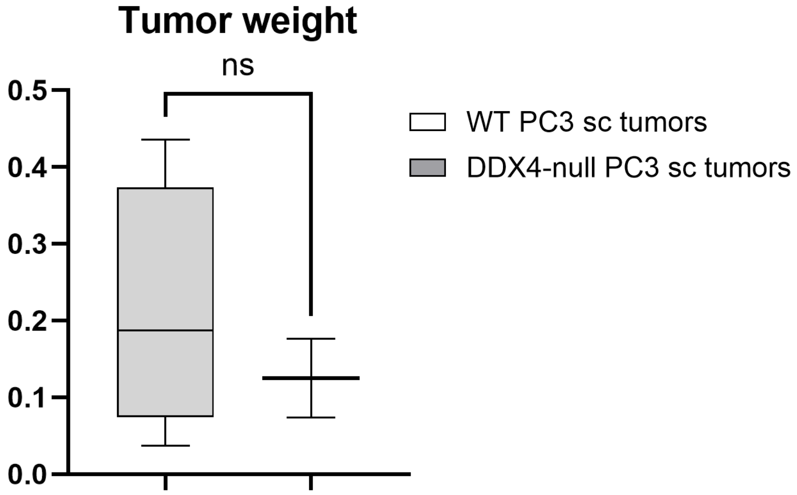

Figure 10. No significant difference in weight of primary orthotopic tumors was observed (p-value 0.5333; Mann-Whitney U test).

2.2 Deletion of *DDX4* likely affects the formation and growth of UT-SCC-14 tumors

To further validate the changes seen in *DDX4*-null PC3 tumors, we repeated the experiment in another cell line, UT-SCC-14. To understand if the deletion of *DDX4* affects UT-SCC-14 tumor formation and growth *in vivo*, we performed subcutaneous inoculation of *DDX4*-null UT-SCC-14 tumor cells into immunodeficient male mice. Each mouse (*DDX4*-null, n=5; WT, n=5) carried two tumors, one on each flank. Tumor formation and growth was followed for 4 weeks with weekly measurements of mouse weight and tumor volume by ultrasound. During weekly measurements, it was clear the formed *DDX4*-null tumors were visibly smaller, firm to touch and disc-like in shape when palpated.

There were no significant differences between body weights of mice carrying subcutaneous UT-SCC-14 WT tumors or UT-SCC-14 *DDX4*-null tumors (Figure 11).

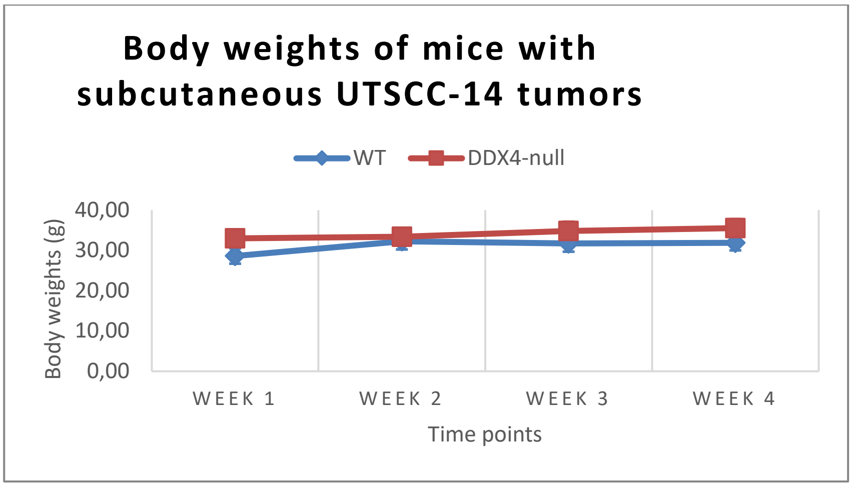

Figure 11. The 4-week progression of body weight (g) of mice inoculated with subcutaneous WT (blue, n=5) and DDX4-null (red, n=5) UTSCC-14 cells depicted in a line graph. Error bars represent a 95% confidence interval.

The experiment was terminated after 4 weeks, and the tumors were dissected. The dissected tumors were weighed and snap freezed or placed in formalin for subsequent RNA isolation or histological analysis. However, due to time constraints the UT-SCC-14 tumor samples were not analyzed for this thesis.

2.3 Setting up the RT-qPCR for validation of RNA-Seq results

Previously obtained RNA-sequencing data by the group indicated that *DDX4*-deletion causes major transcriptomic changes in *DDX4*-null vs WT PC3 xenograft tumors (data not shown). To further study the transcriptomic changes previously seen in RNA-sequencing of *DDX4*-null compared to WT PC3 cells, we performed quantitative RT-PCR. In total of 76 genes for dysregulated genes were chosen and corresponding primer pairs ordered for further validation with RT-qPCR. According to literature, the aberrant expression of the chosen genes has been associated with one or several types of cancers, and might carry prognostic value or therapeutic potential. Out of the chosen 76 genes, 58 were upregulated, and 18 were downregulated in the previously obtained RNA-sequencing results, and they are listed in Table 2.

Table 2. Differential expression of chosen genes based on previously obtained RNA-sequencing data

Upregulated (58)			**Downregulated (18)**
ANKH	HLF	PDE10A	ADAM12
B3GALT1	HSD17B2	RGS17	CDH6
CACNA1H	IGFBP2	RNF43	CDH7
CAMK2N1	IGSF9B	SERPINB2	CTTNBP2
CCND2	JAM3	SHISA2	CYP27C1
CD33	KIRREL3	SLFN11	EPHA4
CDH2	KLK11	SMOC1	EVC2
CEACAM1	LOX	STC1	GLIS1
CHRD	LPAR6	TCN1	HORMAD2
CLEC2B	MGP	TGM2	IL31RA
COL17A1	MUC2	TMEM74B	MYLK
COL2A1	NDN	TRPV3	NNMT
CSPG4	MUC2	VCAN	SAMSN1
CXADR	NDN	WDR72	SFRP1
DCC	NDUFA4L2	ZFPM2	SLC44A3
DIRAS1	NEIL1	ZNF415	TGM2
FAM107A	NELL2	ZNF516	TNFRSF11B
FFAR4	NFATC4	ZPLD1	WASF3
FXYD6	NLGN4X		
GJB2	OLFM2		

To ensure the primer pairs yield the correct product in subsequent RT-qPCR, all primer pairs were first tested with RNA isolated from PC3 and UTSCC-14 cell lines. After performing qPCR, the products were run on gel to define the amplicon size of the PCR product (Appendix 1). Based on the initial results, primer pairs for 11 genes produced a wrong amplicon size, no amplicon at all or multiple PCR products. New primer pairs were designed and tested for these 11 genes. From the original 76 primer pairs 67 were producing a correct sized amplicon and were selected for subsequent RT-qPCR validation experiments. These primer pairs are listed in Table 3.

Table 3. List of genes further validated with RT-qPCR (with functioning primer pairs)

ANKH	FXYD6	RNF43
ADAM12	GJB2	SAMSN1
B3GALT1	GLIS1	SERPINB2
CACNA1H	HLF	SFRP1
CAMK2N1	HORMAD2	SHISA2
CCND2	HSD17B2	SLC44A3
CD33	IGFBP2	SLFN11
CDH2	IGSF9B	SMOC1
CDH6	IL31RA	STC1
CDH7	KLK11	TCN1
CEACAM1	LOX	TGM2
CLEC2B	LPAR6	TMEM74B
COL17A1	MGP	TNFRSF11B
CSPG4	MUC2	TRPV3
CTTNBP2	MYLK	WASF3
CXADR	NDN	VCAN
CYP27C1	NDUFA4L2	ZFPM2
DCC	NEIL1	ZNF415
DIRAS1	NELL2	ZNF516
EPHA4	NFATC4	ZPLD1
EVC2	NNMT	
FAM107A	PDE10A	
FFAR4	RGS17	

Before starting RT-qPCR validation, the RNA samples were measured with a spectrophotometer to determine the concentration and assess the purity of RNA. The concentration of RNA correlated with tumour size and the yield of RNA was consistent with published literature. For RNA 260/230 ratio of 2-2.2 and 260/280 ratio of ~2.0 is considered acceptable. All measured samples were in an acceptable range, as depicted in Tables 4 and 5.

Table 4. Results from spectrophotometric measurement for subcutaneous WT and DDX4-null PC3 tumors.

Sample ID	ng/µl	260/280	260/230	Total volume, µl	Total RNA, µg
PC3sc WT1	1278.66	1.95	2.02	200	255.7
PC3sc WT3	1423.25	1.94	1.78	300	427.0
PC3sc WT4	377.13	1.88	2.2	200	75.4
PC3sc null1	274.53	1.89	2.3	50	13.7
PC3sc null2	2303.09	1.92	2.17	50	115.2
PC3sc null5	2735.55	1.85	2.07	50	136.8

Table 5. Results from spectrophotometric measurement for orthotopic WT and DDX4-null PC3 tumors.

Sample ID	ng/µl	260/280	260/230	Total volume, µl	Total RNA, µg
PC3ot WT1	2870.13	1.90	2.01	200	574.0
PC3ot WT3	2721.49	1.90	1.81	400	1088.6
PC3ot WT11	1045.56	1.98	1.93	100	104.6
PC3ot WT13	2827.15	1.87	1.96	200	565.4
PC3ot null30	2229.54	2.00	2.06	100	223.0
PC3ot null38	2914.98	1.89	1.84	200	583.0

However, to further determine the integrity of RNA and ensure repeatability of subsequent experiments, an automated electrophoresis was performed for the subcutaneous tumour RNA samples (Figure 12). To interpret the electropherogram, sample integrity is determined by the entire electrophoretic trace of the RNA sample, instead of the ratio of ribosomal bands alone. In general, RIN values above 7-8 are considered acceptable for example RNA-Seq. Higher RIN values indicate intact RNA, lower values indicate degradation of RNA. All subcutaneous tumour RNA samples had RIN values ≥7.5 (Table 6).

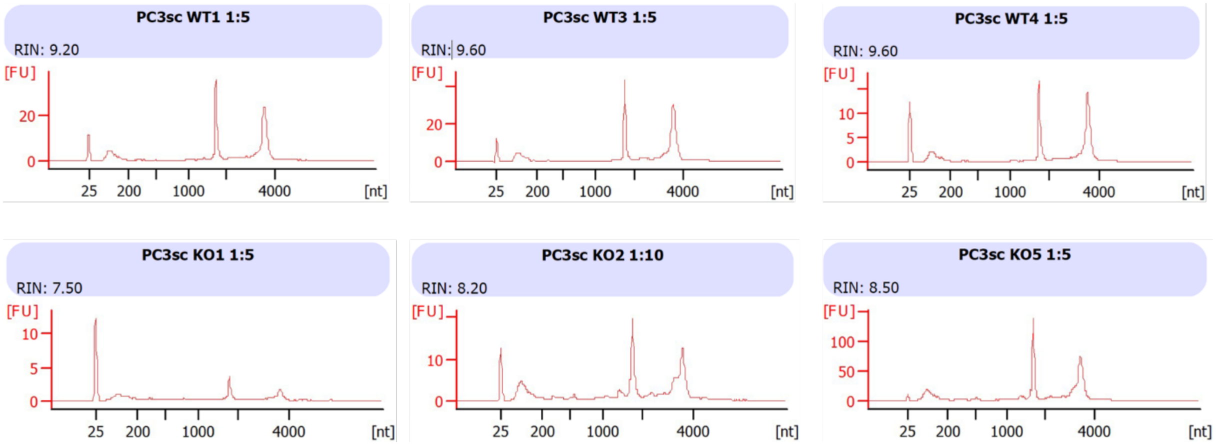

Figure12. Electropherogram results for subcutaneous WT and DDX4-null PC3 tumors.

Table 6. RNA Integrity Values for subcutaneous tumour RNA.

Sample ID	RIN value
PC3sc WT1	9.20
PC3sc WT3	9.60
PC3sc WT4	9.60
PC3sc null1	7.50
PC3sc null2	8.20
PC3sc null5	8.50

2.4 Deletion of DDX4 affects the tumor transcriptome

To further validate the RNA-sequencing results of selected genes previously obtained by the group (data not shown) we performed RT-qPCR of the DDX4-null and WT PC3 tumor RNA. In total, 76 differentially expressed genes were selected for RT-qPCR validation experiments and their primer pairs are listed in Appendix 2. The fold change in gene expression was calculated using hRPL19 as a reference gene. For each tumor type, *DDX4*-null and WT, three biological replicates, meaning three tumors from three different mice, were used. Due to the large number of genes, only two technical replicates were run on one plate.

2.4.1 *DDX4*-deletion has significant effects on the transcriptome of PC3 cells in subcutaneous xenograft tumors

Out of the 67 genes, only seven were significantly upregulated upon *DDX4*-deletion in the subcutaneous xenograft tumor mRNA: CEACAM1, CLEC2B, KLK11, MGP, MUC2, NNMT and TCN1 (Figure 13). Out of these, KLK11 showed the highest relative expression in *DDX4*-null tumors. Data from human prostate cancer tissues show increased levels of KLK11 (Nakamura et al., 2003). However, low expression of KLK11 has been reported to correlate with higher tumor stage in prostate cancer (Fuhrman-Luck et al., 2014). CEACAM1 has been reported to associate with cancer progression and metastasis, and is currently being investigated in Phase II clinical trials (Götz et al., 2023). Despite the majority of data supporting this view, there have also been conflicting findings regarding the role of CEACAM1 in cancer (J. Xu et al., 2018). While the expression of MUC2 has been associated with better survival and decreased metastasis in colorectal cancer (Hsu et al., 2017), TCN1 overexpression in colorectal cancer associates with tumorigenesis (Zhu et al., 2023). Aberrant expression of MGP has been reported in various cancer types, but seems to be tumor dependent: MGP shows negative correlation with tumor progression in e.g. prostate carcinomas, whereas upregulation associates with poor prognosis in breast cancer (Caiado et al., 2023).

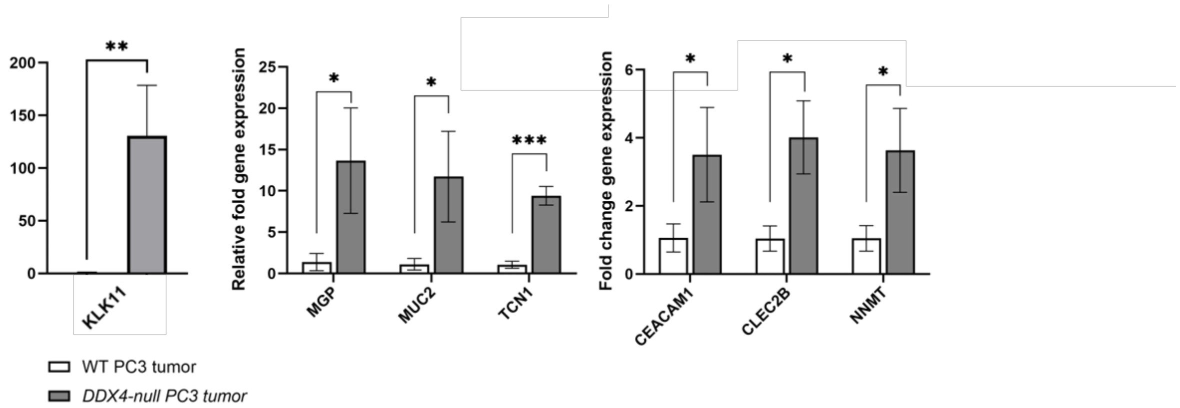

Figure 13. Upregulated genes grouped by their relative expression: KLK11 (p-value 0.0094), MGP (p-value 0.0303), MUC2 (p-value 0.0294), TCN1 (p-value 0.0003), CEACAM1 (p-value 0.0429), CLEC2B (p-value 0.0105), NNMT (p-value 0.0271). *P<0.05, ** P<0.01, ***P<0.001, unpaired t-test, two-tailed. The error bars represent the standard deviation.

Out of the 67 genes, 21 were significantly downregulated upon *DDX4*-deletion in the subcutaneous xenograft tumors: B3GALT1, CAMK2N1, CCND2, CDH6, CDH7, CTTNBP2, CXADR, CYP27C1, DCC, EVC2, FFAR4, LPAR6, RNF43, SAMSN1, SERPINB2, SHISA2, SLFN11, VCAN, ZFPM2, ZNF415 and ZNF516 (Figure 14). However, the Ct –values of DCC for both DDX4-null as well as WT were above 35, indicating such low expression that calculating relative expression is unreliable.

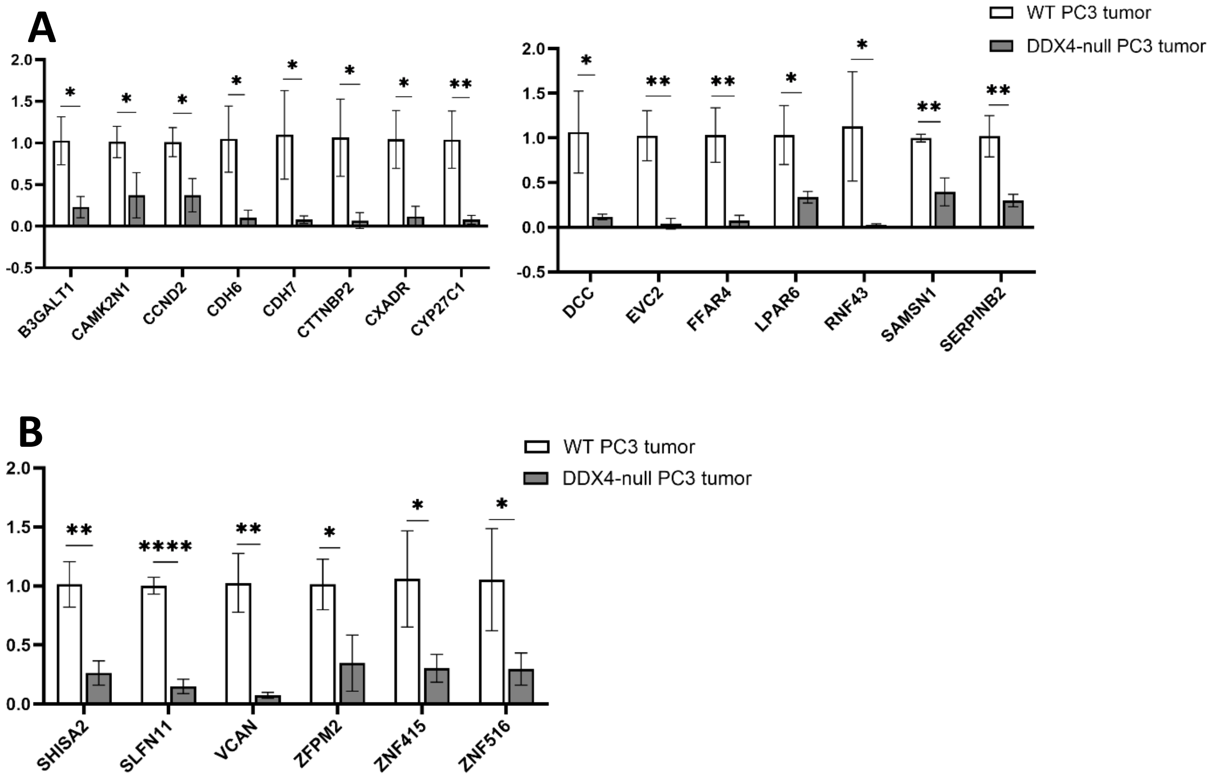

Figure 14. Upregulated genes grouped by their relative expression. **A)** B3GALT1 0.0118), CAMK2N1 (p-value 0.0285), CCND2 (p-value 0.0141), CDH6 (p-value 0.0161), CDH7 (p-value 0.0298), CTTNBP2 (p-value 0.0222), CXADR (p-value 0.0123), CYP27C1 (p-value 0.0088), DCC (p-value 0.0233), EVC2 (p-value 0.004), FFAR4 (p-value 0.006), LPAR6 (p-value 0.0229), RNF43 (p-value 0.0352), SAMSN1 (p-value 0.0029), SERPINB2 (p-value 0.0066). **B)** SHISA2 (p-value 0.004), SLFN11 (p-value <0,0001), VCAN (p-value 0.0027), ZFPM2 (p-value 0.0224), ZNF415 (p-value 0.0447) and ZNF516 (p-value 0.0365). *P<0.05, ** P<0.01, ***P<0.001, unpaired t-test, two-tailed. The error bars represent the standard deviation.

Eight genes were upregulated in DDX4-null vs. WT subcutaneous tumors but the difference was not significant or the variation was too high: ANKH, COL17A1, CSPG, PDE10A, SMOC1, TGM2, TRVP3, and WASF3. Twenty-six genes were downregulated without significant difference: ADAM12, CD33, CDH2, DIRAS1, EPHA, FAM107A, FXYD6, GJB2, GLIS1, HLF, IGFBP2, IGSF9B, IL31RA, LOX, MYLK, NDN, NDUFA4L2, NEIL1, NFATC4, RGS17, SFRP1, SLC44A3, STC1, TMEM74B, TNFRSF11B, and ZPLD1. One gene, NELL2, had no difference between *DDX4*-null and WT mRNA, and HORMAD2 was undetectable in both.

Compared to previously obtained RNA-Sequencing results, the upregulated genes in *DDX4*-null tumors matched the expression in RNA-Seq, except for one, NNMT. However, in the downregulated genes the differences were more notable (Table 7). Despite the drastic difference in results, direct comparison of RNA-Seq results with qPCR is problematic as they are based on different scales. However, this does indicate that RNA-Seq results should always be validated with another method like RT-qPCR.

Table 7. List of genes with opposite expression in RT-qPCR from subcutaneous tumors compared to RNA-sequencing.

GENE	RT-qPCR	RNA-Seq	GENE	RT-qPCR	RNA-Seq
NNMT	↑	↓	*RNF43*	↓	↑
B3GALT1	↓	↑	*SERPINB2*	↓	↑
CAMK2N1	↓	↑	*SHISA2*	↓	↑
CCND2	↓	↑	*SLFN11*	↓	↑
CXADR	↓	↑	*VCAN*	↓	↑
FFAR4	↓	↑	*ZFPM2*	↓	↑
LPAR6	↓	↑	*ZNF415*	↓	↑
OLFM2	↓	↑	*ZNF516*	↓	↑

2.4.2 *DDX4*-deletion has drastic effects on the transcriptome of PC3 cells in orthotopic xenograft tumors

Out of the 67 genes, 29 were significantly upregulated in the orthotopic xenograft tumor mRNA**.** The highest differential expression was seen in TCN1, SLFN11 and VCAN (Figure 15A). The rest of the upregulated genes were ANKH, CACNA1H, CAMK2N, CCND2, CD33, CDH6, CDH7, CLEC2B, CSPG, CXADR, CYP27C1, EPHA4, FXYD6, GJB2, HLF, HSD17B2, IGFBP2, IGSF9B, IL31RA, LOX, MGP, NEIL1, NELL2, SLC44A3, SLFN11, SMOC1, STC1, TCN1, TNFRSF11B, VCAN, ZFPM2 AND ZNF516 (Figure 15B-F). Interestingly, only one of the genes, FFAR4, was significantly downregulated (Figure 16). CEACAM1, KLK11, MUC2 or NNMT were not significantly upregulated in orthotopic tumors, unlike in subcutaneous tumors. However, known cell adhesion proteins CDH6 and CDH7 were significantly upregulated in the orthotopic primary tumors. The expression of the only downregulated gene in orthotopic tumors, FFAR4, matched the expression of subcutaneous tumors.

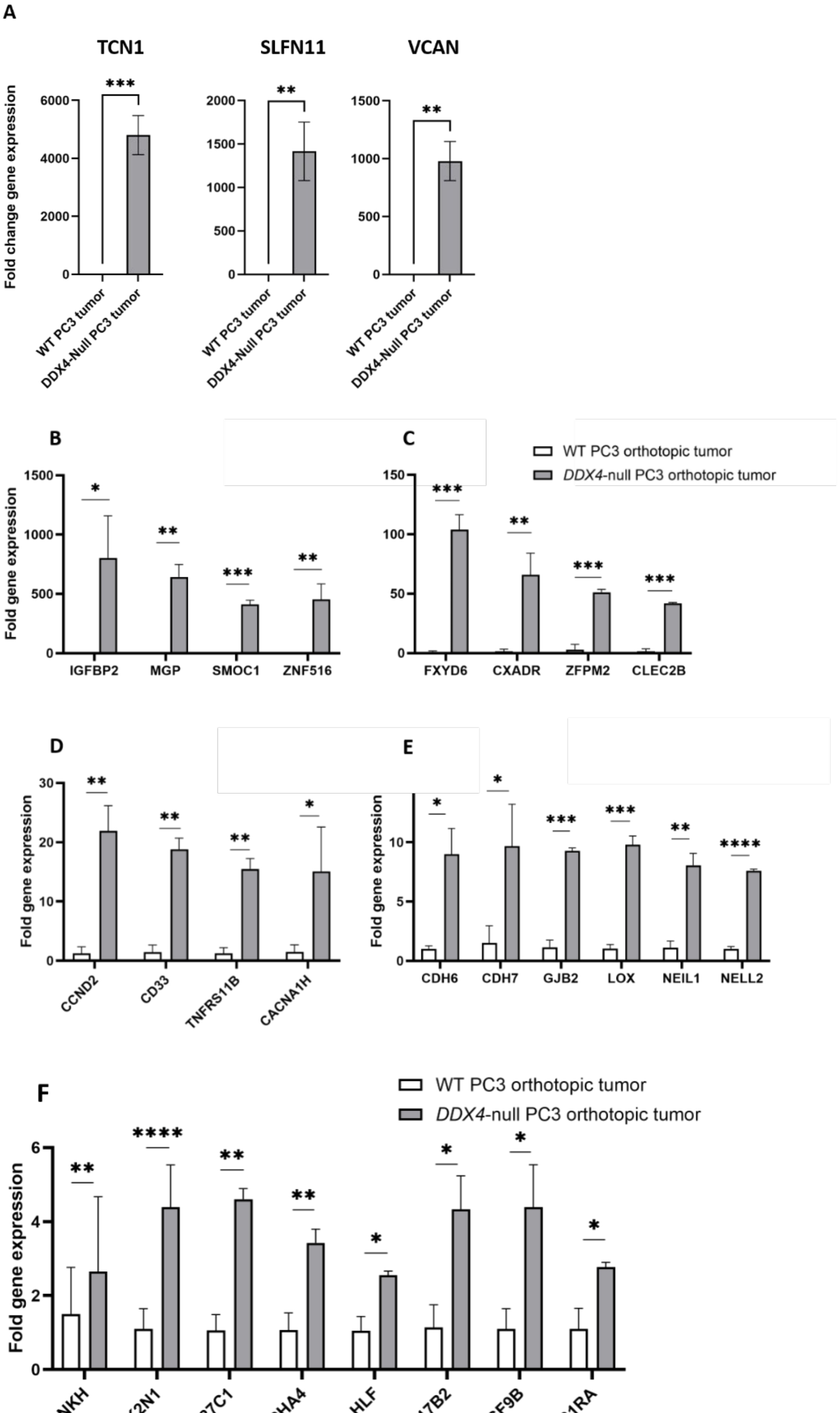

Figure 15. Upregulated genes grouped by their relative expression. **A)** TCN1 (p-value 0.0009), SLFN11 (p-value 0.0041), VCAN (p-value 0.0016). **B)** IGFBP2 (p-value 0.0233), MGP (p-value 0.0013), SMOC1 (p-value 0.0002), ZNF516 (p-value 0.0071). **C)** FXYD6 (p-value 0.0006), CXADR (p-value 0.007), ZFPM2 (p-value 0.0008), CLEC2B (p-value 0.0001). **D)** CCND2 (p-value 0.0033), CD33 (p-value 0.001), TNFRS11B (p-value 0.0012), CACNA1H (p-value 0.0442). **E)** CDH6 (p-value 0.0063), CDH7(p-value 0.0322), GJB2 (p-value 0.0004), LOX (p-value 0.0003), NEIL1 (p-value 0.0021), NELL2 (p-value <0,0001). **F)** ANKH (p-value 0.0087), CAMK2N1 (p-value <0,0001), CYP27C1 (p-

value 0.0021), EPHA4 (p-value 0.0095), HLF (p-value 0.0151), HSD17B2 (p-value 0.0168), IGSF9B(p-value 0.0201), IL31RA (p-value 0.0284). *P<0.05, ** P<0.01, ***P<0.001, unpaired t-test, two-tailed. The error bars represent the standard deviation.

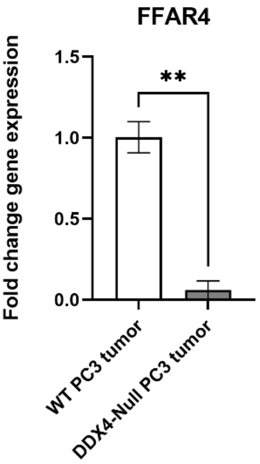

Figure 16. FFAR4 was the only gene significantly downregulated by DDX4-deletion in orthotopic PC3 tumors (p-value 0.0012). *P<0.05, ** P<0.01, ***P<0.001, unpaired t-test, two-tailed. The error bars represent the standard deviation.

However, mRNA expression of three of the genes, SLFN11, IGFBP2 and ZNF516 were undetectable in the WT PC3 tumors. For these genes, a Ct-value of 40 was used in calculating fold change. Consequently, the calculation is not accurate and was done to illustrate that the expression of these genes went from undetectable to detectable. In the case of SLFN11, the expression was detected at cycle 26, hence the increase in expression can be considered significant. Regarding IGFBP2 and ZNF516, the expression was detected at around cycle 30 and can be considered to have gone from undetectable to low, but detectable.

Twenty genes were upregulated in *DDX4*-null vs. WT orthotopic tumors but the difference was not significant or the variation was too high: ADAM12, CDH2, CTTNBP2, DIRAS1, EVC2, GLIS1, LPAR6, MUC2, MYLK, NDN, NFATC4, NNMT, PDE10A, RNF43, SAMSN1, SFRP1, SHISA2, TGM2, TMEM74B and ZNF415. Five genes were downregulated without significant difference: B3GALT1, CEACAM1, DCC, FAM107A and HORMAD2. Seven genes, COL17A1, KLK11, NDUFA4L2, RGS17, SERPINB2, TRPV3 and ZPLD1, had no difference between *DDX4*-null and WT mRNA.

Remarkably, out of all the differentially expressed genes only five were similarly expressed in both subcutaneous and orthotopic tumors. The genes upregulated in both subcutaneous and orthotopic PC3 tumors were CACNA1H, CLEC2B, MGP and TCN1. The only significantly downregulated gene in orthotopic tumors, FFAR4, was also significantly downregulated in subcutaneous tumors.

Compared to previously obtained RNA-Sequencing results, the significantly upregulated genes in *DDX4*-null tumors matched the expression in RNA-Seq, except for 5 genes: CDH6, CDH7, CYP27C1, IL31RA, and TNFRSF11B. However, in the significantly downregulated genes opposite expression was seen in only one gene, FFAR4 (Table 8). Despite the drastic difference in results, direct comparison of RNA-Seq results with qPCR is problematic as they are based on different scales. However, this does highlight that RNA-Seq results should always be validated with another method like RT-qPCR. Table 9 lists all genes (n=3) sharing the same significantly different expression pattern across all samples (RNA-seq and RT-qPCR of subcutaneous and orthotopic tumor types).

Table 8. List of genes with opposite expression in RT-qPCR from orthotopic tumors compared to RNA-sequencing.

GENE	**RT-qPCR**	**RNA-Seq**
CDH6	↑	↓
CDH7	↑	↓
CYP27C1	↑	↓
IL31RA	↑	↓
TNFRSF11B	↑	↓
FFAR4	↓	↑

Table 9. The only genes with same direction of expression upon *DDX4*-deletion across all three sample types.

GENE	**RT-qPCR subcutaneous**	**RT-qPCR orthotopic**	**RNA-Seq**
CLEC2B	↑	↑	↑
MGP	↑	↑	↑
TCN1	↑	↑	↑

2.5 Validating the transcriptomic changes on protein level

To see whether the changes observed in tumor transcriptome were translated to protein level, we performed SDS-PAGE and western blotting of SHISA2, NNMT, HORMAD1 and Mucin2. The antibodies were first tested on cell culture samples of both PC3 and UT-SCC-14 cell lines. Unfortunately, only the antibody for SHISA2 seemed to work properly (Figure 17). The western blotting was repeated multiple times with little success, and there was not enough time to order new antibodies. Therefore, no emphasis was put on the western blotting results and hence not quantified for this thesis.

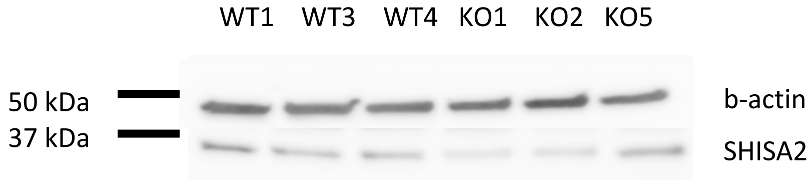

Figure 17. The expected protein size for SHISA2 is 42kDa and

Western blotting image of SHISA2 protein expression in WT PC3 tumors and DDX4-null (three biological replicates each). β-Actin was used as the loading control. Bar chart shows the quantification of the SHISA2 protein level normalized by β-Actin signal. No significant difference of SHISA2 protein level was observed, even though SHISA2 protein level was slightly higher in DDX4-null compared to WT.

3 Discussion

In this thesis, we aimed to gain novel information of the role of DDX4 in the formation, growth and invasive properties of PC3 and UT-SCC-14 xenograft tumors. In addition, we aimed to elucidate the effects of *DDX4*-deletion on PC3 tumor transcriptome. As has previously been discovered by the group, DDX4 is one of the CG antigens consistently found in germ granules participating in RNA regulation. Furthermore, its aberrant expression was found in several human cancer tissue samples, including PC3 and UTSCC-14 cell lines. The previous results from 2D and 3D cultures indicated that the deletion of *DDX4* affects tumor formation only in an authentic TME. As already reported, DDX4 has been associated with tumor progression and growth in ovarian cancer and SCLC through microarray, immunofluorescence and mass spectrometry analyses (Hashimoto et al., 2008; K. H. Kim et al., 2014; Noyes et al., 2023). However, these studies have not focused on the effects of *DDX4* deletion on tumor transcriptome. In blood cancer-derived cell lines, *DDX4* knockout has been shown to downregulate certain oncogenes/cell cycle regulators contributing to cell proliferation and migration, consistent with its role in the germ line, but the extent of the study was fairly limited (Schudrowitz et al., 2017). Since the role of DDX4 in cancer has not been exhaustively studied, its effects might largely vary across tumor types. Nonetheless, the effects of *DDX4*-deletion called for further investigation.

In the first subcutaneous xenograft tumor experiment, it was obvious that the *DDX4*-null tumors were forming slower, seemingly smaller in size as well as more compressed in nature as compared to WT tumors. The trend was seen in both PC3 and UTSCC-14 subcutaneous xenograft tumors. This finding was further validated by weekly ultrasound measurements of tumor volume as well as in weight of the dissected tumors where we saw significant hindrance in the growth of *DDX4*-null tumors. To see how the growth is affected when tumors grow in an orthotopic tumor environment, we repeated the xenograft tumor experiments in the prostate of mice. Unsurprisingly, the results were in line with the previous subcutaneous xenograft tumors: upon *DD4X*-deletion, the growth was compromised. In addition, it seems that the invasive capabilities of DDX4-null PC3 cells was also affected. To validate the findings that *DDX4*-deletion comes with transcriptomic changes we ran RT-qPCR with 67 primer pairs. The transcriptomic changes were drastic, but surprisingly mostly differing between the subcutaneous and orthotopic tumors.

From the 67 investigated genes, only two genes, MGP and TCN1, were significantly upregulated based on all three experiments: RNA-sequencing and RT-qPCR of both subcutaneous and orthotopic tumors. MGP, Matrix Gla protein, is an extracellular matrix protein synthesized mostly in cartilage and bone where it regulates calcification (X. Li et al., 2020). Overexpression of MGP has been found in various human cancers, including prostatic carcinomas, testicular and ovarian cancer, glioblastoma, and colon and colorectal cancer (Caiado et al., 2023; Gheorghe & Craciun, 2016). Wang et al reported in 2020 that the overexpression of MGP correlates with poorer prognosis and that the inhibition of MGP resulted in reduced cell proliferation, migration and invasion. The overexpression of MGP in ovarian cancer has been reported as necessary for tumor initiation and progression, but also contributing to resistance against common chemotherapies paclitaxel and topotecan (Nieddu et al., 2023; Sterzyńska et al., 2018). Huang et al found similar association between MGP expression and resistance to oxaliplatin in colorectal cancer in 2021. In contrast, a statistically significant inverse correlation of MGP expression and tumor size, grade and metastasis was found in renal cell and prostatic carcinomas. Notably, high expression of MGP was only found in primary prostatic carcinomas, but not in prostate cancer metastasis derived cell lines indicating that the loss of MGP expression could associate with cancer progression and metastasis. (Levedakou et al., 1992). The PC3 cell line is one of the most commonly used prostate cancer cell lines and it is derived from lymph node and bones metastasis (Tai et al., 2011). This might explain the low MGP expression in both subcutaneous and orthotopic WT PC3 tumors. The significant upregulation of MGP in our results suggest that the expression of MGP may be triggered and sustained upon *DDX4*-deletion in primary tumors throughout cancer progression. This could indicate beneficial changes in tumorigenesis of prostate cancer as a result of *DDX4*-inhibition. However, since the expression of MGP is likely tumor-type dependent and its role still unclear (Caiado et al., 2023) further investigation is needed.

TCN1, transcobalamin 1, is a vitamin B12 –binding protein that participates in cobalamin metabolism and homeostasis. Its higher expression levels have been associated with at least pancreatic, colorectal and colon cancer as well as lung adenocarcinoma (Chu et al., 2014; H. Li et al., 2022; G. Liu et al., 2020; Wu et al., 2018; Zhu et al., 2022). Kim et al reported a correlation of TCN1 with the progression, stage, metastasis and poor prognosis in gastric cancer. (B. Kim et al., 2006). In 2014 Chu et al found TCN1 among novel genes related to colorectal cancer calling for further investigation into their roles in cancer (Chu et al., 2014). In a more recent study, Zhu et al reported that a loss-of-function of TCN1 reduced cell proliferation and invasion in colorectal cancer xenograft tumors, providing more evidence that TCN1 has a role in the tumorigenesis of colorectal cancer. Moreover, TCN1 upregulates ITGB4 which subsequently upregulates the Notch signaling pathway, a regulator of self-renewal and differentiation in several cell and tissue types. (Zhu et al., 2023). In gastric cancer TCN1 might be useful as marker for progression, but the impact of TCN1 on survival is unclear (Zhu et al., 2022). In 2020, Liu et al. found TCN1 expression could predict chemosensitivity in a neoadjuvant setting, and could serve as a negative prognostic biomarker in colon cancer (G. Liu et al., 2020). In addition, TCN1 may play a role in lung adenocarcinoma and could be used as a prognostic and diagnostic marker (H. Li et al., 2022). However, the underlying mechanism behind the oncogenic role of TCN1 is still unclear, as is the mechanism through which TCN1 is significantly upregulated upon DDX4-deletion in both subcutaneous and orthotopic xenograft tumors.

C-type lectins, CLECs, are a superfamily of proteins with prominent roles in immunity and homeostasis (Brown et al., 2018). CLEC2B, C-type lectin–like receptor 2, has been previously identified as a marker for multiple tumor types like melanoma, pancreatic cancer and clear-cell renal-cell carcinoma (Chen et al., 2021; Y. Gao et al., 2020). In 2022, Li, Tao & Ding reported an interesting correlation between CLEC2B and psoriatic arthritis. In this study, CLEC2B showed a significant connection to ferroptosis. Further analysis of CLEC2B suggests it acts as a key link between psoriatic arthritis and cancer through ferroptosis. The association of CLEC2B and psoriatic arthritis could help to understand the high cancer risk in psoriasis patients. (X. Li et al., 2022). Another very recent study by Bian et al found that a certain subtype of effector T cells, CD8+CXCR6+ T cells, is reduced in malignant prostate cancer. This study suggests that pathways including CLEC2B associate with anticancer responses by CD8+CXCR6+ T cells; in the absence of CLEC2B the antitumor responses of CD8+CXCR6+ T cells is inhibited. (Bian et al., 2024). Based on the suggested role of CLEC2B in other cancers, the significant upregulation of CLEC2B upon DDX4-deletion might indicate higher immune cell infiltration and reduced tumor progression. However, this is all speculative and further research is needed.

The only downregulated gene in both subcutaneous and orthotopic tumors was FFAR4, free fatty acid 4. Expression of FFARs have been found from several cancers, and like many others, their effect depends on the cell and tumor type (L. Zhang et al., 2022). In breast and colon cancer FFAR4 has been found to promote cell proliferation and migration (Takahashi et al., 2018; M. Zhang & Qiu, 2019), whereas in pancreatic cancer and osteosarcoma the role of FFAR4 seems to have opposite effects (Fukushima et al., 2015; Takahashi et al., 2017). A recent study in lung adenocarcinoma revealed a possible role of FFAR4 in connecting genetics and environmental factors, which could partly explain the difference in the role of FFAR4 in several cancers (Z. Wang et al., 2024). With such varying roles, the downregulation due to DDX4-deletion needs further clarification.

Some of the most interesting genes dysregulated in either DDX4-null subcutaneous or orthotopic tumors include the cell adhesion molecules cadherin 6 and cadherin 7 (CDH6 and CDH7), both of which have long been associated with cancer invasion and metastasis (Takeichi, 1993). As an adhesion molecule, CDH6 has a major role in epithelial-mesenchymal transition and its aberrant reactivation has been found in different cancers (Gugnoni et al., 2017). The role of CDH 7 has been less studied, but it has been associated with, for example, progression and metastasis of malignant melanoma (Winklmeier et al., 2009). The expression of both CDH6 and CDH7 was opposite in the two tumor types of this study: upregulation was seen in orthotopic tumors, downregulation in subcutaneous. Nevertheless, this proposes an interesting question of whether these genes are only promoted in a suitable TME.

Even though the results from these xenograft tumors suggest that deletion of *DDX4* hinders tumor formation, growth and metastasis, the differences in transcriptomes in subcutaneous vs. orthotopic tumors are rather distinct. These variances are likely due to the difference in the TME of the tumors. Compared to subcutaneous xenograft tumors, the advantage of orthotopic tumors is the conditions that mimic the original organ and therefore orthotopic xenograft tumor are clinically more relevant (Talmadge et al., 2007). In a comparative study of PC3 subcutaneous and orthotopic xenograft tumors, differences were found in size, growth rate, tissue histology of inner organs as well as water retention. In addition, the study found that orthotopic tumors have higher vascular perfusion compared to subcutaneous tumors. As the study focused on the drug delivery efficacy of BB2r-targeting agents, no comparison on tumor transcriptome was included. (W. Zhang et al., 2019). The main advantage of a subcutaneous xenograft is its simplicity. Injecting tumor cell suspension subcutaneously is quick and easy and proposes very little stress to the mouse. An orthotopic tumor inoculation, especially to mouse prostate, requires a technically challenging procedure with anesthesia and analgesia, which can be extremely demanding for the animal (Fernandez et al., 2023). In addition, as seen in our experiment as well, measuring subcutaneous tumors is quicker, easier and more accurate compares to orthotopic tumors. Yet, it is obvious that an orthotopic tumor is more reflective of human tumors compared to subcutaneous xenografts (Talmadge et al., 2007).

One of the aims of this thesis was to validate the results from previously obtained RNA-sequencing results. RNA-sequencing has for the most part replaced microarray methods in transcriptome studies. The main benefit of RNA-seq is the capability to measure the full transcriptome in a sensitive and accurate manner. RT-qPCR, another method in measuring gene expression, is tied to a smaller, selected subset of transcripts. Despite its advantages, there are still differing opinions whether RNA-sequencing results need to be validated with another method or not. (Coenye, 2021; Z. Wang et al., 2009). Many studies propose the robustness of RNA-seq methods do not require validation (Coenye, 2021). In addition to qPCR, there are various computational validation strategies to analyze RNA-seq data (Conesa et al., 2016; Koch et al., 2018; Pardo-Palacios et al., 2024). However, results of this thesis together with comprehensive analyses comparing RNA-seq and qPCR suggest technical validation, especially for genes with inconsistent expression measurements, is still needed (Everaert et al., 2017).

Most limitations of this study are associated with animal work. The first encountered limitation was the difficulty in performing orthotopic inoculation to the mouse prostate. Even with the help of skilled and experienced technicians from the animal facility, the operation proved demanding not only to us but also to the animals. Hence, we lost one animal on the day following the procedure. In addition, inexperience in performing ultrasound on an animal made measuring the forming tumors harder than expected, but only in the orthotopic models.

The aforementioned reasons partly explain one of the biggest limitations of this study: the small number (n=2) of orthotopic *DDX4*-null primary tumors. A minimum of three biological replicates is needed to yield statistically significant results. Even though this threshold was not reached, the results can be considered as giving direction for further studies. From another technical and statistical point of view, the qPCR samples were run as duplicates, however, triplicates is the recommended method. It is also recommended to run two identical qPCR plates to rule out any variances caused by the run itself. As the animal experiments took a lot of time, there was not enough time to perform RT-qPCR of UT-SCC-14 samples.

Future plans and experiments that would address these limitations include repeating the study of orthotopic DDX4-null xenograft tumors, and to use both methods, RNA-seq as well as RT-qPCR, from the same tumors. This could shed light on the differences between RNA-seq and qPCR results found in this study. Validation of transcriptomic changes of the UT-SCC-14 tumors could address questions on whether the changes seen upon DDX4-deletion concern another cell type as well, or whether they are tumor type and/or cell type specific. It would also be interesting to see how the transcriptomic changes that *DDX4*-deletion trigger in secondary tumors vs. primary tumors since metastasized tumors often display diverse transcriptomic features (Riaz et al., 2024). One interesting approach would be to perform *DDX4*-deletion on a patient derived DDX4-expressing tumor sample and see how it affects the growth, progression, invasive properties, and transcriptome after transfer to mice as a xenograft. The transcriptomic changes should be monitored from a patient sample, a xenograft sample, as well as from 2D and 3D cultures.

In conclusion, our data revealed that *DDX4*-deletion hinders tumor formation, growth, progression and likely metastasis in xenograft tumor models. In addition, *DDX4*-deletion has major effects on tumor transcriptome. Based on current literature, some of these effects might have beneficial outcomes in hindering tumor growth and progression. However, the expression of some upregulated genes have been associated with undesirable effects in cancer. Nonetheless, further research on DDX4 and its role in cancer could provide valuable information regarding its utility as a marker for certain cancers or even as a therapeutic target. In addition, the differing results highlight the need to validate RNA-sequencing results, and not just with a computational method. This thesis was part of a larger published study regarding the expression and effects of DDX4 in cancer (Olotu et al., 2024).

4 Materials and methods

4.1 Cell culture

The human cancer cell lines PC3 and UT-SCC-14 used in this thesis were purchased from the American Type Culture Collection (Manassas, VA, USA), grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F12; D8900, Sigma-Aldrich) supplemented with 10% inactivated Fetal Bovine Serum (iFBS; 10270106, Gibco) and 1% Penicillin/Streptomycin (15140-122, Gibco). Cells were cultured at 37 ºC in a humidified 5% CO_2 incubator. Passages from 6 to 22 were used in this study for both cell lines.

4.2 Animals for human tumor xenograft models

All animal experiments were carried out in accordance with the Finnish legislation and EU Directives. All protocols were conducted in agreement with good laboratory practice and approved by the ethical committee of animal experimentation, University of Turku animal care center. Project license number: 21485/2020, development and use of *in vivo* cancer models in research and drug development.

The animals used for xenograft experiments were 5-6 week old Hsd:Athymic Nude-*Foxn1*^{nu} T-cell deficient male mice purchased from Envigo (Gannat, France). The mice were allowed to acclimatize to the facility for minimum 1 week prior to commencing the experiment. Five animals (in total of 10 mice in the PC3 group, 15 in the UTSCC-14 group) were housed in individually ventilated cages containing wood chips and environmental entertainment under controlled conditions of 12/12 hour light/dark cycle, humidity of 55%±15%, and temperature of 21±3 ºC. Mice had *ad libitum* access to autoclaved or UV-treated water and irradiated soy-free high-quality feed (Teklad 2920) with 20% protein (RM3; Special Diet Services, Witham, UK).

4.3 Inoculation of tumour cells and monitoring tumour growth

4.3.1 Subcutaneous (s.c.) inoculation

UT-SCC-14 cells or PC3 cells were collected in a state of exponential growth and cells in culture medium (DMEM/F12) without supplements were mixed in 1:1 ratio with Matrigel (BD

Matrigel Matrix; 354234, BD Biosciences). Tumour cell based xenografts were established by subcutaneous injection of UT-SCC-14 or PC3 cells (1×10^6 cells in 50 µl per flank) into the right and left flank of generally anaesthetized (isoflurane) mice. Tumour growth and burden was assessed by weekly weighing's and ultrasound measurements where the transverse and longitudinal length of the tumour was recorded, and tumour volume was estimated by $(\text{length}\times\text{width}^2)/2$ (Euhus et al., 1986; Tomayko & Reynolds, 1989). Following tumour growth, after 4 weeks animals were euthanized by carbon dioxide, followed by cervical dislocation. Tumors were resected and placed into liquid nitrogen for protein/RNA analysis or in 10% formalin for histological analysis.

4.3.2 Orthotopic (o.t.) inoculation

PC3 cells were collected in a state of exponential growth and cells in culture medium without supplements were mixed in 1:1 ratio with Matrigel (BD Matrigel Matrix; 354234, BD Biosciences). Green food coloring in 1:100 ratio was added to detect successful intraprostatic injection. Buprenorphine 0.3 mg/kg (Temgesic, Schering-Plough Nv, Brussels, Belgium) was given 30 min before inoculation and the mice were anesthetized by inhaled isoflurane throughout the procedure. The anesthetized animals were placed in supine position for intraprostatic inoculation of the PC3 cells. After midline laparotomy, the prostate was mobilized to guarantee free access to the dorsal lobe. The injection of 20 µl of tumor cell suspension was performed under sterile surgical conditions with an insulin syringe and a 2x-gauge needle (Tuomela et al., 2008). Post-operationally, carprofen 5 mg/kg (Vet Rimadyl, Pfizer SA, Louvain-La-Neuve, Belgium) was administered subcutaneously every 24 h for three days. Tumour growth and burden was assessed by weekly weighing's and ultrasound measurements where the transverse and longitudinal length of the tumor was recorded, and tumor volume was estimated by $(\text{length}\times\text{width}^2)/2$. After 8 weeks animals were euthanized by carbon dioxide, followed by cervical dislocation. Tumors were resected and placed into liquid nitrogen for protein/RNA analysis or in 10% formalin for histological analysis.

4.4 Western blotting

4.4.1 Lysate preparation

For lysate preparation, the frozen tumors were transferred to ice cold isotonic non-denaturing lysis buffer (150mM sodium chloride (NaCl), 5mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 50mM tris(hydroxymethyl)aminomethane (Tris-HCl), pH 8.0, with 1x protease inhibitor cocktail (PIC), 0.2 mM phenylmethylsulphonyl fluoride (PMSF) and 1mM dithiothreitol (DTT), and lysed with a dounce homogenizer. For thorough disruption the tumours were further lysed with TissueLyser LT (69980, Qiagen), 50 oscillations/second for 4x2 min. After homogenization the lysates were incubated on ice for 30 min and centrifuged at 16 400 rpm at +4°C for 15 min. The supernatant was collected to a new tube and protein concentrations were determined using Pierce BCA Protein Assay Kit (23227, Thermo Scientific) and Victor X4 Multilabel Plate Reader (PerkinElmer) according to manufacturer's instructions. The samples were stored at -20 °C and upon use, thawed on ice.

4.4.2 SDS-PAGE and immunoblotting

After complete thawing, the samples were centrifuged at 13 000 rpm at +4 °C. Equal amounts (30 µg) of protein lysates were mixed with Laemmli buffer and heated at 95°C for 5 minutes before being separated by a 4–20% precast polyacrylamide gel (#4561094, Bio-Rad Laboratories) and transferred onto a PVDF membrane using Trans-Blot® SD semi-dry transfer cell (Bio-Rad Laboratories). After protein transfer to avoid non-specific binding the membranes were blocked with 5% skimmed milk in Tris buffered saline with Tween 20 (TBS-T) for 1h at room temperature and then incubated overnight with primary antibodies (dilution 1:500 or 1:1000 in 2.5% skimmed milk in TBS-T). The used primary antibodies . After primary antibody incubations the membranes were washed 3 x 10 minutes with TBS-T. Secondary antibody incubations were performed at room temperature with the appropriate horseradish peroxidase-coupled secondary antibody (dilution 1:5000 in 2.5% skimmed milk in TBS-T) for 1-2 hrs. The signal was visualized using Western Lightning™ ECL Pro kit (NEL122001EA, Perkin Elmer) or Immobilon® ECL Ultra Western HRP Substrate (WBULS0500, Millipore). The membranes were imaged with LAS-4000 (Fujifilm Life Science) and the signal was quantified by densitometry using ImageJ software. The densitometry readings were normalized to the expression of β-actin.

4.4.3 Isolation of total RNA from cells/tumors and RT-qPCR

Total RNA was isolated from cells and tumors using TRIsure (BIO-38033, Meridian Bioscience) according to the manufacturer's instructions. To remove any DNA contamination, DNase I (AMPD1, Sigma-Aldrich) was used. The concentration and quality of total RNA were measured with NanoDrop™ 2000 spectrophotometer and further verified with 2100 Bioanalyzer (Agilent Technologies) using Agilent RNA 6000 Nano Kit (5067-1511, Agilent

Technologies). cDNA synthesis and qPCR was performed using SensiFAST cDNA Synthesis Kit (BIO-65054, Meridian Bioscience) and DyNAmo Flash SYBR Green qPCR Kit (F415L, Thermo Scientific) according to the manufacturer's instructions. qPCR was run on CFX384 Real-Time PCR System (Bio-Rad) and the conditions were: initial denaturation 95°C for 7 min, and subsequently for 40 cycles 95°C for 10 sec, 60°C for 15 sec, 72°C for 10 sec, followed by final elongation at 72°C for 10 min. The PCR products were run on 1,5% agarose gel and the amplified products were visualized using Midori Green Advance DNA Stain (MG04, Nippon Genetics). The primers used for qPCR were ordered from Integrated DNA Technologies and are listed in Appendix 2. Fold gene expression was calculated using *hRPL19* as a reference gene.

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Lastly, with the words of a great poet (Snoop Dogg): “I wanna thank me for believing in me, for doing all this hard work, and for never quitting.”

Abbreviations

ATP	Adenosine triphosphate
CB	Chromatoid body
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CG	Cancer-germline
CT	Cancer-testis
Ct	Cycle threshold
CTLA	Cytotoxic T-lymphocyte associated protein 4
DDX4	DEAD-box helicase 4
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
IMC	Intermitochondrial cement
KRAS	Kirsten rat sarcoma virus
MAGE-A1	Melanoma associated antigen 1
mRNA	Messenger-RNA
NK-cell	Natural killer cell
NY-ESO-1	New York esophageal squamous cell carcinoma 1
ot	Orthotopic
PC3	Prostatic carcinoma 3
PCG	Primordial germ cell
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PGD2	Prostaglandin D2
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
sc	Subcutaneous
SCLC	Small cell lung cancer
SKIP	Ski-interacting protein
Sry	Sex-determining region y
TGFβ	Transforming growth factor beta
TME	Tumor microenvironment
TNBC	Triple-negative breast cancer
UT-SCC-14	University of Turku squamous cell line 14
VEGF	Vascular endothelial growth factor
WT	Wild-type
ZNF	Zinc finger transcription factor

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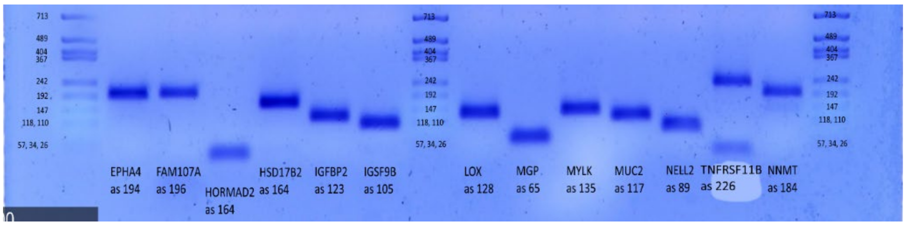
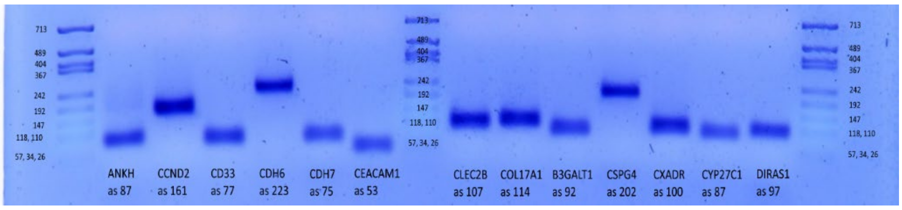
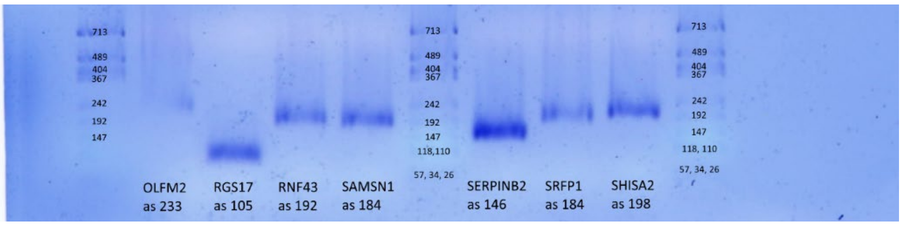
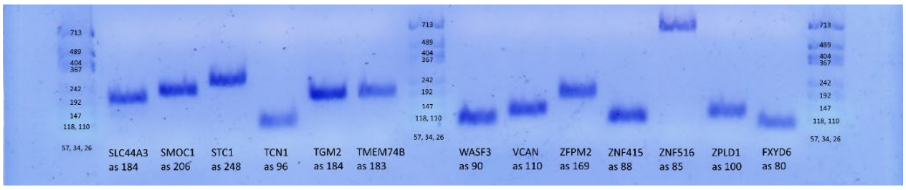
Appendices

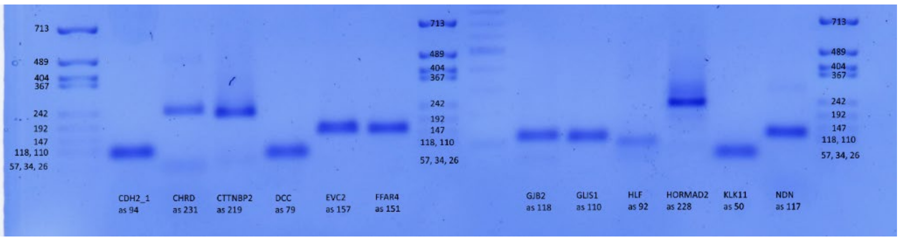
Appendix 1 PCR products from primer pairs and their sizes

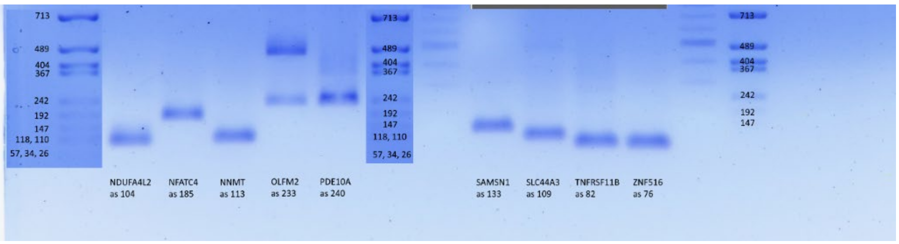












Appendix 2 List and sequence of primers for RT-qPCR

Name	Primer sequence	Amplicon size
ADAM12	Fw 5'-CGAGGGGTGAGCTTATGGAAC-3' Rev 5'-GCTTTCCCGTTGTAGTCGAATA-3'	144
ANKH	Fw 5'-CATCACCAACATAGCCATCGAC-3' Rev 5'-GCCAGCATCTCGACTGCAT-3'	87
B3GALT1	Fw 5'-CGCCCTACTTCTTCTTACACTG-3' Rev 5'-GGTCGAGTTCTTATGTTGCCA-3'	92
CACNA1H	Fw 5'-ATGCTGGTAATCATGCTCAACTG-3' Rev 5'-AAAAGGCGAAAATGAAGGCGT-3'	121
CAMK2N1	Fw 5'-GACACCAACAACTTCTTCGGC-3' Rev 5'-TCATCTTCAATAACAACCCGCTT-3'	92
CCND2	Fw 5'-ACCTTCCGCAGTGCTCCTA-3' Rev 5'-CCCAGCCAAGAAACGGTCC-3'	161
CD33	Fw 5'-GGCCACTCCAAAAACCTGAC-3' Rev 5'-GACAACCAGGAGAAGATCGGG-3'	77
CDH2	Fw 5’-TCAGGCGTCTGTAGAGGCTT-3’ Rev 5’-ATGCACATCCTTCGATAAGACTG-3’	94
CDH6	Fw 5'-AGAACTTACCGCTACTTCTTGC -3' Rev 5'-TGCCCACATACTGATAATCGGA-3'	223
CDH7	Fw 5'-TCAAATACATCTTGTCAGGCGAA-3' Rev 5'-TGGCATGAATATCCCCAGTGT-3'	75
CEACAM1	Fw 5'-TGCTCTGATAGCAGTAGCCCT-3' Rev 5'-TGCCGGTCTTCCCGAAATG-3'	53
CHRD	Fw 5'-TTCGGCGGGAAGGTCTATG-3' Rev 5'-ACTCTGGTTTGATGTTCTTGCAG-3'	160
CLEC2B	Fw 5'-GTTCCACTCAACATGCCGAC-3' Rev 5'-TGCCATCTTCAGTCCAATCCA-3'	107
COL17A1	Fw 5'-ACCAGCAATGGCTATGCTAAAA-3' Rev 5'-GCCTCGTGTGCTTCCAGTT-3'	114
COL2A1	Fw 5'-TGGACGATCAGGCGAAACC-3' Rev 5'-GCTGCGGATGCTCTCAATCT-3'	244
CSPG4	Fw 5'-CTTTGACCCTGACTATGTTGGC-3' Rev 5'-TGCAGGCGTCCAGAGTAGA-3'	202
CTTNBP2	Fw 5'- ACTCTCAGTAAATCCGAGCTGC -3' Rev 5'- GGGGTTGGTACAAACTGGCT -3'	219
CXADR	Fw 5'-GTGCTCCTGTGCGGAGTAG-3' Rev 5'-ATGGCAGATAGGCAGTTTCCC-3'	100
CYP27C1	Fw 5'-AGAAACCGTGACCAATGTCAAT-3' Rev 5'-CAGCCCAAACGACTCTCATAAA-3'	87
DCC	Fw 5'-ACCCAAGCTGGCTTTTGTACT-3' Rev 5'-TGTGACGGCATCAGAAGGTTC-3'	124
DIRAS1	Fw 5'-ATGCCGGAACAGAGTAACGAT-3' Rev 5'-GGAACGTGCCCTTCACGAA-3'	97
EPHA4	Fw 5'-TTCGCCCTATTTTCGTGTCTC-3' Rev 5'-TGGTAGGTTCGGATTGGTGTAT-3'	194

EVC2	Fw 5'-ACCACTTGGAATGAAATTGGACA-3' Rev 5'-GCGGTGTGTTATAGGAGACTCT-3'	157
FAM107A	Fw 5'-GCAGCGTGTCCTAGAGCAC-3' Rev 5'-CCGCAGGTTTTCCCTGACT-3'	196
FFAR4	Fw 5'-TGGAGCCCCATCATCATCAC-3' Rev 5'-TGCACAGTGTCATGTTGTAGAG-3'	151
FXYD6	Fw 5'-GTGCAAGTGCAGTTTCAATCAG-3' Rev 5'-CATTGGCGGTGATGAGGTTCT-3'	80
GJB2	Fw 5'-TCGCATTATGATCCTCGTTGTG-3' Rev 5'-GGGGAAGTAGTGATCGTAGCAC-3'	118
GLIS1	Fw 5'- CGTCTCTGGTCACCTGTGTAA -3' Rev 5'- CTCATGGCTGTCCGTCGAT -3'	110
HLF	Fw 5'-CCACCTTTATCCCGCCTCC-3' Rev 5'-TTTACTAAATGCGTCTTCGTGGT-3'	92
HORMAD2	Fw 5'- ATGGAGAACGCCATTTGGATG -3' Rev 5'- CCATAGTGGCTCCTTCTTTCG -3'	228
HSD17B2	Fw 5'-TCTTCTCGGTGTCATGCTTCC-3' Rev 5'-CAAAACTCCGGCAAATACCGT-3'	164
IGFBP2	Fw 5'-GACAATGGCGATGACCACTCA-3' Rev 5'-CAGCTCCTTCATACCCGACTT-3'	123
IGSF9B	Fw 5'-AGAGGAGCCCGAGTTTGTGA-3' Rev 5'-CACTCTACGACATAGGGTGGG-3'	105
IL31RA	Fw 5'-AACATAGCGAAAACTGAACCACC-3' Rev 5'-GCCAACTCAGGCTTTATCCATTC-3'	98
JAM3	Fw 5'-CGGCTGCCTGACTTCTTCC-3' Rev 5'-TGGGGTTCGATTGCTGGATTT-3'	84
KIRREL3	Fw 5'-CAGCTCGATCTGCTCTTCGTC-3' Rev 5'-ATTCTCCGAAACTTGTCCTTGG-3'	113
KLK11	Fw 5'-TTCAAGGCATTATCTCCTGGG-3' Rev 5'- CTTTCGGGTGATCGCACAC -3'	140
LOX	Fw 5'-CGGCGGAGGAAAACTGTCT-3' Rev 5'-TCGGCTGGGTAAGAAATCTGA -3'	128
LPAR6	Fw 5'-TTGTATGGGTGCATGTTCAGC-3' Rev 5'-GCCAATTCCGTGTTGTGAAGT-3'	193
MGP	Fw 5'-TCCGAGAACGCTCTAAGCCT -3' Rev 5'-GCAAAGTCTGTAGTCATCACAGG-3'	65
MUC2	Fw 5'-GAGGGCAGAACCCGAAACC-3' Rev 5'-GGCGAAGTTGTAGTCGCAGAG-3'	117
MYLK	Fw 5'-CCCGAGGTTGTCTGGTTCAAA-3' Rev 5'-GCAGGTGTACTTGGCATCGT-3'	135
NDN	Fw 5'- AGTTTGCGCTGGTCAAAGC -3' Rev 5'-GGCCCTTCACGTAGATGAGG-3'	117
NDUFA4L2	Fw 5'-ATGATCGGCTTAATCTGCCTG-3' Rev 5'-TCCGGGTTGTTCTTTCTGTCC-3'	104
NEIL1	Fw 5'-GCCCTATGTTTCGTGGACATC-3' Rev 5'-CGCTAGGTTTCGTAGCACATTC-3'	129
NELL2	Fw 5'-GCACAAGCTCTCCTTAGCCAT-3' Rev 5'-AGGGCTTTTCTACTACCCTTTCA-3'	89

NFATC4	Fw 5'- GACACAGCCCTATCTTCAGGA -3' Rev 5'- GAGCTTGACTACGGGGTGA -3'	185
NLGN4X	Fw 5'-GGTTTACCGCCAATTTGGATACT-3' Rev 5'-CCGTGGGCACGTAGATGTT-3'	87
NNMT	Fw 5'- ATATTCTGCCTAGACGGTGTGA -3' Rev 5'- TCAGTGACGACGATCTCCTTAAA -3'	113
OLFM2	Fw 5'-TCCTTGAGTTGCGGACGTATC-3' Rev 5'-GCCGGAGAGATTCCTCACC-3'	233
PDE10A	Fw 5'-GGACCTTCTAATAATGCGAGCTG-3' Rev 5'-TCCCTGCATATTCGTATCTTGGT-3'	240
RGS17	Fw 5'-CAGAGGCCCAACAACACCTG-3' Rev 5'-TGTGGGTCTTCCCGCATTTT-3'	105
RNF43	Fw 5'-CATCAGCATCGTCAAGCTGGA-3' Rev 5'-TTACCCCAGATCAACACCACT-3'	192
SAMSN1	Fw 5'- AGATCCCACAAATGGAAGTGGA -3' Rev 5'- CTCAGAAAGGGCTTTGATGTACT -3'	133
SERPINB2	Fw 5'-CAGCACCGAAGACCAGATGG-3' Rev 5'-CCTGCAAAATCGCATCAGGATAA-3'	146
SFRP1	Fw 5'-ACGTGGGCTACAAGAAGATGG-3' Rev 5'-CAGCGACACGGGTAGATGG-3'	184
SHISA2	Fw 5'-GGAGACCATCCCCATGATCC-3' Rev 5'-AGCACAGAGAAATTCGTGGGC-3'	198
SLC44A3	Fw 5'- GTCCAAAAGCAGACTCACTGT -3' Rev 5'- GCAAATAGGGAGTAGCACTCAGG -3'	109
SLFN11	Fw 5'-AACCCCAACGCCCGATAAC-3' Rev 5'-TCATGCAAGCATAGCCATAGAG-3'	81
SMOC1	Fw 5'-AGGTCCTACGAGTCCATGTGT-3' Rev 5'-CACTGCACCTGGGTAAAGG-3'	206
STC1	Fw 5'-GTGGCGGCTCAAAACTCAG-3' Rev 5'-GTGGAGCACCTCCGAATGG-3'	248
TCN1	Fw 5'-CCCCTAGTGGGGCTCTTACT-3' Rev 5'-CAGAGGTTTTAGGCGGATGTAG-3'	96
TGM2	Fw 5'-GAGGAGCTGGTCTTAGAGAGG-3' Rev 5'-CGGTCACGACACTGAAGGTG-3'	184
TMEM74B	Fw 5'-AGAGGAGCATGAGACCCATTT-3' Rev 5'-AGGGCGGAAACAAAGCCAT-3'	183
TNFRSF11B	Fw 5'- GTGTGCGAATGCAAGGAAGG -3' Rev 5'-CCACTCCAAATCCAGGAGGG-3'	82
TRPV3	Fw 5'-GGTAACTGTGATGACATGGACTC-3' Rev 5'-ACCAGCAACTCTACCAACTCC-3'	188
WASF3	Fw 5'-AAGGGATTACCAGCGAACTTG-3' Rev 5'-CTTCAGCATGTTTGCTCAGACT-3'	90
VCAN	Fw 5'-GTAACCCATGCGCTACATAAAGT-3' Rev 5'-GGCAAAGTAGGCATCGTTGAAA-3'	110
WDR72	Fw 5'-TGATGACCAGCGAACGATTGT -3' Rev 5'-CACGCACTGTCCATTGGTG-3'	223
ZFPM2	Fw 5'-ATGTCCCGGCGAAAGCAAA-3' Rev 5'-AGCTCAGATTTTCAGGCCCAA-3'	169

ZNF415	Fw 5'-TGCCTGAACTCTACACAGAGG-3' Rev 5'-AGTTACGAGACAGATCCAGGG-3'	88
ZNF516	Fw 5'-GGACCCGGTCAACAGCTAC-3' Rev 5'-GGCCCCGTACTTGAGGTACT-3'	85
ZPLD1	Fw 5'-AACCTCCACAGTAGATTTCCTGC-3' Rev 5'-CCGAGAAAAGTACCGTGCAAA-3'	100
Reference gene		
RPL19	Rev 5’-CCATGAGAATCCGCTTGTTT-3’ Fw 5’-AGGCACATGGGCATAGGTAA-3’	