Androgen Receptor as a Determinant of Targeted Drug Resistance in Melanoma

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Androgen receptor as a determinant of targeted drug resistance in melanoma

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Abstract

Metastatic melanoma is the most aggressive form of skin cancer. Activating $BRAF^{V600}$ mutations contribute to approximately 50% of melanomas. Specific BRAF/MEK inhibitors (BRAF/MEKi) rapidly regress patient tumors. Yet nearly all patients relapse within the first two years. Most BRAFi/MEKi-resistant tumors are also cross-resistant to immunotherapies. This highlights the urgent need to develop approaches to prevent and circumvent resistance. We have recently shown that suppression of androgen receptor (AR) activity in a panel of melanoma cells promotes cellular senescence and suppresses tumorigenicity. Here we find AR signaling drastically increased in BRAFi-resistant (BR) melanoma as well as in the sensitive treatment-naïve melanoma cells shortly after BRAFi exposure. Increased AR expression is sufficient to drive a BRAFi-resistant-like state in melanoma cells, eliciting TGF-B and EGFR transcriptional programs of BR melanoma subpopulations and driving elevated *EGFR* and *SERPINE1* expression of likely clinical significance. Pharmacological inhibition of AR blunts changes in gene expression, depletes melanoma cell growth, and reduces tumorigenicity of BR melanoma cells while enhancing MHC-I expression and CD8+ T cells infiltration. As such, our results point to the importance of AR signaling in the development of BRAF/MEKi resistance and to the use of AR inhibitors to mitigate this process.

Résumé

Le mélanome métastatique est la forme la plus agressive de cancer de la peau. Les mutations activatrices BRAFV600 sont à l'origine d'environ 50 % des mélanomes. Les inhibiteurs spécifiques de BRAF/MEK (BRAF/MEKi) font rapidement régresser les tumeurs des patients. Cependant, presque tous les patients rechutent au cours des deux premières années. La plupart des tumeurs résistantes à BRAFi/MEKi présentent également une résistance croisée aux immunothérapies. Il est donc urgent de développer des approches pour prévenir et contourner la résistance. Nous avons récemment montré que la suppression de l'activité du récepteur des androgènes (AR) dans un panel de cellules de mélanome favorise la sénescence cellulaire et supprime la tumorigénicité. Ici, nous constatons que la signalisation AR augmente considérablement dans les mélanomes BRAFi-résistants (BR) ainsi que dans les cellules de mélanome sensibles naïves de traitement, peu après l'exposition au BRAFi. L'augmentation de l'expression de l'AR est suffisante pour conduire à un état BRAFi-résistant dans les cellules de mélanome, déclenchant des programmes transcriptionnels TGF-B et EGFR dans les sous-populations de mélanome BR et conduisant à une expression élevée de l'EGFR et de la SERPINE1, dont l'importance clinique est vraisemblable. L'inhibition pharmacologique de l'AR atténue les changements dans l'expression des gènes, ralentit la croissance des cellules de mélanome et réduit la tumorigénicité des cellules de mélanome BR tout en augmentant l'expression du CMH-I et l'infiltration des cellules T CD8+. Ainsi, nos résultats soulignent l'importance de la signalisation AR dans le développement de la résistance BRAF/MEKi et l'utilisation d'inhibiteurs AR pour atténuer ce processus.

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List of Abbreviations

3βHSD2	3β-hydroxysteroid dehydrogenase 2
17βHSDs	17-β-hydroxysteroid dehydrogenases
AF	activation functions
AIS	androgen insensitivity syndrome
ANOVA	analysis of variance
AR	Androgen receptor
AR OE	AR overexpressing lentivirus
AREs	androgen response elements
BR	BRAFi-resistant
BRAF/MEKi	BRAF/MEK inhibitor
BUS	B-upstream segment
CAFs	cancer-associated fibroblasts
DAB	Dabrafenib
DBD	DNA-binding domain
DDX3Y	DEAD box 3, Y-linked
DHEA	dehydroepiandrosterone
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
EREs	estrogen response elements
FDA	Food and Drug Administration
GEO	Gene Expression Omnibus
GO	Gene ontology
GR	glucocorticoid receptor
GSEA	Gene Set Enrichment Analysis
HRE	hormone-response elements
HSP	heat shock proteins
IC50	half-maximal inhibitory concentration
IFN	interferons
IGF1-R	insulin-like growth factor receptor
JNK	Jun N-terminal kinase
LBD	ligand-binding domain
LH	luteinizing hormone
LSH	follicle-stimulating hormone
MAPK/ERK	mitogen-activated protein kinases/extracellular signal-regulated kinases
MHC I	major histocompatibility class I

MITF	microphthalmia-associated transcription factor
MR	mineralocorticoid receptor
MSigDB	Molecular Signatures Database
NSG	NOD scid gamma
p450c17	cytochrome P450 17A1
P450scc	cytochrome P450 side-chain cleavage enzyme
PDXs	Patients Derived Xenografts
PFA	paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PR	progesterone receptor
PREs	progesterone response elements
RPMI	Roswell Park Memorial Institute
RQ	relative quantification
RT	room temperature
RT-qPCR	Real-time quantitative PCR
SEER	Surveillance, Epidemiology, and End Results Program
SMC	starved-like
SOX10	SRY-box transcription factor 10
SRY	sex-determining region Y
StAR	steroidogenic acute regulatory
SULT2A1	sulfotransferase
TCGA	The Cancer Genome Atlas
TLR	toll-like receptor
TME	tumor microenvironment
TRA	Trametinib
TSPO	translocator
UNDIF	undifferentiated melanoma cells
UNDIF-NC	neural crest melanoma cells
URPP	University Research Priority Program
URPP	University Research Priority Program
UTY	ubiquitously transcribed tetratricopeptide repeat containing, Y-linked
UV	ultraviolet
WHO	World Health Organization
ZFY	zinc finger protein, Y-linked

Introduction

1. Sex Differences

In the last three decades, clinical and preclinical research has identified sex differences as a major contributor to morbidity and mortality worldwide [1]. Differences between males and females originate at the genetic, epigenetic, and sex hormonal levels and influence various aspects of behavior, health, and disease [1] (Fig.1). Biological sex influences behavior leading to more aggressive or caring phenotypes [1,2]. In its turn behavior has an impact on lifestyle choices and lifestyle choices contribute to disease risk [1]. As such, the elevated risk of cancer among males has been historically linked to risk factors such as diet, exposure to environmental toxins or carcinogens, and risky behaviors like smoking and drinking alcohol [1,3]. Although these differences may cause distinct epigenetic changes that affect gene expression and cellular processes, they alone cannot account for the sex-based differences in cancer risk [1,3-5]. As even after adjusting for these risk factors, adult women still exhibit greater overall protection against cancer than men [3]. For this reason, it is important to gain a deeper understanding of cellular and molecular mechanisms underlying these sex differences. Biological sex guides and differentially affects multiple biological processes, including the stem self-renewal potential, immune response, microbiome composition, cell metabolism, and brain development [1,3-8]. Similarly, sex differences lead to disparities in the prevalence, manifestation, and treatment response of many acute and chronic diseases between males and females [1,8,9]. For instance, research has shown that while women are more prone to autoimmune disorders like lupus and multiple sclerosis, men are more likely to get specific types of cancer, such as prostate and liver cancer [3,8,10]. In addition, even though women and men may experience similar health conditions such as heart disease and stroke, women often face unique symptoms and complications [1,8]. Biological sex differences are complex and multifactorial, and understanding them is critical

for improving health outcomes and developing personalized treatment approaches that consider the unique needs of both sexes.

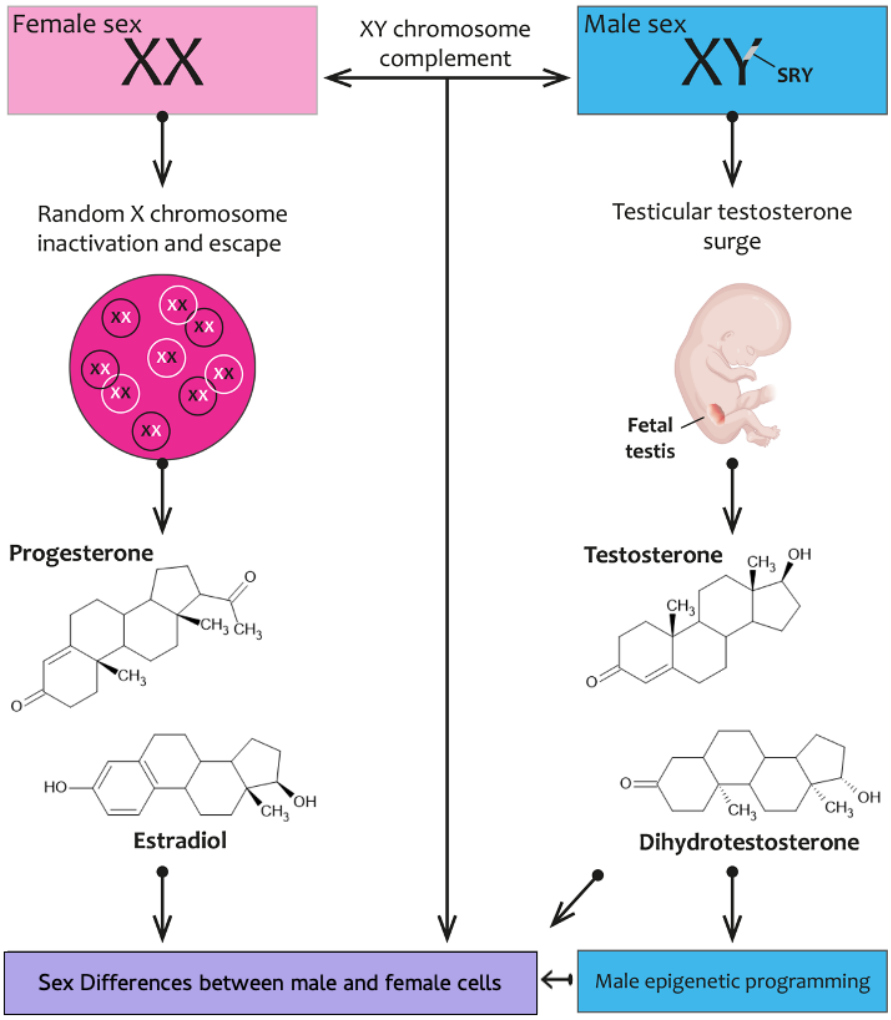

Figure 1. Molecular causes of sex differences. First layer of sex differences comes from a genetic make-up of male and female cells carrying either XY or XX chromosome compliments. Second layer is random X chromosome inactivation and X-linked escapees present in all female cells. In male cells, Y-encoded *SRY* gene instructs testis development. Testicular testosterone surge at the end of first trimester of pregnancy drives broad epigenetic reprogramming in males. Last layer of sex differences is circulating sex hormones. Males and females have significantly different sex hormone levels throughout their lifespan.

1.1.Genetic Sex Differences

The genetic differences between males and females originate at the time of conception with an embryo carrying XY or XX chromosomes, respectively [1]. While the X and Y chromosomes

share some homologous regions, the Y chromosome also carries genes that are unique to it and do not have a homologous copy on the X chromosome [1,11] (Fig.1). For example, the SRY (sex-determining region Y) gene is essential for male sex determination during embryonic development. Other genes on the Y chromosome, such as DDX3Y (DEAD box 3, Y-linked), ZFY (zinc finger protein, Y-linked), and UTY (ubiquitously transcribed tetratricopeptide repeat containing, Y-linked), have counterparts on the X chromosome (DDX3X, ZFX, and UTX, respectively), but exhibit subtle functional differences [1]. For instance, a recent study showed DDX3X and DDX3Y to regulate translation via distinct separation behaviors, suggesting that these two RNA helicases might have different effects on the translation of overlapping mRNA targets and the regulation of distinct RNA components [12]. Besides translation, X and Y homologs are involved in various biological processes, such as chromatin remodelling, gene transcription and protein-protein interactions[13-16]. Thus, the subtle differences in these homologs may have global consequences in various cellular processes.

On the other hand, X chromosome contains more than 1,000 genes responsible for both sex-specific and general physiological functions that affect both sexes [8,17-20]. It is inherited differently between males and females with males only carrying one maternally-inherited copy and females carrying both the maternal and paternal copies of the X chromosome. To minimize the differences in the X-linked gene expression between the two sexes, one X chromosome undergoes X chromosome inactivation early on during female embryogenesis [17-20]. It is a complex multistep process that involves interactions between DNA, RNA, and proteins that lead to the heterochromatinization of the inactive X chromosome also known as the "Barr Body" [18]. As X chromosome inactivation occurs randomly in all female cells, it leads to tissue mosaicism in X-linked allelic expression, which in its turn generates an additional level of sex differences in gene expression [1,18,19]. Nonetheless, X chromosome inactivation is incomplete with up to 15-30% of X-linked genes escaping X inactivation, either in a universal or a tissue-

specific fashion [17,20,21] (Fig.1). Most known escapees lack functional homologs on the Y chromosome and further contribute to sex differences [1,3-5].

Overall, the fundamental differences in the sex chromosome complements create a widespread variation on a molecular level between all male and female cells that not only persist throughout life but also have a detrimental impact on the disease risk and pathogenesis.

1.2.Hormonal Sex Differences

1.2.1. Sex Hormones

Androgens, estrogens, and progesterone are sex hormones produced by the endocrine system that play a crucial role in mediating the proper development and maintenance of sex differences between men and women [7,22-24]. These hormones not only shape the anatomical differences between male and female bodies, but are also major players in immunity, human behavior, and the development of brain structures [1,3,7,23-27]. For example, studies have shown that prenatal exposure to testosterone in males is associated with increased aggression and decreased empathy, while prenatal exposure to estrogen in females is associated with increased verbal fluency and social sensitivity [28-31]. Furthermore, sex hormones continue to play a role in behavior and mood throughout the lifespan. For example, fluctuations in estrogen and progesterone levels during the menstrual cycle can affect mood and cognitive function in women, and reduced testosterone levels in men have been associated with decreased libido, mood changes, and increased risk of depression [32-34].

In conclusion, sex hormones are critical players in mediating sex differences in both sexes, affecting not only physical and sexual characteristics but also cell metabolism, immunity, and brain development (Fig.1). Further research is needed to fully understand the complex

interactions between these hormones and the development of sex differences, as well as their potential implications for health and disease.

1.2.2. Sex Hormone Synthesis Pathways

Testosterone is the primary sex hormone in males, while estrogen is the primary female sex hormone. Both hormones play a crucial role in the development of sexual organs and secondary sex characteristics. Progesterone is another important sex hormone, which is primarily produced in females during the menstrual cycle and pregnancy [7,22,24,35]. Sex hormone synthesis is tightly regulated and requires a complex feedback system involving various hormones, enzymes, and receptors. In ovaries, estrogen synthesis is stimulated by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland. LH also stimulates androgen production in the male testis, and progesterone production in the adrenal glands and female ovaries [22,24,36]. Importantly, both males and females produce all these sex hormones, albeit at significantly different levels [7].

The initial synthesis of sex hormones is the earliest and perhaps the most crucial step which gives rise to the differences in the sex hormone levels between males and females. Sex hormone synthesis is achieved through steroidogenesis, a complex process involving the conversion of lipid cholesterol into an active steroid hormone [22]. Both in males and females, cholesterol serves as a single precursor for biochemical reactions, involving multiple enzymes and several cellular organelles, that eventually lead to the production of desired sex hormones in a specialized cell of a specific tissue [22,24,36]. Any defective or failed enzymatic reaction during this process would generate an excess of one of the sex hormones and a shortage of the other/s. This could have detrimental consequences on the various physiological processes, including the regulation of the menstrual cycle, bone health, and cardiovascular health [37-40].

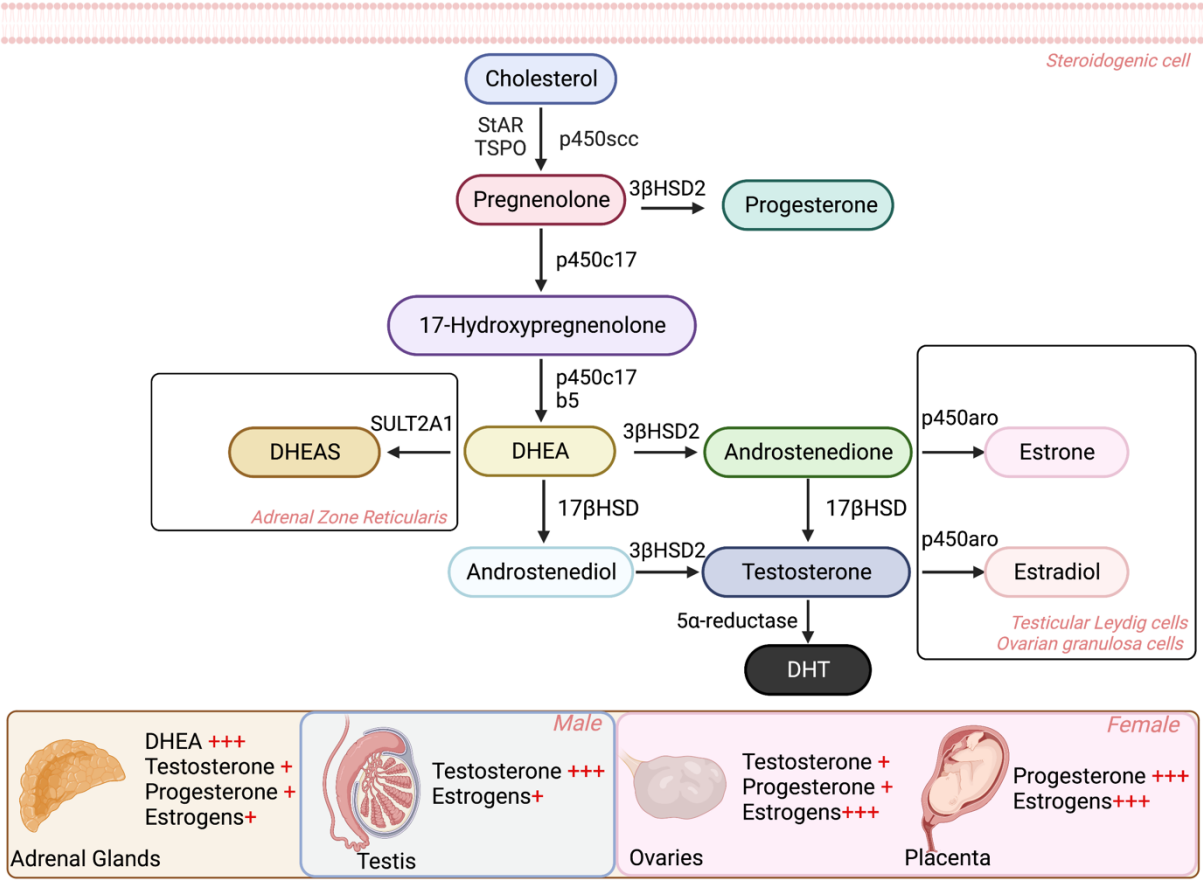

Figure 2. Major steroidogenic pathways of sex hormones in the adrenal glands, ovarian and testicular tissues. The synthesis of sex hormones is initiated in the adrenal zona reticularis, testicular Leydig cells and ovarian theca cells by converting cholesterol into pregnenolone. Pregnenolone can either be converted by 3βHSD2 to progesterone or it can get converted to DHEA via two oxidation reactions. In the adrenal zona reticularis, SULT2A1 converts DHEA to DHEAS. In testicular Leydig cells and ovarian theca cells, DHEA molecules get converted to testosterone molecules by 3βHSD2 and 17βHSD. P450 aromatase converts testosterone and androstenedione to estrogens in Leydig cells and granulosa cells. Steroidogenic cells produce different levels of sex hormones, with DHEA mostly produced by the adrenal grands, testosterone in male testis, progesterone in placental tissue and estrogens both by the ovaries and placenta. *Created using BioRender.com (2020).*

Therefore, it is important to understand the complex interplay between estrogens, androgens, and progesterone during steroidogenesis from cholesterol.

As a first step of steroidogenesis, intracellular cholesterol gets transported inside the inner mitochondrial membrane by steroidogenic acute regulatory (StAR) proteins and translocator (TSPO) proteins (Fig.2). Then, it gets cleaved at the aliphatic tail by the cytochrome P450 side-

chain cleavage enzyme (P450scc, *CYP11A1* gene) [22]. This reaction leads to the production of pregnenolone, which is a common intermediate of all steroidogenic pathways. Pregnenolone can then be converted to progesterone by 3β-hydroxysteroid dehydrogenase 2 (3βHSD2). Alternatively, pregnenolone can enter the endoplasmic reticulum (ER), and get oxidized to 17-hydroxypregnenolone by cytochrome P450 17A1 (p450c17, *CYP17A1* gene) and then to 19-carbon androgen precursor dehydroepiandrosterone (DHEA) by p450c17 and cytochrome b5 (b5) [22,24,36]. These sequential reactions occur in all steroidogenic cells, such as adrenal zona reticularis cells, testicular Leydig cells, and ovarian theca cells [22] (Fig.2), and mutations in any of these enzymes or transporters lead to abnormalities in development. As such, StAR and *CYP17A1* mutations lead to congenital adrenal hyperplasia, while 3βHSD2 deficiency was associated with adrenocortical insufficiency and genital ambiguity [41-43]. These clinical cases highlight the essential nature of these enzymes and their requirement not only for steroid hormone synthesis but also for the appropriate physiological development of males and females.

In the adrenal gland, cytosolic sulfotransferase (SULT2A1) sulfates DHEA to DHEAS, which then gets secreted into the circulation. DHEA are weak androgens, which get converted to testosterone or to estrogens in the testis, ovaries, or other non-reproductive organs, such as the brain or the adipose tissue (Fig.2). 17-β-hydroxysteroid dehydrogenases (17βHSDs) catalyze the conversion of DHEA to androstenediol, androstenedione to testosterone, and estrone to estradiol in different tissue [22]. Testicular Leydig cells produce DHEA and then directly convert it to testosterone via androstenedione and androstenediol intermediates (Fig.2). Testosterone can be further reduced by the 5α-reductase to yield 5α-dihydrotestosterone (DHT), which is a more biologically active form of testosterone. Alternatively, testosterone can also be aromatized to yield estradiol in several male adult tissues, including the testis, adipose tissue, brain, and bone [22,24]. In ovaries, the complete synthesis of estrogens is divided

between the granulosa and theca cells. Theca cells produce testosterone and androstenedione, which then get taken up by granulosa cells to produce estradiol and estrone (Fig.2). The enzymes expressed by granulosa cells responsible for this conversion are 17βHSD and P450 aromatase. Progesterone gets produced in female ovaries from pregnenolone during the luteal phase of the cycle [22,24,36].

In conclusion, androgens, estrogens, and progesterone are critical players in mediating sex differences in both sexes. Steroidogenesis drives the sustained production of sex hormones from cholesterol molecules, both in the gonads and in the adrenal glands. Further research is needed to fully understand the complex interactions between these hormones, abnormal steroidogenesis, the development of sex differences, as well as their potential implications for health and disease.

1.2.3. Lifespan-associated Changes in Sex Hormones

Testosterone, estrogen, and progesterone levels are not constant throughout a life of a male and female [7,44,45]. In males, the earliest testosterone level surge takes place at the end of the first trimester of pregnancy. This surge in testosterone drives gene expression programs and tissue architecture via epigenetic remodeling in multiple organs to permanently masculinize the developing embryo [1]. During early childhood, both girls and boys undergo "minipuberty" due to a rapid and transient rise of LH and FSH shortly after birth [46-48]. This rise in gonadotrophins increases testosterone levels in boys and estrogens in girls during the first 6 months of life [46]. The biological significance and the exact role of minipuberty remain unclear, but it could potentially play a role in future reproductive health and disease. Throughout childhood, androgen and estrogen levels remain low until they start escalating at adolescence [46-48]. At the end of puberty, androgen synthesis in males reaches its maximum and remains relatively constant until the fourth decade of life. In adult women, estrogen and progesterone

levels pulsate due to different phases of the menstrual cycle ranging from 30-800 ug/day for estrogens and 1,000-24,000 ug/day for progesterone [7,22,46]. Nonetheless, adult males and females differ significantly in the levels of estrogens and androgens. The total free testosterone range for an adult man is between 9 to 12 ng/dl, while for a woman it ranges between 0.3-0.7 ng/dl on average [7,44,45]. Estradiol concentration range between 25-54 pg/ml and 30-800 pg/ml for men and women, respectively [7]. These differences in circulating sex hormones persist throughout adulthood and start to decline only with age.

Aging is a natural process that leads to changes in multiple organs and systems. One of the major features of aging is a decline in physical function, which is often attributed to the loss of skeletal muscle mass [34,49,50]. However, the role of hormones in regulating muscle metabolism is often overlooked. Hormones, such as androgens and estrogens, play a key role in human muscle metabolism and their decrease with aging may be responsible for muscle loss, weakness, and decreased functional performance [49,51]. Studies reported that the levels of circulating testosterone and DHEA begin to decline in males around 40-50 years old of age [44,51]. Testosterone levels drop by 1-2% annually, while DHEA reduce by 2% on average [44]. This age-related decline is primarily due to testicular dysfunction and due to a decreased responsiveness of the hypothalamic-pituitary-testicular axis [44,49,51]. Changes in the levels of sex hormones similarly occur in aging females. During the first few years of menopause, 80% of circulating estrogens are depleted [49,51]. Testosterone and DHEA also decline in females with age, with the steepest decline observed during early reproductive years [45]. These changes in sex hormone levels directly contribute to the aging process by affecting metabolism, muscle, and bone mass in aging individuals in a sex-specific manner.

Overall, sex hormone levels fluctuate throughout the lifespan of males and females. Hormonal surges occur in several stages of life and directly affect epigenetic memory, cell

metabolism, and organ tissue development [1,34,45,51]. These surges can potentially intensify the male-female sex differences beyond the level of the sex chromosomes or the sex hormone levels. Thus, it is important to consider these fluctuations as well as the age-related changes in hormones as potential modifiers of physiology or even as critical players of pathogenesis. Future studies should address the relationship between these changes and the development of cancer, cardiovascular, neurodegenerative, and metabolic diseases in both sexes.

1.3.Sex Hormone Receptors

1.3.1. Structural Differences and Similarities of Sex Hormone Receptors

Once sex hormones are synthesized and released into the circulation, their actions are mediated by and are dependent on the presence of steroid hormone receptors in target tissues [22]. The signaling of sex hormones, like estrogen, progesterone, and androgen, is similar in a way that it involves the binding of a specific steroid hormone to a specific receptor, resulting in changes in gene expression and cellular responses [22,24,52-54]. However, there are also important differences in the specific mechanisms and functions of each receptor and its signaling pathway.

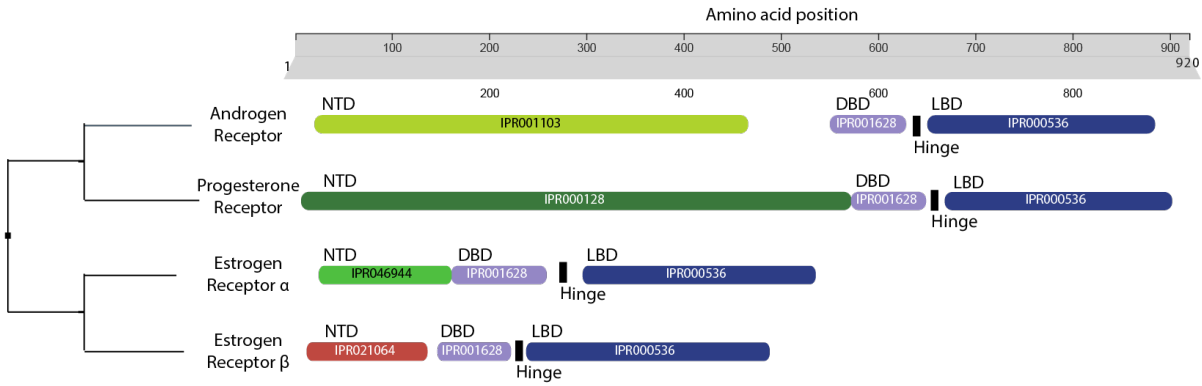

Figure 3. Structural differences and similarities of sex hormone receptors. All sex hormone receptors consist of four functional domains: NTD, DBD, hinge and LBD. To determine the degree of structural similarity, amino acid sequences of AR, PR, ERα and ERβ were analyzed using Clustal Omega. The hierarchical clustering of the proteins is depicted by

a dendrogram (left). The proteins were then aligned and analyzed using InterProScan. The annotated domains along with their identifier codes are depicted with colored boxes. Each colored box stands for a unique domain. Ruler on top represents amino acid position and guides the distribution of different domains.

Androgen receptor (AR), estrogen receptor (ER), and progesterone receptor (PR) are all members of the nuclear Class I receptors superfamily [22,35,52-54], which was suggested to have evolved due to gene duplications and exon shuffling from a common ancestor nearly 500-800 million years ago [55,56] (Fig.3). As a result, AR, ER, and PR have a similar overall structure consisting of 4 major functional domains: an NH2-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) [54,56-59]. Moreover, all nuclear hormone receptors contain two activation functions (AF) domain: AF-1 is located inside NTD, and AF-2 is in embedded in LBD. AFs serve as surfaces for interactions of sex hormone receptors with their co-activators and co-repressors [54,56-58,60,61]. The overall sequence homology was also confirmed with using a multiple sequence alignment Clustal Omega tool (Fig.3). The tool revealed that AR and PR share the largest sequence homology, when compared with ERs. NTDs of different steroid hormone receptors bear the least sequence homology (15%) when compared to other regions [54]. NTDs contribute to transcriptional transactivation, and AR is highly dependent on its NTD [54,58,62]. The DBD facilitates the binding of the nuclear receptor to the chromatin and facilitates the homodimerization of two nuclear receptors. Across different steroid hormone receptors, DBD is approximately 80 amino acids long and comprises two zinc finger structures (Fig.3). The zinc fingers allow the direct binding of sex hormone receptors to gene promoters and enhancers [63]. Strikingly, AR, PR, glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) contain nearly identical DBD residues [64]. However, each receptor recognizes its unique sequence. AR is able to bind to specific DNA sequences known as androgen response elements (AREs), while the estrogen and progesterone receptors bind to specific DNA sequences known as estrogen response

elements (EREs) and progesterone response elements (PREs), respectively [7,22,54,58]. The exact mechanism of sequence recognition remains unknown, although several hypotheses have been put forward. For example, the higher affinity of AR to the AREs was suggested to come from the two amino acid substitutions in the DBD, which lead to a markedly stronger dimer-DNA interface [58,65]. The hinge region separates DBD from LBD, and it varies in length between different nuclear receptors. It plays a role in nuclear import, export, and DNA transactivation [66]. LBD consists of a twelve α-helical sandwich with a core pocket that permits the binding of a specific ligand to its receptor. Upon binding, the ligand-binding pocket becomes closed, and a hydrophobic cleft is formed on the receptor's surface. The hydrophobic cleft is recognized by the coactivators of sex hormones [52,57,61,65,66]. Despite overall structural similarities, there are also important differences in AR, ER, and PR, which deserve further attention.

Estrogen receptor (ER) has two receptor isoforms, ERα and ERβ, which are encoded by chromosome 6's *ESR1* gene and by chromosome 14's *ESR2* gene, respectively [53]. ERα and ERβ are found in a wide variety of tissues throughout the body. ERα is mostly expressed in the uterus, ovaries bones, white adipose tissue, kidney, liver, and breast [53,57,59,67,68]. Meanwhile, ERβ is found in the male reproductive system, central nervous system, cardiovascular system, immune system, lung, colon, and kidney [53,68,69]. Structurally, ERβ has a shorter NTD than ERα leading to different DNA transactivation properties [53]. DBDs of two receptor isoforms are nearly identical and are predicted to therefore bind estrogen-response elements (ERE) sequences with similar affinity and specificity [53,59,67]. LBDs of two receptors share 56% sequence homology [59]. Interestingly, the ligand binding pockets of ERα and ERβ are only different by two amino acids: Leu384 and Met421 in ERα are substituted by Met336 and Met421 in ERβ, respectively. These differences may be responsible for differences in the binding and affinity of different ER agonists and antagonists [70]. In addition, ERβ has a more

compact ligand binding pocket, and sequence differences in the other parts of LBDs alter the binding of various coactivators to ERα and ERβ [53,70].

Progesterone receptor (PR) also has two receptor isoforms, PR-A and PR-B, which are encoded by the same gene [61]. The two different isoforms are derived from the differential gene promoter usage. PR-A has truncated NTD, while PR-B is a full-length protein. PR-A misses the first 164 amino acids from exon 1 of PR, which are referred to as the B-upstream segment (BUS) [71,72]. Not surprisingly, BUS contributes to the major differences observed in promoter selectivity, transcriptional activity, and coregulator binding preferences between the two PR isoforms. PR-A and PR-B are mostly co-expressed in reproductive tissues, such as the uterus and mammary gland [25]. Progesterone binding to these receptors leads to changes in gene expression and cellular responses, including the regulation of cell proliferation and differentiation [73]. Progesterone receptor signaling is essential for the maintenance of pregnancy, and progesterone is often used as a therapeutic agent in reproductive medicine [61,71-75].

Lastly, androgen receptor (AR) signaling is mediated by a single receptor isoform, AR. AR has an extensive NTD, which constitutes approximately half of the protein size. NTD contains polymorphic polyglutamine (CAG) and polyglycine (GGC) repeat sequences, which vary in length in the human population [54,58]. The CAG and GGC repeated sequences are unstable and originate due to strand-slippage replication and recombination [76]. Variations in CAG repeat lengths have been shown in Caucasian, Asian, and African populations, and are associated with human neurodegenerative disorders and cancers [77-79]. The average length of CAG repeats is estimated between 9-36 repeats, whereas in pathological conditions like Kennedy disease it ranges between 38-62 repeats [76]. Meanwhile, an increased risk of prostate cancer is associated with fewer CAG repeats [80]. The length of the repeats significantly modulates AR function, with

individuals having shorter polymorphic repeat regions having increased AR transactivation function [77-79]. One study has shown that the deletion of polyglutamine repeats increased AR activity four-fold [81]. Outside of polymorphic repeats, NTD is structurally disorganized, and this structural plasticity of NTD provides a platform for multiple protein-protein interactions, that are of paramount importance for AR activity [54,58,65]. The NTD's AF-1 is flanked by two transcription activating units (tau), namely tau-1 and tau-5. These units are crucial for AR transactivation, as they enable the ligand-dependent interdomain NTD-LBD interactions [65]. Recent structural studies revealed that AR dimer binds to AREs in a head-to-head and tail-to-tail fashion. The two DBDs and LBDs form a core of the ARE-AR complex, while the two NTDs wrap around LBDs to form intimate contact with each other [82]. Mutations in the DBD affect AR binding to DNA and lead to complete or partial androgen insensitivity syndrome (AIS) [83]. Mutations in the sequence of LBD have also been identified and similarly lead to AIS and to cancer. In the first case, loss of function mutations lead to a reduced ligand binding affinity, accelerated ligand-receptor dissociation, or prevent the ligand-dependent dimerization [58]. On the other hand, LBD mutations found in prostate cancer expand the ligand-binding repertoire of AR by allowing the binding of additional molecules such as estrogens, adrenal androgens, and glucocorticoids [54,58,62,84] .

In summary, AR, ER, and PR share many structural similarities, but they also have distinct differences that reflect their specific physiological roles and hormone-binding preferences. The largest structural diversity comes from the NTDs of different sex hormone receptors. The intrinsically disorganized NTD structure offers each sex hormone a range of unique and flexible conformations, which provide binding surfaces for a great variety of activators, repressors, and non-coding RNAs. The plethora of actions of the circulating sex hormones is driven by specialized sex receptors in target tissues. Genetic alterations affecting gene expression, structure, or function of steroid hormones lead to a great number of diseases

affecting sex development, fertility, neurodegeneration and even promoting cancer development. Future research is needed to better understand the mechanisms driving the cell- and tissue-specific expression of sex hormone receptors, both in physiological as well as pathophysiological conditions.

1.3.2. Signaling of Sex Hormone Receptors

The signaling of sex hormone receptors can be broadly divided into direct genomic, indirect genomic, and hormone-independent signaling pathways [53,54,58,62,67]. Similar to sex hormone synthesis pathways and to the sex hormone receptors structures, there are a lot of commonalities and evolutionary conservation in the overall signaling of AR, ER, and PR. For this reason, the following chapter will focus on the AR signaling pathways and the parallel will be drawn to ER and PR signaling.

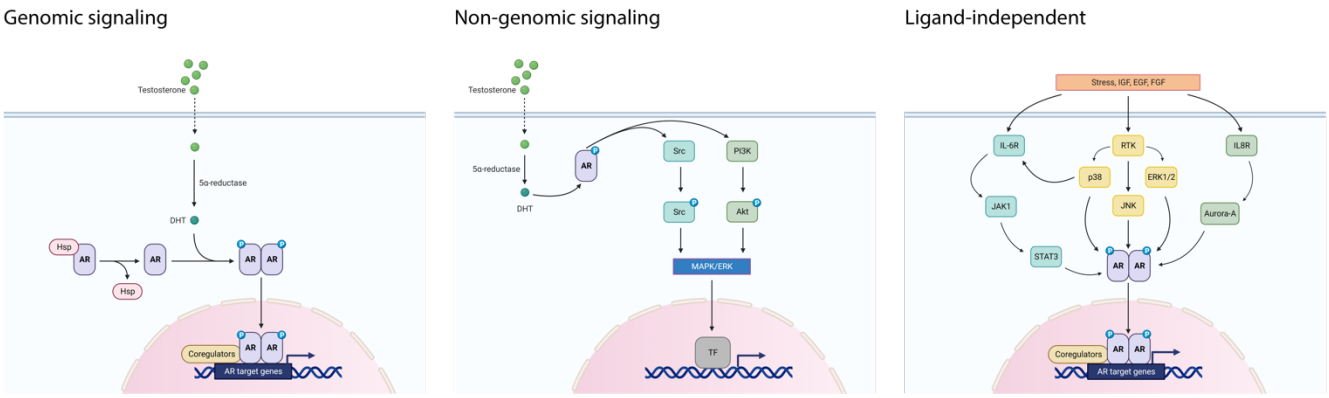

Figure 4. AR signaling pathways. Direct genomic AR signaling is initiated upon DHT binding to the receptor (left). HSP detaches, two receptors dimerize to translocate into the nucleus, and transcription of target genes is initiated. Non-genomic AR signaling is initiated following DHT binding (center). Active AR form facilitates PI3K and Src activation to promote transcription. Ligand-independent signaling is initiated in the absence of the ligand (left). AR phosphorylation is promoted by other signaling pathways, including STAT3, p38, JNK, ERK, and Aurora-A. Active AR dimers translocate into the nucleus and induce transcription of downstream targets. *Created using BioRender.com (2020).*

Direct genomic AR signaling is initiated once the ligand binds to the receptor [54]. In AR inactive state, the hinge region containing NLS is bound by the heat shock proteins (HSP) HSP-90, HSP-70, or HSP-56 that sequester AR in the cytoplasm [62] (Fig.4). Upon testosterone or DHT binding, a conformational change in the receptor is induced that drives LBD helices 3, 4, and 12 to form the AF-2 surface [58]. Nuclear receptors utilize AF-2 to bind coactivators containing LxxLL-motives. However, AR does not bind coactivators via the LBD, it rather favors the binding of NTD containing FxxLF motif [54,58,85]. Once the HSPs dissociate from AR, the NLS surface becomes exposed to importin alpha, and the two AR molecules dimerize. The dimerized complex translocates into the nucleus where it can bind to the promoters or other gene regulatory elements of target genes via hormone-response elements (HRE) and ARE sequences [58].

Indirect genomic or nongenomic action of steroid hormone receptors often encompasses the generation of second messengers, and activation of various signal transduction cascades, such as ion fluxes, cyclic AMPs, and protein kinase pathways [25,35,54,67,84,86-90] (Fig.4). While the genomic signaling pathway is considered a classical route for the activation of sex hormone receptors, it might take several hours before the successful response reaches its peak and the transcriptional targets get transcribed [53]. In stark contrast, the action of androgens, estrogens, or progesterone might be more effectively and rapidly transmitted via the nongenomic signaling pathways. As such, the binding of androgens leads to the association of AR to the Src kinase and Phosphoinositide 3-kinase (PI3K), which then mediates mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) cascade to promote cell proliferation and survival [89]. In prostate cancer cells, upregulation of phospho-ERK can be observed within the first 5-30 minutes following the androgen exposure [90]. Similarly, ER signaling leads to the activation of multiple downstream signaling pathways, including the MAPK and PI3K pathways to regulate gene expression and cellular responses [91]. It has been

shown that 30% of ER binding sites lack ERE sequences further supporting that ER also mediates transcription via indirect genomic signaling [92]. The nongenomic signaling of sex hormones could be fundamental under adverse environmental conditions and/or when the ligand is limited, and rapid signal transduction is required to assure cell growth and survival [86].

Hormone-independent or ligand-independent signaling takes place when nuclear hormone receptor signaling is activated in the absence of a specific ligand [35,84,93] (Fig.4). Mechanistically, ligand-independent signaling can be initiated either due to the phosphorylation of specific residues in nuclear receptors or due to interaction with individual coregulators [35,52-54,84,91,93,94]. NTDs of AR, ER, and PR contain multiple phosphorylation sites accessible for binding of MAPKs [25,95,96] and CDKs [95,97,98]. AR and PR also contain additional phosphorylation sites in the hinge [25]. The phosphorylation site on the sex hormone receptor has a dramatic influence on its transcriptional activity, target gene binding affinity, protein stability, and localization [25,95,97,99]. For instance, AR phosphorylation at serine 81 and serine 213 residues in castration-resistant prostate cancer leads to the upregulation of different target genes, including genes involved in oxidative damage response, proliferation, and aldosterone synthesis [99]. Meanwhile, phosphorylation of AR at serine 650 residue by stress signaling p38 and Jun N-terminal kinase (JNK) cascades compromises AR transcription by increasing AR nuclear export [100]. The crosstalk between MAPK and the AR-AR coactivator pathways has also been experimentally demonstrated to drive the expression of androgen-induced genes even in testosterone-depleted conditions. Specifically, HER2, a member of the epidermal growth factor receptor (EGFR) family, promotes AR transactivation via the MAPK signaling leading to the downstream AR interaction with its coactivators, such as ARA70 and ARA55 [101]. Importantly, the crosstalk of sex hormone receptors with MAPK signaling drives forward powerful feed-forward signaling loops that can cause persistent, ligand-independent sex hormone activation [25]. This is especially crucial for the maintenance of sustained biological

responses that are relevant to cancer cell growth and metastatic dissemination. Ligand-independent signaling of ER has been shown to be an important driver of hormone-independent and resistant breast tumors. Experimentally, EGFR, HER2, and insulin-like growth factor receptor (IGF1-R) have been shown to activate ER to regulate breast cancer cell proliferation [35]. Similarly, PR and GR ligand-independent signaling has been shown to contribute to the growth and survival of cancer cells, especially in the hormone-depleted environments [25].

In summary, estrogen, progesterone, and androgen receptor signaling share some similarities, but there are also important differences in their specific mechanisms and functions. These differences reflect the unique roles that each signaling pathway plays in regulating gene expression and cellular responses in different tissues throughout the body. On a transcriptional level, each signaling pathway induces a unique battery of genes involved in the development and maintenance of sexual characteristics [25,35,52-54]. On non-genomic and ligand-independent signaling axes, the 3D protein structure and amino acid sequence of each receptor allow a variety of post-transcriptional modifications, which lead to distinct protein stability, cellular localization, coactivator engagement, and target gene transcription [35,52-54,67,88]. One crucial unresolved issue is that the identity of the proximal kinases for many sites of sex hormone receptors remains unknown. Furthermore, the roles of specific residue phosphorylation and its interplay with other phosphorylation sites remains limited both in normal physiology as well as in the disease state [95]. Lastly, sex hormone receptors are subjected to additional modifications such as acetylation, ubiquitination, sumoylation, and methylation [53,102]. The contribution and crosstalk of these numerous post-translational modifications during genomic, non-genomic, and ligand-independent signaling and in the context of sex differences remain an unresolved issue, which should be investigated in the future.

1.4.Impact of Sex Chromosomes, Sex Hormones, and Sex Hormone Receptors in Immunity

Sex differences are vast and span a range of genetic, epigenetic, and sex hormone actions[4]. Not surprisingly, sex is an important biological determinant of immunity [1,8]. Epidemiological data support that sex differences are evident in multiple aspects of immunity, including autoimmunity, response to pathogens, and cancer immunity [8]. The female sex is consistently associated with more robust immune responses towards many viruses, including influenza, HIV-1, and herpes simplex viruses [103]. Moreover, both clinical and preclinical data demonstrate that the number and activity of innate immune cells, including monocytes, macrophages, and dendritic cells, as well as inflammatory immune responses, are higher in females compared to males [4,5,8,10,103,104]. As a result, these differences contribute to the disparities in the disease outcomes observed between males and females. Specifically, recent studies of COVID-19 patient cohorts showed that males had a significantly higher risk of being admitted to the intensive care unit and had increased mortality rates compared to females [8,104-106]. Autoimmune diseases exhibit a different pattern than infectious diseases, as they tend to affect females at a higher rate [8,10]. On average, women are 2-3 times more likely to experience autoimmune conditions, including scleroderma, multiple sclerosis, rheumatoid arthritis, and others [10]. As a matter of fact, 80% of autoimmune patients are females [8,10,27]. Sex differences are also hypothesized to mediate improved immune surveillance and enhanced immune editing in females during multiple stages of tumorigenesis [4,5,7,8]. The sex dimorphism in immunity is a result of a multifaceted interplay between genetic, epigenetic, and sex hormone mechanisms.

On the genetic level, the X chromosome encodes a great number of genes involved in T cell activation, cytokine signaling, toll-like receptor (TLR) pathways, and antiviral type I

interferons (IFN), such as *TLR7*, *TLR8*, *CXCR3*, *CD40LG*, *IL13RA1*, and *FOXP3* [8,10,27,107,108]. Although some of these immune-related genes, such as *CXCR3* and *FOXP3,* are transcriptionally silenced in females through X chromosome inactivation, others escape this process and lead to a female-biased expression [17,20]. The immune cells exhibit a stronger tendency for female-biased gene expression, and some of the X chromosome escapees are associated with an increased risk of scleroderma, systemic lupus, and Sjögren's syndrome [10,109]. For instance, *TLR7* encodes for a single-stranded RNA sensor and is a variable escapee with a most pronounced sex-biased expression in plasmacytoid dendritic cells, monocytes, and B cells [10]. TLR7 stimulation promotes the induction of genes related to antiviral type I interferon (IFN) responses [110], which play a crucial role in maintaining inflammatory responses by promoting the maturation of monocytes into dendritic cells [110,111]. This, in turn, enables the activation of CD4+ T cells, which can assist in the differentiation of autoreactive CD8+ T cells and B cells into effector cells [111]. The extent to which TLR7-driven signaling contributes to autoimmunity remains uncertain, as the sex hormones could also play equally important in this regard. However, lupus and scleroderma are rarely reported in patients with monosomy X (XO, Turner's syndrome), while individuals with Klinefelter's syndrome (47, XXY) or trisomy X (47, XXX) are at an increased risk of developing of these conditions compared to individuals with a normal chromosome count of 46, XX or 46, XY [8,10,108].

On the level of sex hormones, activity, differentiation, and expansion of both adaptive and innate immune are governed by the endogenous and exogenous sex hormone levels [7,8,10,26,104,112-114]. For example, several studies have shown that the estradiol-ER signaling axis regulates the activation of IFN and TLR signaling in plasmacytoid dendritic cells [115,116]. Mechanistically, estradiol significantly enhanced IFN-α and TNF-α production by pDCs in response to TLR9 and TLR7 stimulation via the intrinsic ERα signaling. This effect was specific to the plasmacytoid dendritic cells and not to other innate immune cells [115,116].

Importantly, plasmacytoid dendritic cells from postmenopausal women had a diminished response to TLR stimulation compared to premenopausal women [115]. Furthermore, another study demonstrated a trend toward a lower frequency of IFN-α–producing plasmacytoid dendritic cells in postmenopausal women compared to premenopausal women [117]. Meanwhile, the testosterone-AR signaling axis has been shown to affect T cell differentiation, exhaustion, and cytokine production [112-114,118]. Specifically, AREs are found in the promoter and enhancer elements of multiple immune-related genes, including *FOXP3* and *AIRE* [8,119,120]. Upon testosterone binding, AR was reported to bind to the *FOXP3* gene locus and induce epigenetic changes driving differentiation and maintenance of regulatory T cells [119]. Moreover, androgen-driven upregulation of AIRE in the thymic epithelial cells assures more effective negative selection self-reactive T cells in the thymus [120]. Cumulatively, these findings suggest that female and male sex hormones play seemingly opposite roles in immune regulation, with female hormones promoting immune cell activation and inflammation while male hormones promote self-tolerance. Hence, a balance between male and female hormones is crucial in determining an individual's susceptibility to immune-related disorders.

Overall, the observed fundamental differences in innate and adaptive immune responses between males and females are influenced by various factors, including sex chromosomes, sex hormones, and sex hormone receptors. Additionally, the close interplay of these factors is likely to further contribute to the variations in immune responses observed between males and females [4,5,8]. The genetic differences between males and females, determined by the X and Y chromosomes, impact the expression of immune-related genes. Meanwhile, sex hormone receptor-mediated epigenetic modifications, such as DNA methylation and histone modifications, have a varying effect on immune cell function between the sexes. Lastly, sex hormones themselves, such as estrogen and testosterone, regulate immune responses by controlling the expression of cytokines, chemokines, and other immune-

related factors. These factors collectively contribute to the observed sex differences in immune function and susceptibility to certain infections and autoimmune diseases.

1.5.Impact of Sex Chromosomes, Sex Hormones, and Sex Hormone Receptors in Major Chronic Diseases

The impact of sex on various chronic diseases has become a topic of interest in the last several decades [1]. The well-established genetic and hormonal differences between males and females play a significant role not only in the normal physiological processes but also in pathogenesis [1,3-5,7,8,104]. Sex appears to be a critical determinant in the development and progression of chronic diseases such as cardiovascular disease, neurodegenerative disease, and cancer [1,3-5,7].

Cardiovascular diseases are a leading cause of death worldwide with an estimated 24.2% mortality for males and 21.8% mortality for females [1]. Heart disease and heart failure are major contributors to the observed mortality, and they bear important sex differences. Males are more often affected by obstructive coronary artery disease, while females suffer more from coronary microvascular dysfunction [1]. The sex differences may be due in part to the protective effects of estrogens on the cardiovascular system and inflammatory fibrosis [40]. The protective role of estrogens is further supported by the clinical data demonstrating that premenopausal women are protected from heart diseases when compared to age-matched males. These differences gradually disappear in postmenopausal women. Estrogens have been found to reduce the risk of atherosclerosis, thrombosis, and hypertension [1,40,107]. In addition, estrogen has been found to improve the lipid profile and glucose metabolism [121-123], which are important cardiovascular disease risk factors. These protective effects of estrogen may in part explain why women have a lower risk of cardiovascular diseases compared to men.

Neurodegenerative diseases, such as Alzheimer's and Parkinson's, are characterized by the progressive loss of neurons and cognitive decline [124]. Alzheimer's disease is the fifth leading cause of death in women, while in men it is the seventh [1,125]. In Parkinson's disease, men have a greater incidence and higher prevalence of advanced symptoms, such as speech impairment [126-129]. The underlying causes of these diseases are not fully understood, but several studies have shown that sex hormones may contribute to their development and progression [125-131]. Specifically, clinical data highlight the importance of estrogens, as early bilateral oophorectomy and early menopause associate with an increased risk of Alzheimer's disease [1,125.] Estrogens are also suggested to maintain low blood-brain-barrier permeability to sustain brain health [130.] On the other hand, testosterone has been found to have neuroprotective due to its ability to suppress inflammatory cytokines and guard against oxidative stress damage [132].

Cancer is the second leading cause of death globally, with lung cancer being the most common type [1]. The fundamental sex differences in the cancer risk are evident, as men are twice as likely to develop cancer [3-6,9]. The role of androgens and estrogens is well established in reproductive cancer where: the ERα stimulates breast cancer cell growth, and the AR promotes the proliferation of prostate cancer cells. These findings have led to the development of hormone therapies that target these receptors, providing targeted treatment for hormone-sensitive cancers. However, the contribution of sex hormones, as well as the sex chromosomes, expands beyond the reproductive tissues and their role will be discussed in the following sections.

In conclusion, biological sex plays a significant role in various chronic diseases, including cardiovascular disease, neurodegenerative disease, and cancer. The impact of sex hormones and sex chromosomes on disease development and progression is multifaceted and not fully understood. However, it is obvious that these sex differences should be considered in

the diagnosis, treatment, and prevention of chronic diseases. Future research is needed to fully appreciate the molecular mechanisms underlying these differences and to develop and/or adopt new therapies that target sex-specific factors to improve health outcomes in both men and women.

1.6. Impact of Sex Chromosomes, Sex Hormones, and Sex Hormone Receptors in Cancer

According to the World Health Organization (WHO), cancers account for nearly 10 million deaths annually and these numbers are predicted to surge by 47% by 2040 [9] (Fig. 5). The SEER (Surveillance, Epidemiology, and End Results Program) data from 2008–2012 revealed that the excess risk of developing any type of cancer is 20.4% higher in US males compared to US females [133]. Moreover, men are more likely to develop cancer than women across the globe, with most non-reproductive cancers showing a 2:1 male prevalence [1,3,6,133] (Fig. 5A). By 2040, incidence of cancer in men will remain disproportionally higher for most nonreproductive cancers, including leukemias, liver, lung, oesophagus, and stomach cancers. While geographically the incidence rates of cancer vary, the clear sexual bias remains unchanged. The exceptions are meningiomas, thyroid cancers, and lung cancers in non-smokers, which are more prevalent in women [1,3] (Fig. 5A) For oropharynx, larynx, esophagus, and bladder cancers, the male versus female incidence ratios can be higher than 4:1[1]. The overall mortality rates are similarly higher and for men than for women across multiple cancer types and extend beyond the socioeconomic and regional constraints [1,3,7]. Similar to incidence rates, higher mortality rates in men are predicted to persist globally by year 2040 (Fig. 5B). Historically, the higher cancer incidence and mortality in men were attributed to gender constructs and risk behaviors, such as work environment, dietary habits, smoking, and alcohol

consumption [1,3-5]. However, more recent data show that even after adjusting for these risk factors, adult men still have a higher cancer risk than women [3,6].

Sex-specific differences, including sex chromosome complements, incomplete X-inactivation in females, X chromosome escapees, Y chromosome-encoded oncogenes, and chromatin remodeling effects of sex hormones, play a fundamental role in cancer biology [3-8]. All these disparities shape multiple hallmarks of cancer, including growth, metabolism, angiogenesis, and immunity [1,3-5]. For example, the *DDX3X* gene is an X-linked gene responsible for RNA splicing and translation and is found frequently mutated in human cancers [16,134]. Along with other X-linked genes, *ATRX*, *CNKSR2*, *KDM5C*, *KDM6A*, and *MAGEC3,* the *DDX3X* gene was demonstrated to harbor inactivating mutations in male rather than female cancer cells [134]. Mechanistically, loss-of-function *DDX3X* mutations in male patient-derived melanoma lines were shown to reduce proliferation, while increasing cell migration and inducing targeted therapy resistance [15]. These findings suggest that sex-biased mechanisms can alter the course of tumor progression and offer novel resistance mechanisms in cancer patients.

Cancer treatment and prevention can be improved by sex-specific approaches [1,4,5]. For instance, immune checkpoint inhibitors can improve survival rates for men with advanced melanomas more than for women [135]. Sex-specific transcriptomes can help molecularly subtype different cancer types and enhance therapies in a sex-specific manner. Meanwhile, a deeper understanding of sex-specific mechanisms involved in immune escape and metastases formation could guide the development of personalized treatment strategies.

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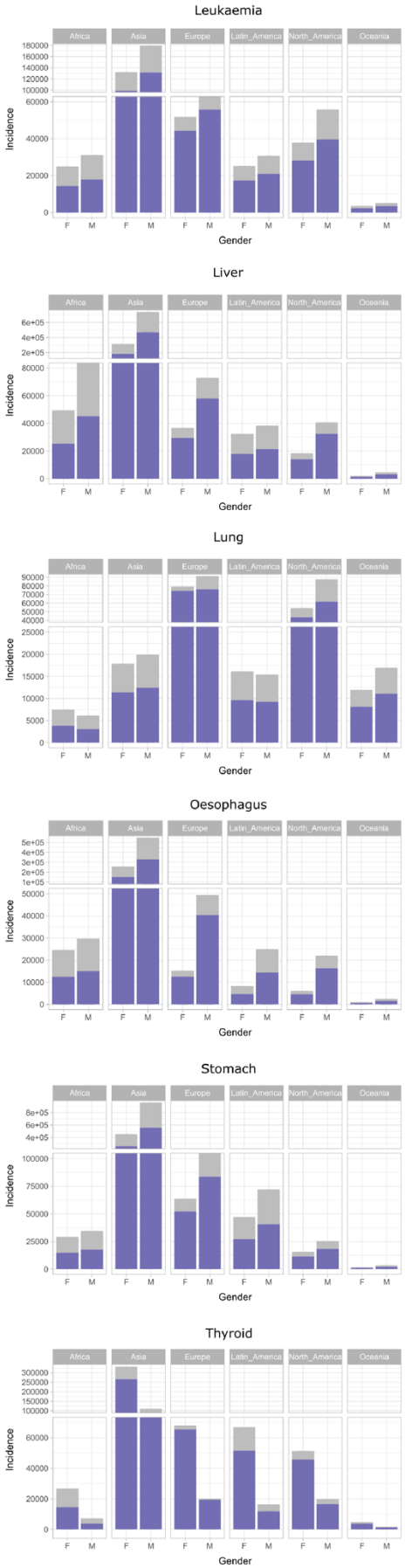
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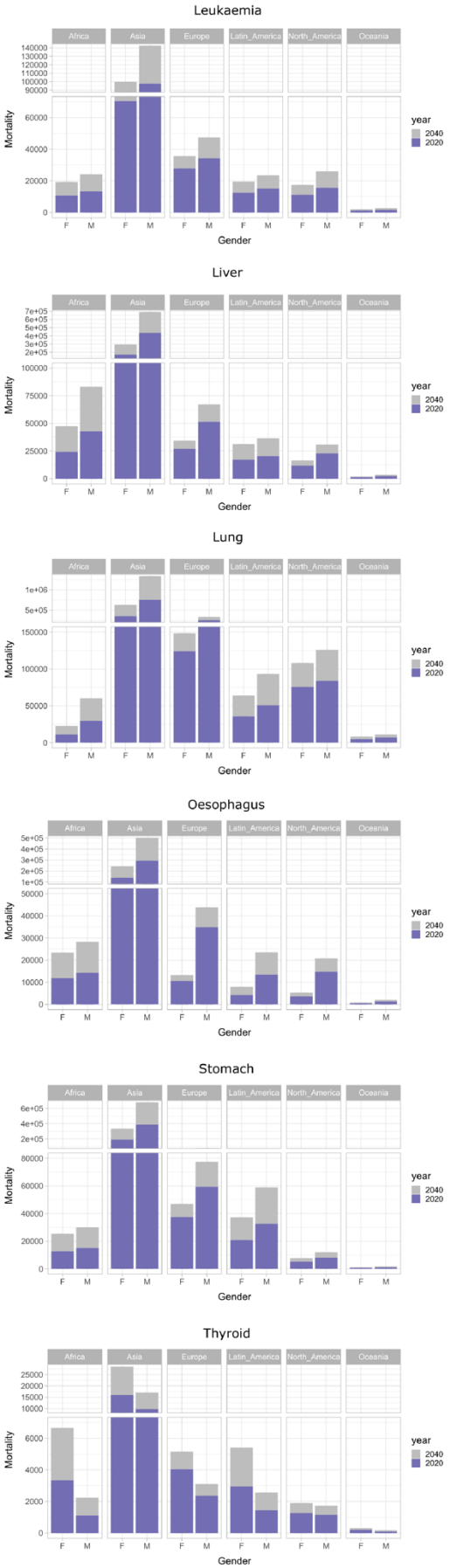
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Figure 5. Incidence and mortality rates of major nonreproductive cancer types stratified by sex for the year 2020 and predicted for year 2040. Incidence (A) and mortality (B) rates of leukemia, liver, lung, oesophagus, stomach, and thyroid cancer occurred in year 2020 and predicted for year 2040. The data are stratified by sex (gender) and geographical region. The data were retrieved from World Health Organization's Agency for Research on Cancer's GLOBACAN 2020 project. The plots were generated using ggplot2 package in R version 4.2.2.

2. Melanoma

2.1.Epidemiology of Melanoma

Melanoma, the most aggressive form of skin cancer, has become increasingly prevalent in the Caucasian population over the last few decades [136]. From 1986 to 2006, White males and females aged 50 years or older experienced an incidence rate increase of 178% and 142%, respectively, in the United States [137]. In year 2020, there were approximately 324,525 new cases and 57,043 deaths from melanoma globally [9]. Europe and North America have the highest melanoma incidence rates, and Europe has the highest mortality rates (Fig. 6). The male sexual bias in melanoma incidence and mortality is mostly and will be observed in Europe, North America, and Oceania (Fig. 6), where cutaneous melanoma is most prevalent [138]. Interestingly, melanoma incidence rate is comparably high between European males and females (76,309 and 74, 318 cases, respectively) in year 2020. However, by year 2040 European males are predicted to experience a 20% increase in newly diagnosed cases, while European females will only experience a 5% increase [9] (Fig. 6A). Compared with other forms of skin cancer, melanoma is rare, but it accounts for most skin cancer deaths [136,138]. The high mortality rates are associated with the high propensity of melanoma cells to metastasize [138,139], where even small tumors (<1mm thick) were shown to metastasize [140].

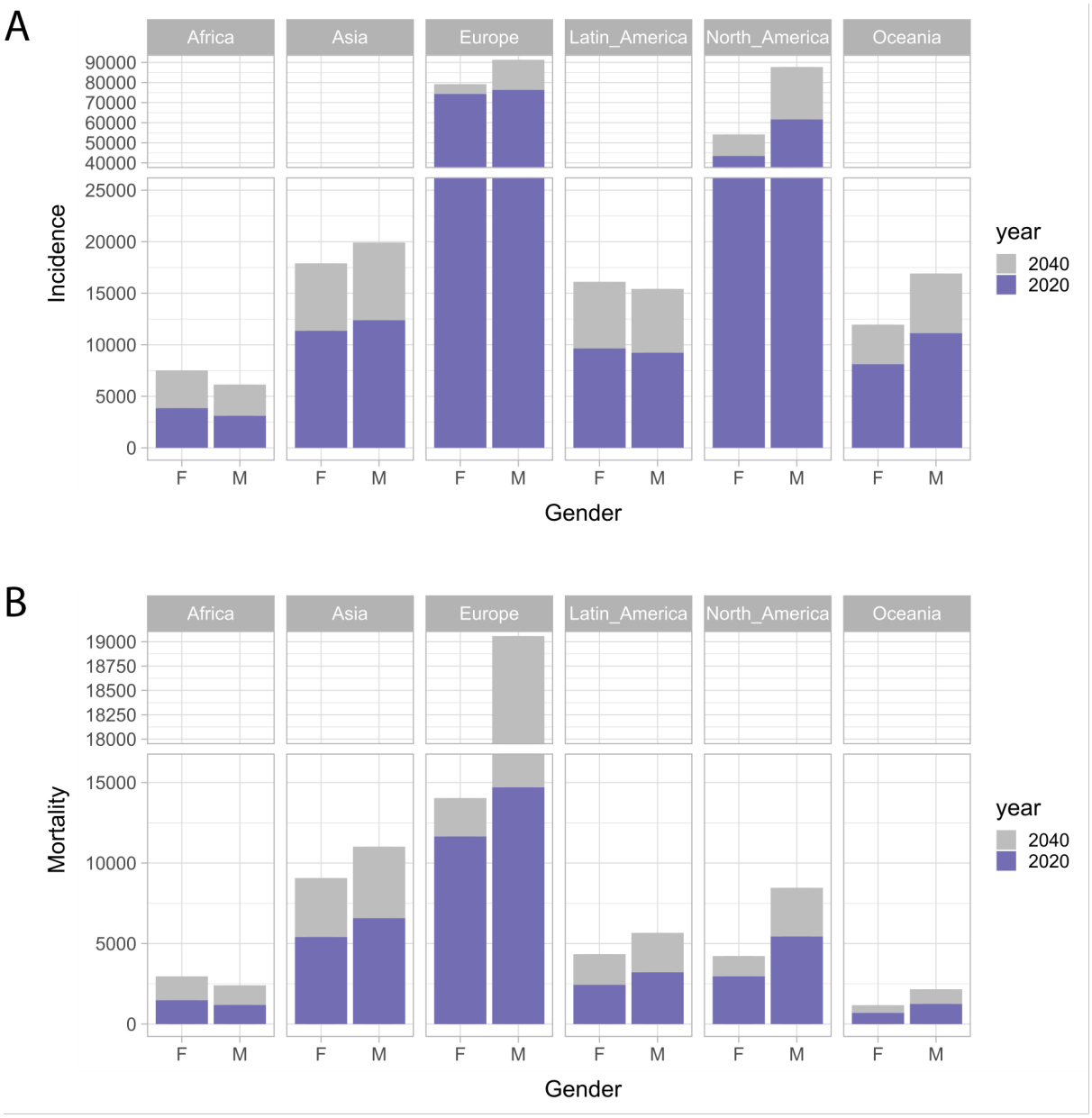

Figure 6. Melanoma incidence and mortality rates stratified by sex for the year 2020 and predicted for year 2040. Incidence (A) and mortality (B) rates in year 2020 and predicted for year 2040 stratified by sex (gender) and geographical region. The data were retrieved from World Health Organization's Agency for Research on Cancer's GLOBACAN 2020 project. The plots were generated using ggplot package in R version 4.2.2.

Cutaneous melanoma disproportionally affects the White population, while mucosal and acral melanoma are more prevalent in Black and Asian populations [138]. In fact, the annual cutaneous melanoma incidence rate is the highest in Australia and New Zealand with 60 diagnosed cases per 100,000, followed by Northern Europe and the United States at the rates

of 30 cases per 100,000 [138,141]. The development of cutaneous melanoma is influenced by several important risk factors, including the number of common nevi and atypical nevi, skin phenotype, family history of melanoma, actinic damage, history of sunburns, and exposure to ultraviolet (UV) radiation, particularly during childhood [141]. Indeed, the increased incidence of melanoma is believed to be associated with significant behavioral changes, such as exaggerated tanning habits and the use of sunbeds [138]. The Cancer Genome Atlas (TCGA) analysis of cutaneous melanoma revealed a distinct mutational pattern that reflects the misrepair of covalent bonds induced by ultraviolet radiation. As a result, melanomas are among the most hypermutated tumors, with a somatic mutation frequency that exceeds 100 mutations per megabase [142].

The lifetime probability of developing invasive melanoma further stratifies melanoma patients into two age-dependent intervals: 1) comprises individuals aged up to 49 years; 2) individuals from 50 years on [138,143]. Interestingly, the risk of invasive disease is higher for young females than for young males, and it is mostly attributed to the lifestyle differences (i.e., use of sunbeds) [138]. Meanwhile, in aged individuals this trend is reversed, whereby 1 in every 38 males and 1 in every 88 women will develop aggressive melanoma [143]. Clinically, the most frequent body sights of cutaneous melanoma are the back in males and the lower extremities in females [138]. Remarkably, women have a survival advantage compared to men at all stages of the disease [144,145], although the reason for this is yet to be fully understood.

To summarize, cutaneous melanoma represents a prime example of non-reproductive cancer driven by sex differences in incidence, aggressiveness, and mortality. The reasons for this bias remain largely unexplored and further research is needed to fully understand the contribution of sex to the multiple stages of melanoma progression. Preventative measures and more effective therapy options are urgently needed for both sexes. Early detection is vital, as

it assures high survival rates for patients with early local disease. Once it has spread, survival rates drop drastically. Although significant progress was made in managing metastatic melanoma, improvement in overall survival rates has been challenging due to the high rate of metastasis and therapy unresponsiveness in advanced disease [136,137].

2.2.Genomic and Nongenomic Diversity of Melanoma

2.2.1. Genomic Classification of Cutaneous Melanomas.

The genomic landscape of cutaneous melanoma is extremely heterogenous and includes mutations the well-known oncogenes and tumor suppressors, such as *BRAF, NRAS, CDKN2A, TP53*, *PTEN, IDH1, NF1, DDX3X,* and *KIT* [146,147]. Despite this genomic heterogeneity, melanomas can be broadly classified into four different subtypes: *BRAF* mutant, *NRAS* mutant, *NF1* mutant, and a triple wild type (including *KIT* mutations). $BRAF^{V600E/K}$ mutations are detectable in approximately 50% of all melanomas [146,147]. *NRAS* hotspot mutations, specifically $NRAS^{Q61R}$ or $NRAS^{Q61K}$, are found in approximately 20% of melanomas, while *NF1* loss-of-function mutations are present in 14% of patients. Lastly, triple wild-type melanomas are characterized by a lack of hotspot mutation in *BRAF, NRAS* or *NF1.* This subtype is extremely heterogenous and includes driver mutations in *KIT, GNAQ, GNA11, CTNNB1,* and *EZH2* genes [146]. Importantly, these subtypes have distinct genomic, molecular, clinical, and pathological features. For example, the *BRAF* mutant subtype is frequently accompanied by MITF amplification, while the *NF1* subtype is characterized by older patients and an increased mutational burden [146] . *BRAF, NRAS,* and *NF1* subtypes are predicted to be sensitive to MAPK inhibition, while the triple wild type is suggested to benefit from the pan-receptor tyrosine kinase inhibition. Despite these differences, the composition of the tumor immune microenvironment seems to be independent of mutation status [146] and reflects the similarities in tumor cell-intrinsic mechanisms shared between all melanomas.

2.2.2. Cell of origin.

Melanoma cells are derived from the melanocytic lineage in the skin [136,148-151]. During embryological development, neural crest stem cells give rise to the melanocytic lineage (Fig. 7A). The neural crest cells possess high migratory potential as they form many tissues and structures during embryogenesis by migration and differentiation of precursors [150,151]. However, which cell type from either the melanocytic or the neural crest lineages gives rise to melanoma remains highly debated. Some studies indicated that melanogenesis is driven by the terminally differentiated melanin-producing melanocytes [152], while others demonstrated that only melanoblasts and neural-crest cells can undergo oncogenic transformation [153,154]. These studies highlight the complex and highly adaptable nature of the melanocytic lineage, and that more mechanistic insights are needed to fully understand the melanocyte differentiation pathways in humans. Differentiation trajectories of melanocytes could offer new therapeutic approaches for pigmentation disorders and melanoma [150]. Indeed, multiple studies have shown a relationship between genes that regulate neural crest/melanocyte development and those that contribute to melanoma [153,155-161]. In recent years, multiple groups demonstrated the intrinsic potential of melanoma cells to interconvert between different transcriptional programs and differentiation states in response to various adverse microenvironmental conditions is signified by the re-expression of multiple neural crest and melanoblast marker genes [157,159-165].

2.2.3. Tumor heterogeneity and dedifferentiation

Melanomas possess a high degree of heterogeneity at both the inter-tumor and intra-tumor levels, meaning that there are differences not only between different tumors but also within individual tumors [138,149,166]. At the genetic level, melanoma cells within different areas of a tumor may have distinct mutations [166], but there can also be nongenetic heterogeneity, which is characterized by the presence of small subpopulations of malignant cells that can resist

cancer treatments [157,160,167-172]. While genetic heterogeneity may provide a selective advantage for melanoma cells in a certain stress condition, it cannot fully account for the metastatic dissemination and therapy resistance [173]. On the other hand, nongenetic heterogeneity allows melanoma cells to strive in continuously changing tumor microenvironment via transcriptional plasticity and epigenetic remodeling [136,173]. In fact, this phenotypic plasticity closely resembles the differentiation of stem cells, where multiple transcription factors and chromatin remodelers act in concert to modify the epigenetic landscape [136,150,151,158,173].

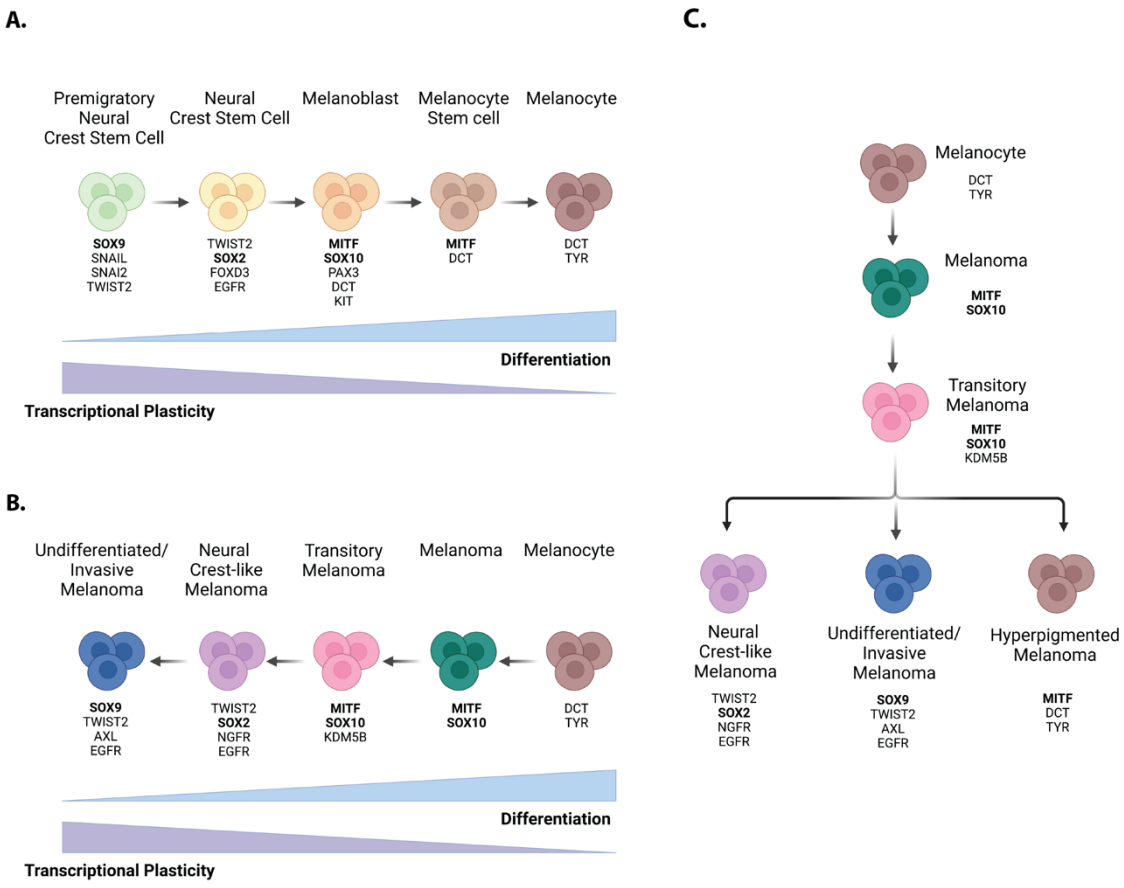

Figure 7. Proposed paths of melanocyte differentiation, melanoma dedifferentiation and tumor heterogeneity. A) Melanocyte differentiation pathway from premigratory neural crest stem cell involves a cascade of intermediate cell states, including more differentiated neural crest stem cell, melanoblast, melanocyte stem cell and finally a terminally differentiated melanocyte. Each cell state is defined by a defined set of marker genes and specific level of transcriptional plasticity. B) Melanoma cell dedifferentiation involves a similar set of cell states to enter an undifferentiated/invasive cell states characterized by expression of stem cell-like markers of neural crest lineage and enhanced transcriptional plasticity. C) Distinct melanoma cell subpopulations can also emerge from a common transitory melanoma cell, which can give rise to neural crest-like, undifferentiated and hyperpigmented melanoma cells. In this scenario,

all cells are described to have high degree of transcriptional plasticity. Markers shared during melanocyte differentiation (A), melanoma dedifferentiation (B) and disctinct melanoma subpopulations are shown in bold. *Created using BioRender.com (2020).*

Nongenetic tumor heterogeneity is mediated through the presence of different cancer cell subpopulations, and it is propagated through dedifferentiation. Cancer cells tend to hijack developmental pathways and dedifferentiate [136,159,161]. The tendency of melanoma cells to dedifferentiate can be attributed to the neural crest origin of melanocytes [136,173]. In fact, interesting parallels can be drawn between the differentiation of neural crest cells into melanocytes and melanoma cell dedifferentiation (Fig. 7A, B). Firstly, pigmented melanoma cells share several similarities with melanoblasts, as they express crucial transcription factors of melanocytic lineage [136,150,153,161]. One of these factors is the microphthalmia-associated transcription factor (MITF), a master regulator involved in melanocyte differentiation and cell cycle regulation. The MITF expression is altered in melanoma [136,138,149,150]. On a genetic level, *MITF* gene amplification ranges between 4-21% of all melanomas [138]. Another crucial reprogramming factor that primes neural crest for melanocyte differentiation is SRY-box transcription factor 10 (SOX10) [150]. Similarly, it is implicated in the progression of melanoma and in giant congenital nevi [138,149,150,153,154,161]. In melanoma, SOX10 and MITF expression control multiple transcription programs that converge on several biological processes, including cell differentiation, pigmentation, proliferation, and cell survival [149,164,174-176]. Secondly, SOX2 is highly expressed by neural crest cells, where it has been shown to suppress the SOX10 and MITF expression [150]. SOX2 expression is limited to neural crest-like melanoma cells, but its function remains largely unexplored [161]. At the same time, SOX2 is a well-established marker of neural stem cells, where it has been shown to limit the activity of repressive chromatin regulatory complexes and maintain a permissive epigenetic state [177]. The possibility that SOX2 could similarly mediate a highly plastic epigenetic state in neural crest-like stem cells needs to be further evaluated. Thirdly, SOX9 is a common transcriptional

denominator of pre-migratory neural crest cells and undifferentiated melanoma cells. Single-cell RNA-sequencing studies have revealed that melanoma cells have two distinct phenotypic cell states, one marked by the SOX10 expression and the other by SOX9 expression [178]. Comparable to pre-migratory neural crest cells, SOX9 was enriched in a mesenchymal-like melanoma cell subpopulation. Importantly, this subpopulation was driven by the transforming growth factor beta (TGF-β) signaling [178], which plays an essential role during neural crest differentiation [150,179].

The differentiation of melanocytes is a linear process [150,151,158], whereas dedifferentiation does not appear to follow the same trend (Fig. 7A, C). Studies of treatment naïve melanomas demonstrated that two distinct cell states exist: a proliferative MITF-driven state and a mesenchymal-like state [157,172,178]. Meanwhile, studies of targeted therapy-resistant melanoma cells revealed the existence of additional transcriptional states, including a starved-like transitory state, hyperpigmented and a neural crest-like state [136,160,161,173] (Fig. 7C). Together with the mesenchymal and proliferative states, these states were shown to either follow linear reprogramming similar to melanocyte differentiation [161] or rather to arise as independent drug-tolerant melanoma subpopulations from a common ancestral starved-like melanoma subpopulation [160].

Melanoma tumors exist in a continuous spectrum of cell state characterized by distinct gene expression programs and biological properties. These states include the pigmented state (MITF+ differentiated cells), intermediate state (CD36+ starved-like state), neural crest-like state (SOX2/SOX10 driven cells), and undifferentiated mesenchymal state (SOX9 and AXL high cells) [157,160,161,173,180] (Fig. 7B, C). This phenotypic heterogeneity is due to cell-intrinsic stresses and microenvironmental factors [157], and different dedifferentiated cell states arise as a result of dynamic epigenetic rewiring of the cancer cell transcriptomes [136,157,159-161,167,171,173,180.

Importantly, this phenotypic plasticity and persistent dedifferentiation of melanoma cells cripple the efficacy of targeted therapies, mediate therapy resistance, and may even contribute to poor immunotherapy response rates [136]. To develop efficient therapies for melanoma, a greater understanding of the specific phenotypic states that promote disease progression is required.

2.3.Therapeutic Avenues for Cutaneous Melanoma

2.3.1. Therapies for metastatic melanoma

For patients with primary melanomas, surgical removal is relatively effective [138]. However, surgery offers little benefit for melanoma that has spread to lymph nodes and distant organs. In the past, metastatic melanoma patients had a very poor prognosis with a median survival time between 6-9 months and 3-year survival of 10-15% [181]. Conventional chemotherapeutic agents, like dacarbazine, showed an overall response rate of about 5% [138,181]. This remained a reality for metastatic melanoma patients until the discovery of targeted therapy agents, such as BRAF and MEK inhibitors, and immune checkpoint inhibitors for the treatment of melanoma [136,138]. These therapeutic agents started to receive Food and Drug Administration (FDA) approval as early as 2011. Since then, both targeted and immunotherapies have transformed the cancer therapy landscape not only in melanoma but also in other cancers [136,138]. The introduction of first-generation and second-generation immunotherapies, such as anti-CTLA-4 and anti-PD-1 checkpoint inhibition, along with BRAF and MEK inhibitors, has led to a doubling of the 3-year relative survival rate for distant-stage melanoma from 20.6% to 39.3% over the last decade [9,137,143].

Historically, PLX4032 (vemurafenib, Zelboraf) was the first targeted therapy agent approved for metastatic melanoma in 2011, and GSK2118436 (dabrafenib, Tafinlar) was approved in 2013. Both agents target BRAF and are presently used in the clinic in combination

with MEK inhibitors for metastatic melanomas harboring *BRAF*$^{V600E/K}$ mutations[136,138,182-185]. These agents have an unparalleled initial response rate of 76%, but the response rate is short-lived [183-185]. The 5-year progression-free survival of patients treated with a BRAF/MEK inhibitor (BRAF/MEKi) combination is only 13% [186]. Both clinical and preclinical data demonstrate melanoma heterogeneity and dedifferentiation are major contributors to therapy resistance [136,160,161,170-173,187]. The phenotypic plasticity of melanoma cells is a major challenge in the field, as disease management becomes difficult and elimination of tumors becomes nearly impossible [136]. It is also important to note that approximately 50% of all melanoma patients do not have activating *BRAF*$^{V600E/K}$ mutations, and therefore alternative treatment options are needed for these patients.

Immunotherapy has shown promising results in the treatment of metastatic melanoma [138,182,188]. Clinical trials demonstrated that the immune checkpoint blockade using anti-PD-1 (pembrolizumab, nivolumab) and anti-CTLA-4 (ipilimumab) alone or in combination can significantly extend survival rates in patients with metastatic melanoma. The overall 3-year survival rates are 34% for the ipilimumab, 52% for the nivolumab, and 58% for the nivolumab-plus-ipilimumab combination [189]. Importantly, immunotherapy is effective in metastatic melanoma patients regardless of the tumor's mutational background (e.i., BRAF, NRAS) [182,188,189]. Half of the immunotherapy-treated patients have durable responses, but the lack of therapeutic efficacy in the other half represents yet another significant challenge to the field. The biomarkers of immunotherapy response are largely missing [182].

Lastly, attempts to combine targeted and immunotherapies have been made [190-192], but they did not show a remarkable improvement when compared to the currently adopted treatment regimen. However, these clinical data indicated that the order of first-line therapy matters, as the patients treated with targeted therapy first followed by anti-PD-1 blockade had

an overall response rate of 29.6% compared to 47.8% observed in patients receiving anti-PD-1 first followed by targeted therapy. Overall, both targeted therapies and immunotherapies extend the lifespans of metastatic melanoma patients. However, most melanoma patients do not respond or experience a relapse. Intrinsic and adaptive resistance are notoriously difficult to address. Identification of markers of response as well as the resistance mechanisms is urgently needed.

2.3.2. Sex differences as a determinant of therapy resistance

Epidemiological studies report male sex to be associated with worse clinical outcomes in cutaneous melanoma [9,143-145]. Significant differences in melanoma death rates exist between males and females even after adjusting for well-known variables (age, Breslow thickness, histologic subtypes, anatomical location, and metastatic status)[3,6,137,138]. Sex differences also seem to prevail in therapy outcomes in metastatic melanoma.

Studies of sex differences in immunotherapy yielded conflicting results. While one meta-analysis study found the immunotherapy efficacy to be sex-independent [193], another meta-analysis found males to have a greater magnitude of benefit with immunotherapies [194,195]. While both studies raise interesting points in our understanding of immunotherapy efficacy, important confounding variables, such as the potential biological and physiological differences, were not included in these analyses. Therefore, future studies are needed to further examine the extent of sex differences during immunotherapy therapy.

The male bias toward worse clinical outcomes is evident in metastatic melanoma patients treated with targeted therapy treatments [185,196,197]. Males have significantly shorter progression-free and overall survivals following BRAF/MEKi. Yet, the mechanisms behind this sexual dimorphism remain poorly studied. A recent study indicated that AR might contribute to the observed male-female differences during targeted therapy in melanoma [198].

AR-positive cells were detected in resistant tumors coming from both male and female subjects, thus raising the possibility that AR might facilitate tumor relapse [198]. However, the molecular mechanisms by which AR promotes targeted therapy remain unknown.

A complex interplay between the sex hormone levels and sex chromosomes is likely to play a key role in sex differences seen in melanoma, as well as in other cancer types [3,7]. Sex hormones can differentially alter the immune system, the tumor microenvironment, and cancer cell-intrinsic properties field [1,3-5,199,200]. Interestingly, the earliest observation about melanoma and hormone responsiveness comes from 1980, when it was then suggested to be an androgen-dependent cancer [201,202]. More recently, free testosterone levels were associated with an increased risk of malignant melanoma development in men [203]. Nonetheless, the experimental evidence for the role of androgens during melanoma development and in therapy resistance remains poorly explored.

3. Aims of the Thesis

In the present thesis work, I aimed to assess the translational relevance of AR suppression in the context of BRAF/MEKi resistant melanoma. First, I aimed to identify whether AR signaling was modulated during the development of BRAF/MEKi resistance. For that, I investigated the timeline of AR upregulation during BRAFi treatment, identified the key transcriptional networks altered in BRAFi-resistant (BR) cells and short-term BRAFi-treated melanoma cells, and investigated the downstream targets of AR in BRAFi-resistant (BR) cells. My second objective was to establish the effects of pharmacological inhibition of AR on the pool of BR melanoma cells. For achieve that, I performed long-term treatments of melanoma cells with a combination of BRAFi and AR inhibitors to assess its effects on the pool of drug-tolerant cells. In parallel, I performed treatments of BR melanoma cells with pharmacological inhibitors of AR to assess proliferation, cell death, and expression of BR markers in these cells.

Lastly, I extended my findings to a mouse model where I assessed the efficacy of AR inhibition on tumor growth and immune cell infiltration.

Results

Androgen Receptor is a Determinant of Melanoma targeted drug resistance

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Statement of Contribution

AS, SG, TP, BT, and MM performed experiments and analyzed the results with GPD. MKY, PO, and GC performed the bioinformatic analysis, MPL provided the clinical samples and critical feedback. AS and GPD designed the study and wrote the manuscript.

Specific Contribution

All the experimental data shown in Figure 1, Figure 2, Figure 5, Figure 6 and Supplemental data were generated by AS. Bioinformatic data shown in Figure 3B-E and Figure 4 B,C,H were generated by AS.

Publication Notice

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1. Abstract

Melanoma provides a primary benchmark for targeted drug therapy. Most melanomas with BRAFV600 mutations regress in response to BRAF/MEK inhibitors (BRAFi/MEKi). However, nearly all relapse within the first two years, and there is a connection between pathways involved in BRAFi/MEKi-resistance and poor response to immune checkpoint therapy. We recently showed that androgen receptor (AR) activity is required for melanoma cell proliferation and tumorigenesis. Here we find that AR expression is markedly increased in BRAFi resistant melanoma cells as well as in sensitive cells soon after BRAFi exposure. Increased AR expression is by itself sufficient to render melanoma cells BRAFi-resistant, eliciting transcriptional changes of BRAFi resistant subpopulations and elevated EGFR and SERPINE1 expression of likely clinical significance. Inhibition of AR expression and activity blunts changes in gene expression and suppresses proliferation and tumorigenesis of BRAFi-resistant melanoma cells, enhances MHC I expression and CD8+ T cells infiltration. Our findings point to targeting AR as a possible co-adjuvant approach for the prevention and management of the disease.

2. Introduction

Significant differences exist in melanoma mortality between men and women across all ages and after adjusting for tumor variables (Breslow thickness, histologic subtypes, body site, and metastatic status) [1]. As for sexual dimorphism in other cancer types [2], even for melanoma, differences in sex hormone levels and/or downstream pathways are likely to play a role [3]. Sex hormone signaling can affect cancer susceptibility through multiple intrinsic and extrinsic mechanisms, impacting cancer stem cell renewal, the tumor microenvironment, the immune system, and the overall metabolic balance of the organism [2,4-6]. As early as 1980, it was proposed that differences in androgen levels could help explain the lower survival of male versus female melanoma patients [7]. Recent epidemiological evidence links elevated free testosterone levels in male human populations with a high risk of melanoma as the only other cancer type besides prostate [8].

In our recent work, we have found that the androgen receptor (AR) is heterogeneously expressed in melanoma cells, both at the single-cell intralesional level and among lesions at various stages of the disease [9]. Irrespective of expression levels, silencing of the *AR* gene and pharmacological inhibition of AR activity suppresses proliferation and induces cellular senescence of a relatively large panel of melanoma cells from both male and female patients [9]. AR plays an essential function in this context by bridging the transcription and DNA repair machinery, maintaining genome integrity. In both cultured melanoma cells and tumors *in vivo*, *AR* gene silencing or treatment with AR inhibitors leads to chromosomal DNA breakage in the absence of other exogenous triggers, leakage into the cytoplasm, STING activation, and a STING-dependent pro-inflammatory cascade [9].

In the present study, we have assessed the translational significance of suppressing AR signaling in the context of melanoma response to targeted drug treatments, specifically BRAF inhibitors. ~50% of all melanomas harbor $BRAF^{V600}$ mutations, with >90% of these expressing

the V600E or K amino acid substitutions. Although >80% of patients with $BRAF^{V600E/K}$ melanomas initially respond to highly specific BRAF and MEK inhibitors (BRAFi/MEKi), nearly all patients relapse after seven months to two years [10]. Most BRAFi/MEKi-resistant melanomas are also resistant to immunotherapies [11], with a cancer cell-instructed mechanism that does not depend on selection by the immune system [12,13]. Initial treatment of melanoma patients with BRAFi/MEKi elicits recruitment and activation of immune cells [14], similarly to what we found in mouse xenografts with melanoma cells with *AR* gene silencing or inhibition [9]. In melanomas with acquired BRAFi/MEKi resistance, an opposite modulation of the immune cell response occurs, which can be attributed, in part, to epigenetic/transcriptional regulatory changes that have the potential of being pharmacologically reversed [14].

We show that increased AR expression and activity are part of the response of melanoma cells with $BRAF^{v600}$ mutations to treatment with BRAF inhibitors and that increased AR expression is sufficient to render these cells resistant to these drugs, inducing transcriptional changes of BRAFi resistant subpopulations of likely clinical significance. Conversely, treatment with AR inhibitors suppresses proliferation and tumorigenicity of BRAFi resistant melanoma cells, enhancing CD8+ T cells infiltration. Our findings raise the exciting possibility that targeting AR signaling, which is a routine treatment of metastatic prostate cancer, can also enhance the efficacy of melanoma targeted therapy.

3. Results

3.1. BRAFi treatment induces AR expression in melanoma cells

Acquisition of BRAFi resistance by melanoma cells can be a dynamic process that is induced in culture by the drug treatment [15,16]. Treatment of primary human melanoma cells (M121224) with multistep increases of the BRAF inhibitor Dabrafenib (DAB) resulted in the emergence of cells actively proliferating in the presence of this compound. RT-qPCR and

immunoblot analysis showed substantially increased AR expression already at lower doses (Fig. 1A). A consistent increase in AR expression was found in additional primary and established melanoma cells selected for BRAFi resistance by immunoblot and immunofluorescence analysis as well as RT-qPCR (Fig. 1B, C, Suppl. Fig. 1A). While AR expression was upregulated in all BRAFi-resistant cell lines relative to parental cells, other genes connected with the acquisition of BRAFi resistance, such as *MITF, SOX9, SOX10, ZEB1,* and *ZEB2* [17] were more unevenly modulated (Fig. 1D; Suppl. Fig. 1B, C). Upregulation of AR expression was also found in the clinical setting, by immunofluorescence analysis of matched lesions arising in the same patients before and after BRAFi/MEKi therapy (Suppl. Fig. 2).

AR upregulation may result from chronic BRAFi treatment or be part of an acute response. In fact, pronounced induction of *AR* expression occurred in SKMEL28 melanoma cells as well as other primary and established melanoma cells already by 48 hours of Dabrafenib treatment (Fig. 1E, Suppl. Fig. 1D). *AR* expression was specifically induced in melanoma cells by treatment with Dabrafenib and other BRAF and MEK inhibitors and not inhibitors of other key signaling pathways, such as NF-κB, STAT3, and AP-1 (Fig. 1F, G).

Expression of the *AR* gene can be positively regulated by the CREB1, c-Myc, LEF/ß-catenin, Foxo3a, Sp1, Twist, and SREBP-1 transcription factors [18]. To gain insights into the mechanisms responsible for the induction of AR expression by Dabrafenib, we probed into the transcriptomic profiles of 3 different melanoma cell lines plus/minus treatment with this compound for 48 hours, as considered in greater detail further below. Of the positive regulators of *AR* gene transcription, *FOXO3* and *CREB1* were consistently upregulated in all three melanoma lines by Dabrafenib treatment and *SP1* in two of the three, while others were more unevenly or not modulated (Suppl. Fig. 3A, Suppl. Table 1).

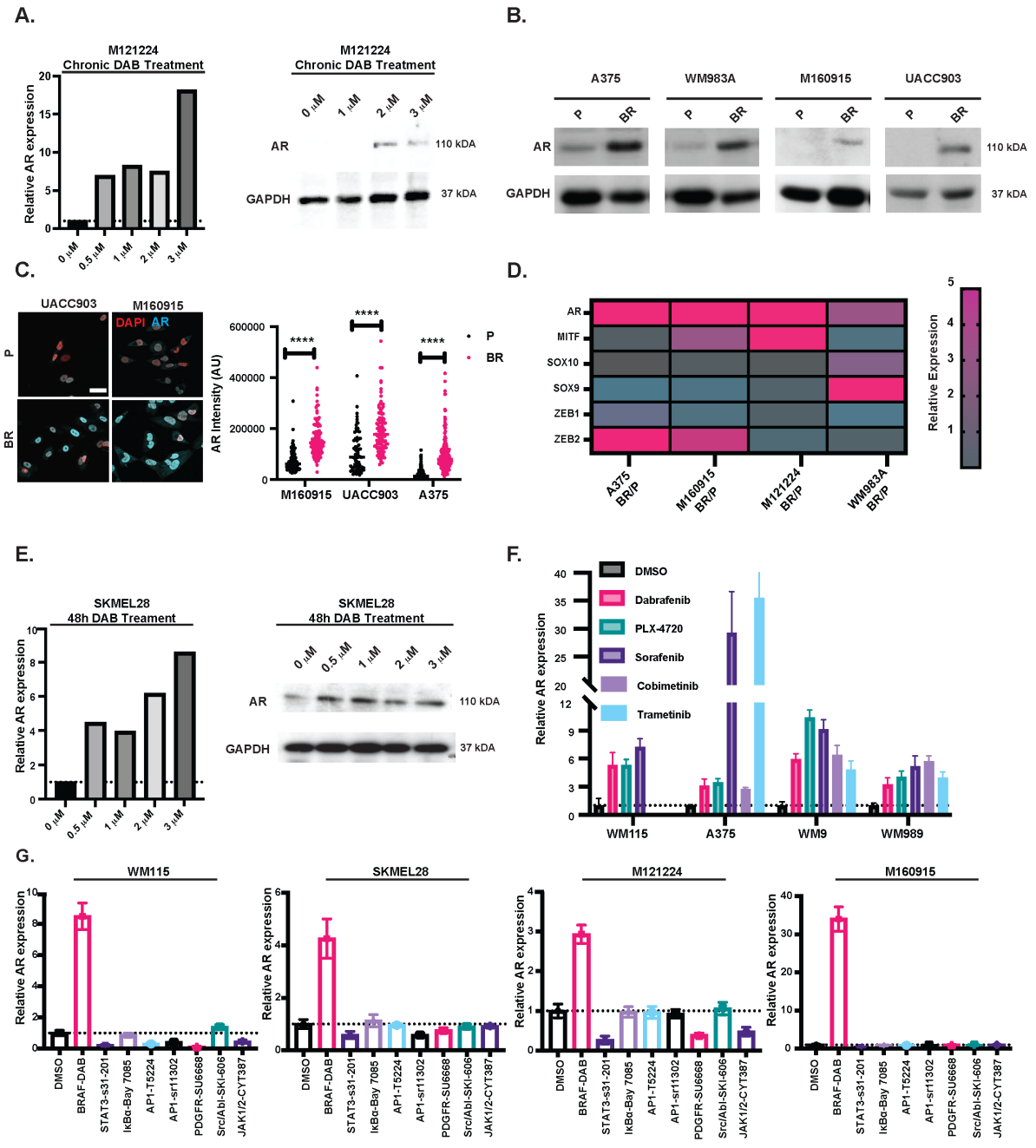

Figure 1: BRAFi treatment of melanoma cells results in increased AR expression

A) RT-qPCR and immunoblot analysis of AR expression in primary human melanoma cells (M121224) cultured with multistep weekly increases of the BRAF inhibitor Dabrafenib (0.5, 1, 2, and 3 μM). Cells were collected at the end of each week of treatment and analyzed together with the untreated parental cells for levels of AR expression by RT-qPCR, with *RPLP0* for internal normalization, and immunoblotting, with GAPDH as an equal loading control. Results of similar independent experiments with additional cell lines are shown in Suppl. Fig. 1A.

B) Immunoblot analysis of AR expression in additional primary (M160915) and established melanoma cell lines (A375, WM983A, and UACC903) selected for BRAFi resistance (BR) by multistep cultivation in increasing amounts of Dabrafenib (up to 3 μM) as in the previous panel,

versus untreated parental cells (P). Immunoblotting for GAPDH was used as an equal loading control.
C) Immunofluorescence analysis of AR expression in primary and established melanoma cells selected for BRAFi resistance (BR) as in the previous panels versus untreated parental cells (P). Shown are representative images and quantification of AR nuclear intensity signal in arbitrary units (AU) per cell (dots) together with a mean, examining >100 cells per sample, unpaired *t*-test, **** $p<0.0001$. Color scale: red, DAPI; cyan, AR. Scale bar: 40μm.
D) Relative expression of the indicated genes in BRAFi resistant (BR) primary (M160915, M121224) and established melanoma cells (A375 and WM983A) versus parentals (P). Results are represented as a heatmap of changes in gene expression as assessed by RT-qPCR analysis with *RPLP0* for internal normalization. Individual bar plots of the results are shown in Supplementary Fig. 1B. Magenta and grey: up- and down-regulated genes, respectively.
E) AR expression in melanoma cells (SKMEL28) for 48 hours with Dabrafenib (0.5, 1, 2, and 3 μM) versus DMSO control. Cells were analyzed by RT-qPCR, with *RPLP0* for internal normalization, and immunoblotting, with GAPDH as an equal loading control.
F) AR expression in the indicated melanoma cells at 48 hours of treatment with various BRAF (Dabrafenib, PLX-4720, and Sorafenib; 0.5 μM) and MEK (Cobimetinib and Trametinib; 5 nM) inhibitors versus DMSO control. RT-qPCR results are expressed as fold changes relative to untreated controls, after *RPLP0* normalization. Results of similar independent experiments with these and additional cell lines are shown in Suppl. Fig. 1D.
G) AR expression in the indicated primary and established melanoma cells at 48 hours of treatment with inhibitors of the indicated molecules/pathways along with corresponding chemical names at concentrations specified in methods. RT-qPCR results are expressed as fold changes relative to untreated controls, after *RPLP0* normalization.

The connection of *FOXO3, CREB1* and *SP1* with *AR* expression was further validated by analysis of the transcriptomic profiles of a large melanoma cohort (TCGA), showing a significant correlation between expression levels of these genes and *AR* (Suppl. Fig. 3B).

Thus, *AR* gene expression is consistently induced in BRAFi-resistant melanoma cells as well as in naïve melanoma cells upon acute exposure to BRAF/MEK inhibitors, with co-regulation by CREB1, Foxo3a, and Sp1 as likely involved.

3.2. Increased AR expression triggers a BRAFi-resistant phenotype

To assess the functional significance of the findings, we infected three different melanoma lines (A375, WM9, and M14) with an AR overexpressing lentivirus (AR OE) versus LacZ-expressing control (CNTRL). In dose-response cell growth assays, the half-maximal

inhibitory concentration (IC_{50}) of Dabrafenib at 72 hours of treatment was drastically increased by AR overexpression in all three cell lines (Fig. 2A). In one-week cell imaging assays (Incucyte), the proliferation of control A375 cells was suppressed by Dabrafenib treatment at all tested concentrations. In contrast, that of AR overexpressing cells was initially reduced, but cultures eventually attained the same density as untreated controls (Fig. 2B). In parallel, Dabrafenib treatment induced cell death to a much greater extent in control than AR overexpressing cells (Fig. 2C).

The findings were expanded by clonogenicity assays. The number of colonies produced by control cells was drastically reduced by Dabrafenib treatment. AR overexpression enhanced the colony-forming ability of cells already under basal conditions and effectively counteracted the decrease caused by this compound (Fig. 2D). Similar protective effects were exerted by AR overexpression in A375 cells treated with Dabrafenib individually and in combination with the MEK inhibitor Trametinib (TRA) (Fig. 2E).

Altogether, the findings indicate that AR overexpression in melanoma cells effectively counteracts growth suppression by BRAF inhibition.

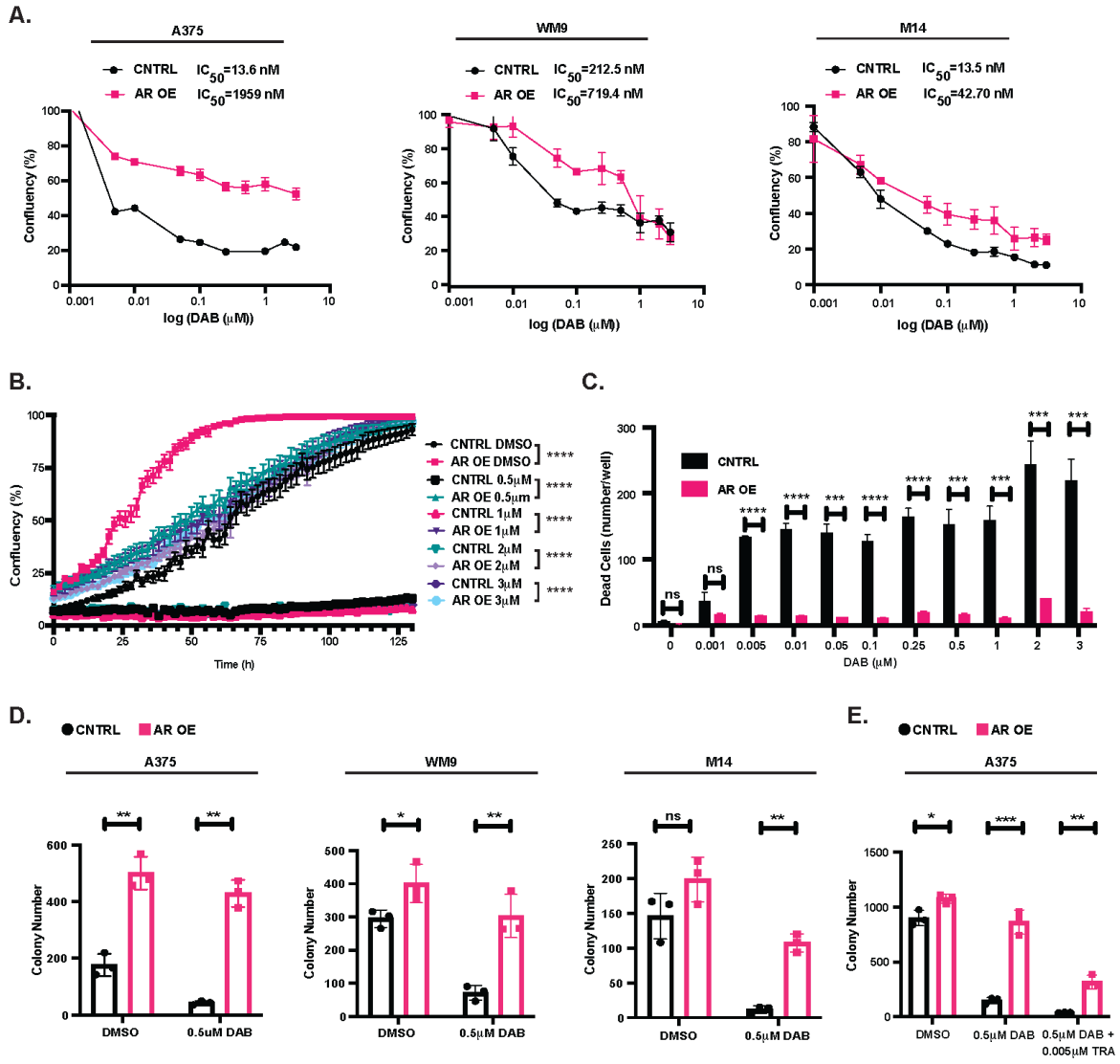

Figure 2: AR overexpression confers BRAFi resistance

A) Cell density assays (CellTiter-Glo) of the indicated melanoma cells stably infected with an AR overexpressing lentivirus (AR OE) versus LacZ expressing control (CNTRL) and treated with the indicated increasing amounts of Dabrafenib for 72 hours. For each condition, cells were tested in triplicate dishes, and results are expressed relative to DMSO control. The calculated IC_{50} for each condition is indicated above.

B) Proliferation by live-cell imaging assays (IncuCyte) of AR overexpressing (AR OE) versus control (CNTRL) A375 melanoma cells (obtained as in the previous panel) cultured with the indicated concentrations of Dabrafenib versus DMSO. Cells were plated in triplicate wells in 96-well plates followed by cell density measurements (four images per well every 4 h for 128 h). cultures, n = 3; Pearson r correlation test. ****, p< 0.0001.

C) BRAFi-induced cell death as detected by live-cell staining (IncuCyte, Cytotox Red) of AR overexpressing (AR OE) versus control (CNTRL) melanoma cells (A375) at 72 hours of treatment with Dabrafenib at the indicated concentrations as in the previous panel. Four images per well cultures, n (cultures) = 3; unpaired *t*-test, ns, non-significant, ***, p< 0.001; ****, p<0.0001.

D) Clonogenicity assays of the indicated melanoma cells transduced with an AR overexpressing lentivirus (AR OE) versus empty vector control (CNTRL) treated with Dabrafenib (DAB, 0.5μM) versus DMSO. Cells were plated in triplicates at clonal density (5000 cells / 6 cm dish) followed by 1-week cultivation. Macroscopically detectable colonies were counted after crystal violet staining. n(dishes)=3, unpaired *t*-test, *, $p<0.05$; **, $p<0.01$, ns, non-significant.
E) Clonogenicity assays of AR-overexpressing versus control A375 melanoma cells as in the previous panel treated with Dabrafenib (0.5μM) individually and in combination with the MEK inhibitor Trametinib (5nM) as in the previous panel. n(dishes)=3, unpaired *t*-test, *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$.

3.3. Increased AR expression perturbs the transcriptional response of melanoma cells to BRAFi.

For mechanistic insights, we undertook a global transcriptomic analysis of the three melanoma cell lines tested above. A large fraction of genes was similarly modulated in control and AR overexpressing cells at 48 hrs of Dabrafenib treatment (Fig. 3A, Suppl. Table 2). Gene families related to cell cycle and DNA replication were commonly downmodulated, consistent with the decreased rate of proliferation that also occurred with AR overexpressing cells at early times of Dabrafenib exposure (Fig. 3B, Suppl. Table 2). By contrast, the mitochondrial pro-apoptotic pathway genes were upregulated by Dabrafenib treatment to a much greater extent in control than AR overexpressing cells, consistent with the differential pro-apoptotic effects (Fig. 3B, Suppl. Table 2).

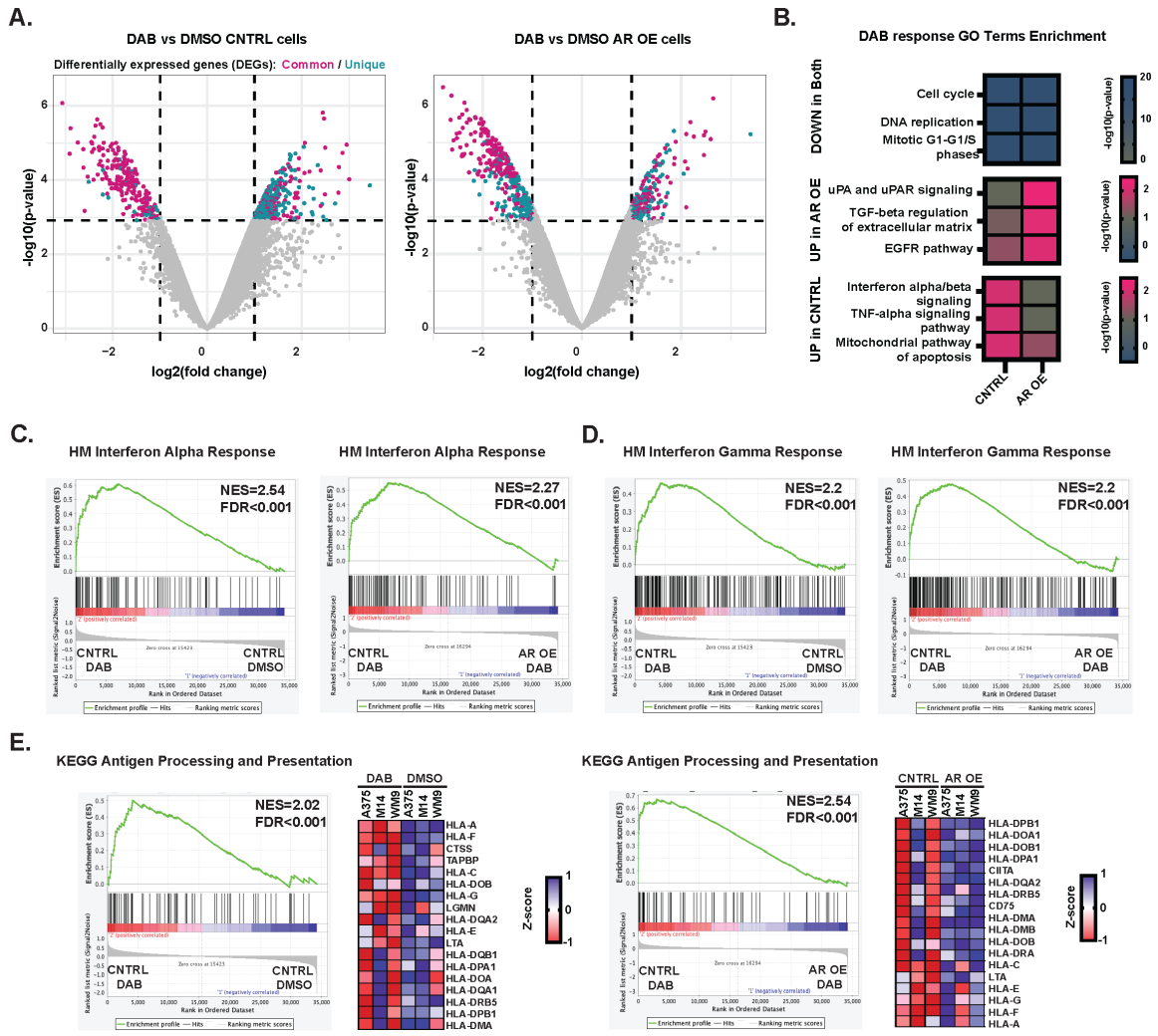

Figure 3: Increased AR expression perturbs the transcriptional response of melanoma cells to BRAFi

A) Transcriptional response of melanoma cells plus/minus AR overexpression to acute BRAFi treatment. Volcano plot of transcriptional changes consistently elicited in A375, M14, and WM9 melanoma cells infected with control (LacZ expressing) (left) or AR overexpressing (AR OE) (right) lentiviruses by 48 hours of treatment with Dabrafenib (0.5 μM) versus DMSO. The x-axis shows the $\log_2$(fold change), and the y-axis shows the $-\log_{10}$(p-value). Each dot represents one gene, with colored dots corresponding to genes with a false discovery rate threshold of < 0.05 and log fold-change threshold of -1 and 1. Magenta and cyan dots correspond to genes similarly and specifically modulated by Dabrafenib treatment in control versus AR overexpressing melanoma cells, respectively. A complete list of modulated genes in the three melanoma cell lines is provided in Suppl. Table 2.

B) Functionally relevant gene ontology families significantly downmodulated by BRAFi treatment in both control and AR overexpressing cells (upper), and gene families modulated only in control (middle) or in AR overexpressing cells (bottom). The $-\log_{10}$(p-value) is indicated by the heatmap color scale. A full list of modulated gene families is provided in Suppl. Table 2.

C-E) Gene Set Enrichment Analysis (GSEA) of transcriptional profiles of control melanoma cells (A375, M14, and WM9) plus/minus Dabrafenib treatment (left panel) and of Dabrafenib-treated control versus AR overexpressing cells (right panel) using predefined gene signatures of interferon alpha (C) and gamma response(D) and antigen processing and presentation (E) derived from the hallmark gene set (HM) [42] and KEGG [43] collections. Genes are ranked by signal-to-noise ratio in Dabrafenib versus DMSO treated melanoma cells; the position of individual genes is indicated by black vertical bars; the enrichment pattern is in green. In (E), GSEA and the leading-edge analysis of the antigen processing and presentation signature are shown in each of the three melanoma lines plus/minus Dabrafenib treatment (left) and of Dabrafenib-treated control versus AR overexpressing cells (right).

Gene families related to pro-inflammatory signaling pathways (interferon α/ß and TNF-α) were significantly induced by Dabrafenib treatment selectively in control cells. Conversely, genes of the EGFR and TGF-ß pathways involved in melanoma progression and targeted drug resistance [15,19] were paradoxically induced by Dabrafenib treatment of AR overexpressing cells, to a much greater extent than in control cells (Fig. 3B, Suppl. Table 2). The findings were expanded by Gene Set Enrichment Analysis (GSEA), showing that signatures of interferon α and γ response were highly induced by Dabrafenib treatment of control cells, with a strong difference in these versus AR overexpressing cells (Fig. 3C, D). Importantly, an antigen presentation gene signature encompassing many major histocompatibility class I (MHC I) genes was also highly enriched in the Dabrafenib-treated control cells, with a profound difference relative to AR overexpressing cells (Fig. 3E).

Thus, increased AR expression in melanoma cells subverts the transcriptional response of melanoma cells to BRAF inhibition, with suppression of pro-apoptotic, immunomodulatory, and antigen presentation pathways and enhancement of pathways implicated in tumor progression and BRAFi resistance.

3.4. Increased AR expression elicits transcriptional changes of clinical significance found in BRAFi-resistant subpopulations.

To identify genes or sets of genes that are permanently modulated by increased AR expression and may account for their long-term BRAFi resistance, we compared the

transcriptional profiles of the three melanoma cell lines plus/minus AR overexpression under basal conditions. Next to the *AR* gene itself, *SERPINE1*, an established TGF-ß target with pro-tumorigenic functions [20,21], was the single most upregulated gene in all three AR overexpressing melanoma cells (Fig. 4A, Suppl. Table 3). Together with a hallmark of AR response, the gene set enrichment analysis (GSEA) [22] showed a strong positive association of the profiles of AR overexpressing cells with predefined gene signatures related to cell proliferation (E2F), epithelial-mesenchymal transition (EMT) and undifferentiated and neural crest melanoma cells (UNDIF and UNDIF-NC) previously connected with BRAFi resistance [23] (Fig. 4B, Suppl. Table 3). Other gene signatures implicated in BRAFi resistance, specifically EGFR and TGF-ß signaling [19], were also positively associated with transcriptional changes elicited by AR overexpression (Fig. 4B, C; Suppl. Table 3).

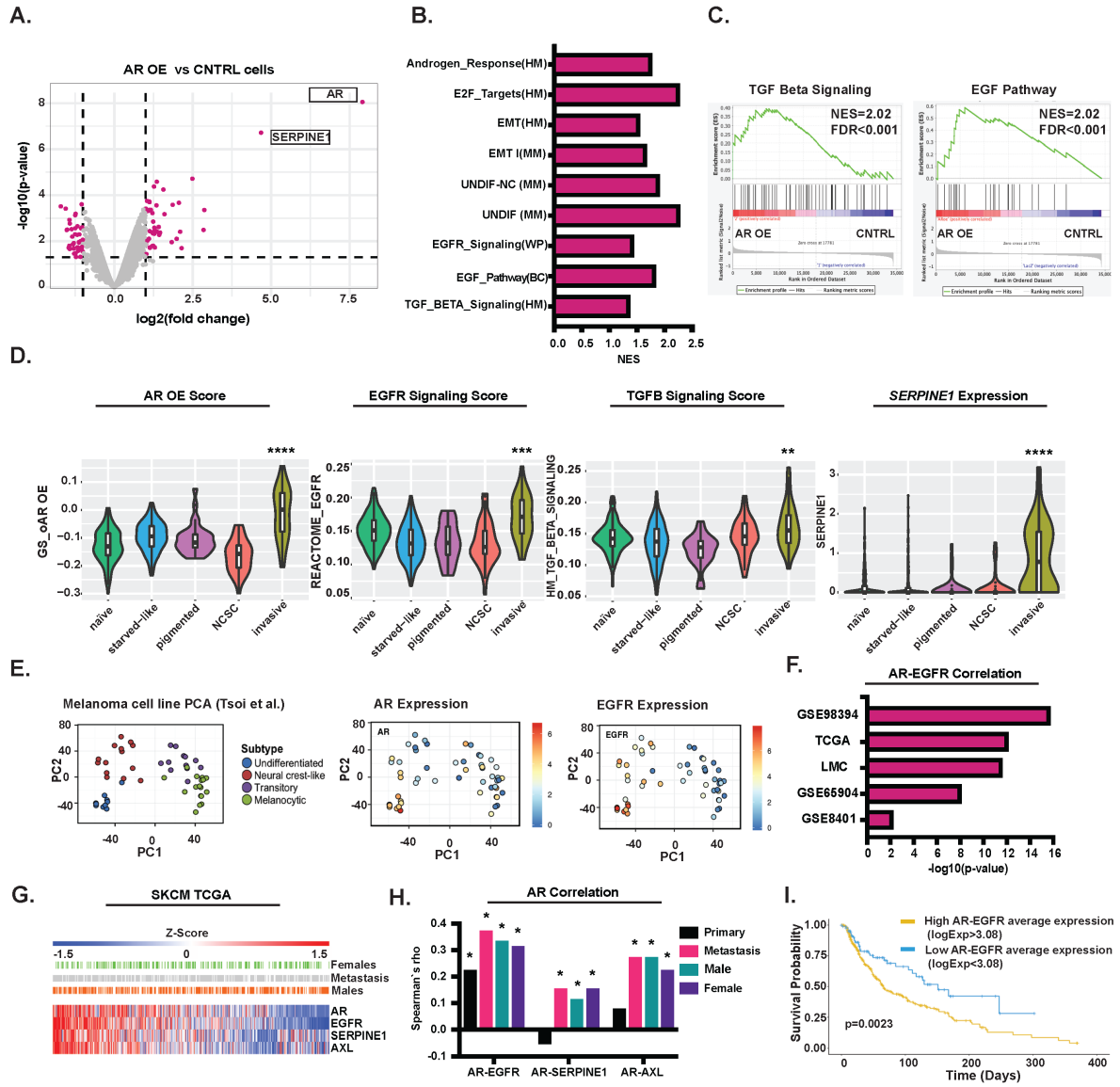

Figure 4: Increased AR expression elicits transcriptional changes of clinical significance found in BRAFi resistant subpopulations.

A) Transcriptional changes elicited in melanoma cells by AR overexpression. Volcano plots of similarly modulated genes in A375, M14, and WM9 melanoma cells stably infected with AR versus LacZ expressing (control) lentiviruses under control conditions (w.o. Dabrafenib treatment). Plotting of differentially expressed genes is as in Fig. 3A. Coloured dots (magenta) correspond to genes with $\log_2$(fold change) threshold of -1 and 1 and p-value<0.05. The complete list of differentially expressed genes is provided in Suppl. Table 3.

B) Gene Set Enrichment Analysis (GSEA) of transcriptional profiles of AR overexpressing versus control A375, M14, and WM9 melanoma cells as in the previous panel using a predefined set of gene signatures as obtained from the Hallmark gene set collection (HM) [42], wikipathways (WP) [44], biocarta (BC) and melanoma-specific studies (MM) [23,37] UNDIF-NC = undifferentiated, neural crest. Shown is a list of selected gene signatures with normalized enrichment score (NES) in profiles of AR overexpressing versus control melanoma cells. A more exhaustive list of signature genes is provided in Suppl. Table 3.

C) GSEA and plot distribution of gene signatures related to EGF (biocarta) and TGF-ß (Hallmark) signaling. Genes are ranked by signal-to-noise ratio in AR-overexpressing versus control melanoma cells; the position of individual genes is indicated by black vertical bars; the enrichment pattern is in green.
D) Scores of *AR* overexpression, EGFR, TGFß gene signatures activity, and *SERPINE1* expression in cell subpopulations identified by single cell RNA-seq analysis of a PDX model of melanoma BRAFi-resistance [17]. A gene signature of 19 upregulated and 39 downregulated genes (absolute FC>1, p-value<0.01) in the AR overexpressing versus control melanoma cells was established (for a list of genes see Suppl. Table 3). The signature was used to calculate scores of AR activity, using AUCell [40], in the scRNA-seq profiles of previously defined populations of the drug-naive melanoma cells and BRAFi-induced starved-like (SMC), pigmented, invasive and neural crest-like subpopulations [17]. Similar score calculations were performed with the Reactome EGFR signaling pathway [45] and the hallmark gene set for TGF-ß activity signatures [42] and single gene *SERPINE1* expression levels. Violin plots show individual cell score distribution within each cell population. The significance of differences in mean score values between invasive versus naïve cell populations (box plots) was calculated by Welch's *t*-test [41]. **, p<0.01; ***, p<0.001; ****, p<0.0001
E) Levels of AR and EGRF expression in multiple melanoma cell lines previously clustered according to multiple differentiation trajectories [23]. Shown is the Principal Component Analysis (PCA) of expression profiles of individual melanoma cell lines (dots) and corresponding subtypes, together with overlapping color-coded indication of *AR* and *EGFR* mRNA levels, as retrieved from http://systems.crump.ucla.edu/dediff/.
F) Positive correlation between AR and EGFR expression calculated from transcriptomic profiles of the indicated studies of melanoma clinical cohorts: GSE98394 (n=51 primary melanomas); TCGA (n=472 primary melanomas and melanoma metastases); LMC (n=703 primary melanomas); GSE65904 (n=214 melanoma metastases); GSE8401 (n=83 primary melanomas and melanoma metastases from xenograft models). Shown is the $-\log_{10}$ (p-value) of the correlation between AR and EGFR expression, as calculated using the *corrplot* v0.92 package with the Spearman`s correlation method.
G) Heatmap of Z-score values for *AR*, *EGFR*, *SERPINE1* and *AXL* expression from RNA-seq profiles of 472 melanoma samples from the TCGA project (TCGA Firehose Legacy, February 2022). Z-scores were obtained by median-centering $\log_2$ (expression values) and dividing them by standard deviation. Shown are score values for each individual tumor, with a corresponding indication of patients' sex, and whether they are from metastatic lesions.
H) Correlation between *AR* an*d EGFR*, *SERPINE1* and *AXL* expression levels calculated from the TCGA melanoma cohort as in the previous panel, using the *corrplot* 0.92 package and the Spearman`s correlation method. Spearman's rho coefficients are reported, with asterisks representing statistical significance (p-value < 0.05).
I) Kaplan-Meier curves of long-term overall survival of melanoma patients from the TCGA dataset. Patients were divided according to high (yellow bar) versus low (blue bar) average expression of AR and EGFR, as calculated using the optimal cutpoint for continuous variables ($\log_2$ (Expression value) = 3.08), obtained from the maximally selected rank statistics from the *maxstat* R package.

A recent study of the BRAFi response at the single-cell level in mouse Patients Derived Xenografts (PDXs) pointed to a transition of drug-naive melanoma cells to a BRAFi-induced starved-like (SMC) subpopulation branching out to three phenotypes [17]. By probing into the

profiles of these distinct subpopulations, we found a highly enriched AR signature score in a specific BRAFi-tolerant subpopulation with elevated AXL expression and invasive features [17] (Fig. 4D). This same population was also found to have a positive enrichment score for the EGFR and TGF-ß gene signatures as well as *SERPINE1* expression (Fig. 4D).

These findings were extended by analyzing the composite transcription profiles of human melanoma cell lines that cluster into four main groups along a two-dimensional differentiation trajectory [23]. Expression levels of the *AR* gene itself were positively associated with those of the *EGFR* gene in the most undifferentiated *AXL*-positive group connected with the targeted drug resistance [23] (Fig. 4E).

To assess the clinical significance of the results, we analyzed the transcriptomic profiles of melanoma cohorts, finding a strong positive correlation between expression levels of the *AR* and *EGFR* genes in multiple data sets (Fig. 4F). In the TCGA repository, we stratified lesions according to expression scores of the *AR*, *EGFR*, *SERPINE1,* and *AXL* genes (Fig. 4G). *AR* expression was positively associated with *EGFR* in both primary and metastatic melanoma lesions from male as well as female patients (Fig. 4H). *AR* expression also positively correlated with *SERPINE1* and *AXL* levels in the metastatic but not primary lesions, in keeping with the complex role played by these genes in melanoma progression. Lesions were subdivided according to optimal cutoff levels of average *AR* and *EGFR* expression, with patients with tumors with higher average expression levels having significantly lower survival than those with negative ones (log-rank test, $p = 0.0026$) (Fig. 4I). The findings remained significant after correcting for age, sex, and primary or metastatic status (multivariate Cox regression, $p = 0.0073$).

Thus, increased AR expression in melanoma cells elicits changes found in a BRAFi tolerant subpopulation and enhanced *EGFR* and *SERPINE1* expression of likely clinical significance.

3.5. Targeting AR overcomes BRAFi resistance

The above results suggested that AR is a positive determinant of melanoma progression and BRAFi resistance, which may be used for therapeutic targeting. Consistent with the transcriptomic results, RT-qPCR and immunoblot analysis showed a marked increase in *SERPINE1* (PAI-1) and *EGFR* levels in all tested melanoma cell lines upon AR overexpression (Fig. 5A, B).

To assess whether targeting of AR in BRAFi resistant melanoma cells elicits the converse effects, cells were treated with two different AR inhibitors, one suppressing AR activity through both ligand competitive and non-competitive mechanisms [24], and the other causing PROTAC-mediated degradation [25]. Immunofluorescence and RT-qPCR analysis showed that treatment with both inhibitors caused effective loss of AR expression, which was paralleled by a decrease of *SERPINE1* (PAI-1) as well as EGFR expression (Fig. 5C, D, Suppl. Fig. 4).

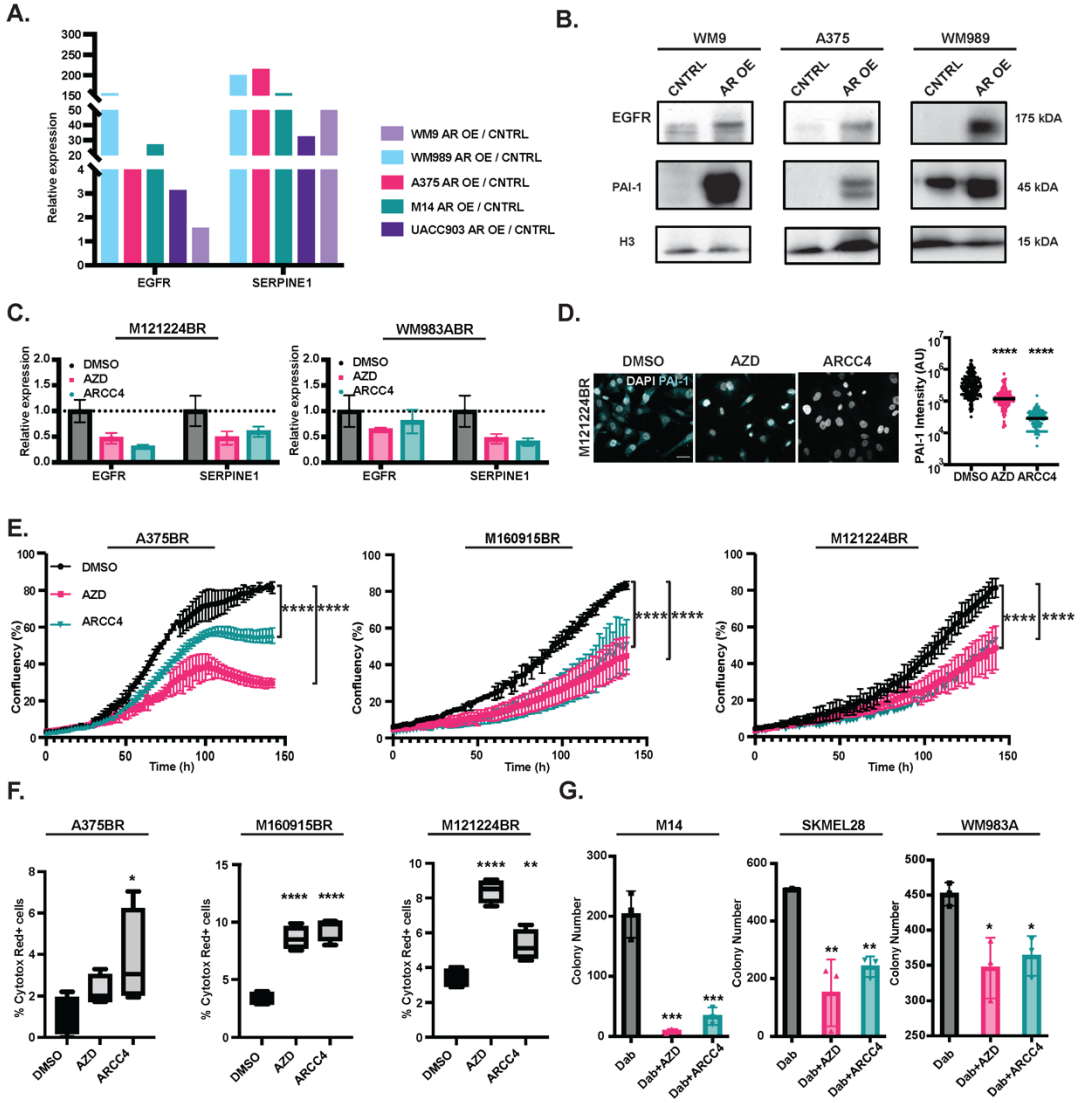

Figure 5: Targeting AR overcomes BRAFi resistance.

A, B) Expression of the *EGFR* and *SERPINE1* genes in multiple melanoma cell lines plus/minus AR overexpression. The indicated melanoma cells stably infected with an AR overexpressing lentivirus versus LacZ expressing control (the same cells as in Fig. 4) were analyzed for expression of the *EGFR* and *SERPINE1* genes by RT-qPCR, with *RPLP0* for internal normalization (A) or immunoblotting with Histone H3 as an equal loading control (B).

C) RT-qPCR analysis of *EGFR* and *SERPINE1* expression in two different melanoma cell lines (M121224BR and WM983ABR) treated with the AR inhibitors. Cells propagated in the presence of Dabrafenib as in Fig. 1 were treated with AZD3514 (10uM) or ARCC4 (1uM) versus DMSO control for 48 hours. Data are represented as the relative expression changes using *RPLP0* for internal normalization.

D) Immunofluorescence analysis of melanoma cells (M121224BR) treated with the AR inhibitor AZD3514 or ARCC4 or DMSO control for 48 hours as in the previous panel with anti-PAI-1 antibodies with DAPI staining for nuclei visualization. Shown are representative images and quantification of the PAI-1signal intensity in arbitrary units (AU) per cell (dots) together with a mean, examining >100 cells per sample, unpaired *t*-test, **** p<0.0001. Color

scale: grey, DAPI; cyan, PAI-1. Scale bar: 40μm. Immunofluorescence analysis of AR expression in parallel cultures is shown in Suppl. Fig. 4.
E) Proliferation live-cell imaging assays (IncuCyte) of the indicated BRAFi resistant cells treated with AR inhibitors or DMSO as in the previous panels. Cells were plated in triplicate wells in 96-well plates, followed by cell density measurements (four images per well every 4 h for 128 h). cultures, n = 3; Pearson r correlation test. ****, p< 0.0001.
F) Cell death as detected by live-cell staining (IncuCyte, Cytotox Red) of the same cultures as in the previous panel at 72 hours of treatment with the AR inhibitors versus DMSO control. Four images per well cultures, n (cultures) = 3; unpaired *t*-test, *, p<0.05; **, p< 0.01; ****, p<0.0001.
G) Clonogenicity assays of three different drug-naive melanoma cell lines treated with Dabrafenib (0.5uM) individually or in combination with AZD3514 (10uM) or ARCC4 (1uM). Cells were plated in triplicates at clonal density (5000 cells / 6 cm dish) followed by 2 weeks of cultivation. Macroscopically detectable colonies were counted after crystal violet staining. n(dishes)=3, unpaired *t*-test, *, p<0.05; **, p< 0.01; ***, p<0.001.

The findings are of functional significance, as live-cell imaging assay showed that treatment with either AR inhibitors blunted melanoma cell proliferation and, at the same time, induced cell death (Fig. 5E, F). To assess whether inhibition of AR activity could also prevent the emergence of BRAFi resistance, drug-naive melanoma cells were cultured in the presence of Dabrafenib alone or in combination with the AR inhibitors. Consistent with previous studies [15,26], a large number of BRAFi-resistant colonies emerged in cultures of parental melanoma cells treated with the BRAFi alone, which was significantly reduced in cultures concomitantly treated with the AR inhibitors (Fig. 5G).

The studies were extended to an orthotopic model of melanoma development based on intradermal Matrigel injection of cells into immunodeficient mice. BRAFi-resistant A375 cells were treated with AZD3514 (10 μM) versus DMSO vehicle alone 24 hours prior to injection. As shown in Fig. 6A, B, this single exposure to the AR inhibitor was sufficient to perturb the tumorigenicity of cells, which formed lesions with significantly decreased cell density and proliferation relative to controls.

An important interconnection has been established in melanoma cells between the acquisition of BRAFi resistance and reduced sensitivity to the immune surveillance [13]. The work was extended to a syngeneic mouse model, whereby BRAFi-resistant mouse melanoma

cells (YUMM1.7) were pretreated with two different AR inhibitors, AZD3514 (10 μM) or ARCC4 (1 μM), versus DMSO were injected into immunocompetent mice. Melanoma cells pretreated with both AR inhibitors produced tumors of significantly smaller size than controls with strongly increased MHC I surface expression and improved CD8+ T cell infiltration (Fig. 6C-F, Suppl. Fig. 5).

Hence, pharmacological inhibition of AR activity in BRAFi-resistant cells provides a tool to effectively suppress *EGFR* and *SERPINE1* expression, proliferation, and tumorigenicity with a concomitant enhancement of immune cell recognition.

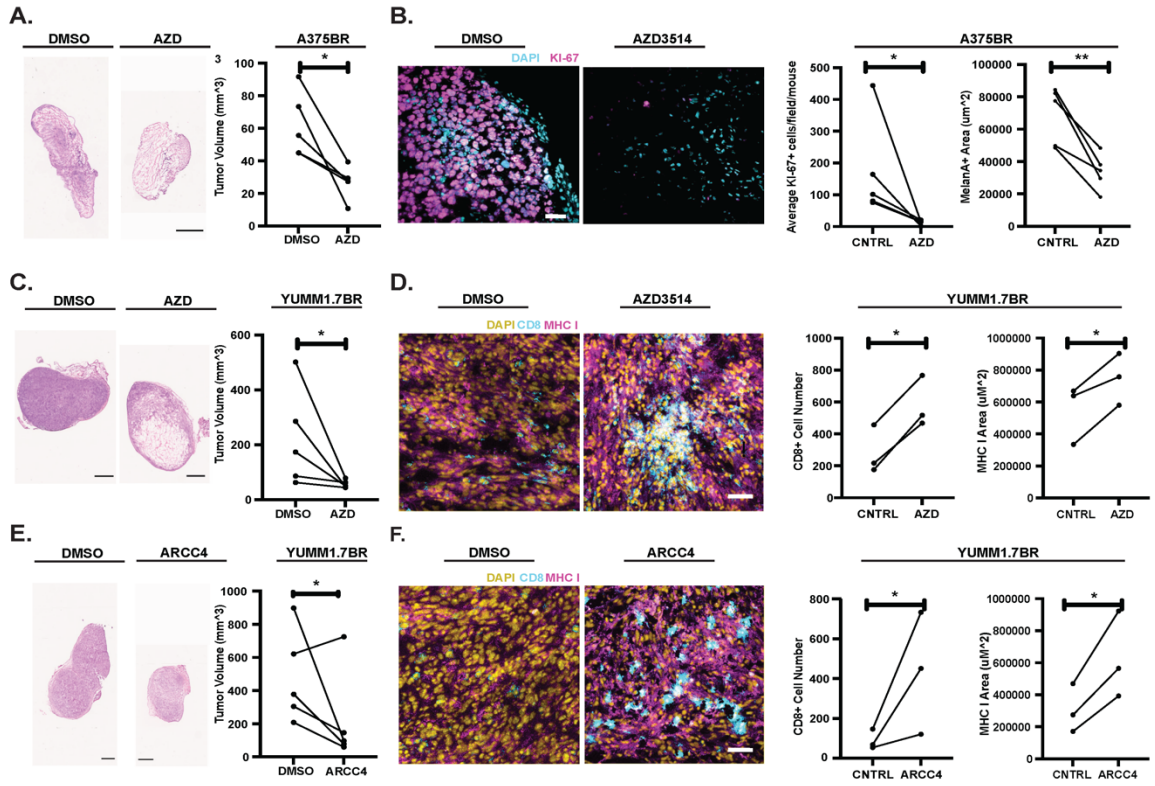

Figure 6: AR inhibition suppresses tumorigenicity of BRAFi-resistant melanoma cells.
A) A375 cells selected for BRAFi resistance (A375BR) were subjected to a single treatment with the AZD3514 (10µM) versus DMSO control followed, 24 hours later, by parallel intradermal injections into 5 male NSG mice. Tumors were retrieved two weeks later. Shown are representative images of H&E stained lesions and quantification of tumor volume by caliper. n (mice) = 5, paired *t*-test, *, $p<0.05$. Scale Bar: 1 mm.
B) Excised tumors, as in the previous panel, were analyzed by immunofluorescence analysis with antibodies against the Ki67 proliferation marker. Shown are representative images and quantification of the Ki67 proliferative index (number Ki67+ cells per field, three fields per lesion). n (mice) = 5, paired *t*-test, *, $p<0.05$. Scale Bar: 40µm.
C-F) Mouse YUMM1.7 melanoma cells selected for BRAFi resistance (YUMM1.7BR), by multistep cultivation in increasing concentrations of Dabrafenib as with the human cells, were subjected to a single treatment with AZD3514 (10µM) (C, D) or ARCC4 (1µM) (E, F) versus DMSO control followed, 24 hours later, by parallel intradermal injections into 5 male C57BL/6JRj mice. C, E): representative images of H&E stained lesions and tumor volume quantification by caliper. n (mice) = 5, paired *t*-test, *, $p<0.05$. H&E Scale Bar: 1mm. D, F): double immunofluorescence analysis of excised tumors with antibodies against MHC I and the CD8+ T cell marker. Shown are representative images and quantification of MHC I + area and the number of CD8+ T cells per field (8 fields per lesion). n (mice) = 3, paired *t*-test, *$p<0.05$. Color scale: yellow, DAPI; magenta, PE-conjugated anti-MHC I antibody (MHC I-PE); cyan, anti-CD8+ antibody. Scale Bar: 40µm.

4. Discussion

Resilience to cancer therapy remains a major challenge even with improved approaches [27]. In concert with modulation of the microenvironment, the resistance of cancer cells to targeted therapies can result from two mechanisms: i) an intrinsic adaptive response, with the expansion of pre-existing cell populations; ii) acquired resistance, through *de novo* genetic/epigenetic events [27]. The adaptive response, which can be very rapid, is the result of compensatory feedback mechanisms of therapeutic interest [28]. Drivers of adaptive responses are typically involved in regulatory circuits of both normal and cancer cells and can be most effectively targeted in the adjuvant therapy [27]. Our combined findings indicate that the AR is one such driver as a key determinant of the adaptive response of melanoma cells to targeted therapy, which may be used to prevent or delay resistance.

The initial sensitivity of melanomas with activating BRAF mutations to BRAF inhibitors can be overcome by several mechanisms, including the compensatory upregulation of the EGFR tyrosine kinase coupled with downmodulation of the MITF and SOX10 transcription factors [15,19,28,29]. In contrast to the negative role played by these transcription factors, we have found that AR is a positive determinant of BRAFi resistance and EGFR expression. We previously showed that basal AR activity is required for sustained proliferation and tumorigenesis of melanoma cells, with AR functioning as a bridge between RNA-Pol II and DNA repair proteins and ensuring the continuous DNA repair process associated with gene transcription [9]. The markedly increased AR expression that is already occurring at early times of BRAFi and MEKi exposure suggested that this molecule can fulfill a second distinct function in melanoma cells as part of an adaptive mechanism leading to targeted drug resistance. In fact, persistently increased AR expression was by itself sufficient to render cells resistant to BRAFi-induced growth suppression and apoptosis, modulating different sets of genes from those affected by *AR* gene silencing [9] (see Suppl. Fig. 6 for a comparison).

Elevated AR expression in melanoma cells did not block but rather subverted the transcriptional response of melanoma cells to BRAF inhibition. Underlying the different sensitivity, apoptosis-related genes were induced by BRAFi treatment to a much greater extent in control than AR overexpressing cells. Efficacy of BRAF inhibitors depends on triggering a cancer cell death program associated with an impact on the tumor immune microenvironment [30]. Gene signatures related to interferon signaling, inflammation, and antigen presentation, which can enhance immune stimulation and response to checkpoint inhibitors [31], were all induced by BRAFi treatment of control but not AR-overexpressing cells. A cross-connection has been established between BRAFi resistance and poor response to immune checkpoint control that does not depend on selection by the immune system and is a cancer cell-instructed [13], in which increased AR expression may be involved. Consistent with this possibility, in a syngeneic mouse model with BRAFi-resistant melanoma cells, low MHC I cell surface antigens expression, which has been linked with poor immune response [32], was strongly enhanced by treatment with AR inhibitors in parallel with CD8+ T cell infiltration.

Besides suppressing induction of pro-apoptotic and immunomodulatory genes, elevated AR expression resulted in a paradoxical upregulation by BRAFi treatment of gene families connected with BRAFi resistance, specifically EGFR and TGF-ß related [19]. Gene signatures of two pathways were also induced by AR overexpression in melanoma cells under basal conditions, with an expression of the *EGFR* gene itself being consistently upregulated. Increased *EGFR* expression was previously connected with TGF-ß activation, with the two inducing cellular senescence of melanoma cells while, in the presence of BRAFi, conferring a growth advantage [19]. The mechanism underlying this dichotomy remains to be established, and an interesting possibility is that AR is involved.

Among TGF-ß responsive genes, *SERPINE1* was prominently induced by increased AR expression. The gene codes for a secreted serine proteinase inhibitor of the SERPIN family

(PAI-1), which exerts complex functions resulting from its binding to several cell surface proteins, promoting tumor development through effects on both cancer cells and the tumor microenvironment [33]. It has been recently shown that elevated *SERPINE1* expression in melanoma cells is associated with a bad prognosis and poor response to immune checkpoint inhibitors [20].

The positive connection between *AR* and *EGFR* and *SERPINE1* expression was validated by single cell analysis of melanoma cells in a PDX model of BRAFi response: increased *AR* and *EGFR* gene signatures were coincidental with elevated *SERPINE1* expression in a specific BRAFi tolerant subpopulation characterized by high AXL expression and invasive features [17]. Similarly, in a study on the heterogeneity of melanoma cell lines and tumors, *AR* expression was found to cluster together with *EGFR* and *SERPINE1* in an undifferentiated AXL positive subgroup connected with targeted drug resistance [23]. The positive association of *AR* with *EGFR*, *SERPINE1,* and *AXL* expression was confirmed in a large patient cohort, irrespective of sex and primary versus metastatic lesions, with poor survival with tumors with elevated *AR-EGFR* levels.

AR has been intensely studied as a driver of metastatic prostate cancer, with resistance to AR-targeting approaches resulting from various mechanisms, including increased *AR* expression [34]. *AR* upregulation is a point of convergence of multiple mechanisms [18], with transcription factors like CREB1 and Foxo3a that we have found to be positively associated with *AR* transcription also in our system. Overall, genetic and epigenetic changes of *AR* resistance are less likely to occur in melanoma, in which other genes drive the disease. Inhibitors targeting AR activity and expression could be employed as co-adjuvants to prevent/delay targeted drug resistance and, as we have shown, suppress tumorigenicity of BRAFi-resistant cells while at the same time inducing CD8+ T cells infiltration. Given the connection between BRAFi resistance and poor immune response [13], as well as the intrinsic

role of AR activation in dampening the T cell activity [12,35], AR targeting may be beneficial in the treatment regimens with immune checkpoint inhibitors.

5. Materials and methods

Cell Culture

A full list of different melanoma cell lines and primary melanoma cells is provided in Supplementary Table S4. Early passage primary melanoma cell cultures (M160915 and M121224) were established from discarded melanoma tissue samples by the University Research Priority Program (URPP) Live Cell Biobank (University of Zurich) following institutional requirements. WM115, WM9, WM983A, WM989, UACC903, and UACC903BR melanoma cells were a gift from Dr. Meenhard Herlyn (The Wistar Institute, US). The YUMM1.7 melanoma cell line [36] was provided by Dr. Ping-Chih Ho (UNIL).

All melanoma cell lines and patient-derived primary melanoma cells were maintained in Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific) supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific). YUMM1.7 melanoma cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific). All cell lines were routinely tested for *Mycoplasma*. Cell morphology and growth characteristics were monitored throughout the study and compared with the previously published reports. No further authentication of these cell lines was performed.

Cell manipulations and treatments

Lentiviral particle productions and infections were performed as described previously [9]. Melanoma cells were transduced with *AR* overexpressing (a gift of Dr. Karl-Henning Kalland, Bergen University, Bergen, Norway) or LacZ expressing control lentiviruses for 6 hours. Two days post-infection cells were selected using 5 μg/ml of Blasticidin for 6 days. RNA or protein samples were collected 7 days after infection.

BRAF resistant (BR) cell lines were established from the parental (P) cells (A375, M160915, M121224, and WM983A) by continuous culturing in Dabrafenib for a period of 4 weeks, with weekly multistep increases in concentration from 0.5 to 3 μM. Resistant cells were thereafter continuously cultured in the presence of 3 μM Dabrafenib.

For *short-term in vitro experiments with various chemical inhibitors*, 24 hours post-seeding cells were treated with the following compounds at the indicated concentrations: Dabrafenib (0. 5 μM), PLX-4720 (0. 5 μM), Sorafenib (0. 5 μM), Trametinib (0.005 μM), Cobimetinib (0.005 μM), s31-201 (50 μM), Bay 7085 (10 μM), T55224 (20 μM), sr11302 (10 μM), SU6668 (10 μM), SKI-606 (10 μM), and CYT387 (10 μM). All inhibitors were purchased from SellectChem and were dissolved in DMSO according to the manufacturer`s instructions. DMSO was used as vehicle control. RNA was collected 48 hours post-treatment.

For *AR inhibition*, 24 hours post-seeding, melanoma cells were treated with 10 μM AZD3514 (SellectChem) or with 1 μM ARCC4 (Tocris). AR inhibitors were dissolved in DMSO according to the manufacturer`s instructions. DMSO was used as vehicle control for all experiments.

Cell proliferation / density assays were carried out by measuring ATP production using the CellTiter-Glo luminescent assay (Promega) as per the manufacturer's instructions. Dabrafenib dose-response curves and IC50 values were attained by fitting the curves to nonlinear regression with variable slope using GraphPad Prism.

For *clonogenicity assays*, cells were plated onto 60 mm dishes (10,000 cells/well; triplicate wells/condition) and treated the next day as indicated in the figure legends. Tissue culture medium was refreshed every 2-3 days. Cells were cultured for 7 days for *AR* overexpressing experiments and 14 days for experiments with AR inhibitors. Colonies were fixed with methanol fixed and stained with 1% crystal violet. The number of clones was counted using Fiji/ImageJ software.

For *IncuCyte cell proliferation and cell death assays*, 1000 melanoma cells per condition were seeded in triplicate in 96-wells plates. Drug treatments were applied 12 hours post-seeding, with cells allowed to proliferate for 5 days. Cell proliferation was monitored using the IncuCyte Zoom Live-Cell Imaging System (Essen Bioscience). Four independent images per well per condition were captured every 2 hours for 5 days. Cell confluence was determined with IncuCyte Zoom software. For cell death measurements, the IncuCyte Cytotox Red Reagent was added to the cells seeded and treated as described above and imaged according to the manufacturer's instructions. Cytotox Red positive cells were quantified using the IncuCyte Zoom software.

Immunofluorescence and immunohistochemistry staining

Immunofluorescences staining of tissue sections and cultured cells was carried out as described previously [9]. In brief, paraffin-embedded sections were deparaffinized and rehydrated prior to a citrate-based buffer antigen retrieval. Frozen tissue sections (8 mm) or cultured cells were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT). Samples were washed with PBS (3x5min) and permeabilized using 0.5% TritonX100 in PBS for 10 minutes. Samples were blocked using 2% bovine serum albumin in PBS for 1 hour at RT. Primary antibodies were diluted in a blocking buffer (PBS/2% bovine serum albumin) and were incubated overnight at 4°C. Following, samples were washed (PBS, 3x5min) and incubated with secondary donkey fluorescence conjugated secondary antibodies (Invitrogen) for 1 hour at RT. DAPI was used to counterstain nuclei. Slides were washed (PBS, 3x5min) and mounted using Fluoromount Mounting Medium (Sigma-Aldrich). Control staining without the primary antibodies was performed in each case to subtract background and set image acquisition parameters. A full list of primary and secondary antibodies and dilutions used for IF is provided in Supplementary Table 5.

Immunofluorescence images were acquired with a ZEISS LSM880 confocal microscope with 20X, 40X, or 63x oil immersion objectives or with a NanoZoomer S60 microscope with a 40X objective. ZEN Blue software was used for image acquisition. Fiji/ImageJ software was used for image processing and analysis. For image analysis, the images were stacked to maximal projections, and immunofluorescent channels were split. A binary mask was then created using a watershed function in the DAPI channel, allowing for the identification of individual nuclei. The mean grey value intensity of channels was measured and summed. The fluorescent intensities are indicated in arbitrary units. On average, >100 cells were analyzed for *in vitro* studies. For *in vivo* studies, five fields were imaged per tumor.

Immunohistochemical staining was performed by the laboratory of pathology in the Department of Biochemistry, UNIL, as previously described [9]. Slides were scanned using a NanoZoomer S60 microscope with a 20X objective. Ndp.View2 and Fiji/ImageJ software were used for the acquisition and processing of images.

Immunoblotting

Cells were lysed using boiling LDS buffer (2%SDS, 50 mM Tris/HCl (pH 7.4) supplemented with 1 mM PMSF, 1 mM Na3VO4, and 10 mM NaF. Total protein content was quantified with a BCA assay (Thermo Fisher Scientific). Equal amounts (20-50 μg) of proteins were subjected to 10% SDS–PAGE followed by immunoblot analysis. All membranes were sequentially probed with different antibodies as indicated in the figure legends. Super Signal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) was used for signal detection. Full details of antibodies used in this study are provided in Supplementary Table 5.

Real-time quantitative PCR (RT-qPCR)

Total mRNA was extracted using TRIzol according to the manufacturer's instructions, followed by cDNA synthesis using the RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific). RT-qPCR was performed using SYBR Fast qPCR Master Mix (Kapa Biosystems) on a Light Cycler 480 (Roche). The relative quantification (RQ) and expression of each mRNA were calculated using the comparative Ct method. All samples were run in technical triplicates and were normalized to an endogenous control, *RPLP0*. A full list of primers used in the study is provided in Supplementary Table 5.

Transcriptomics and bioinformatic analysis

A375, M14, and WM9 melanoma cells infected with an *AR* overexpression lentivirus versus LacZ expressing control virus were treated with Dabrafenib (0.5 μM) versus DMSO vehicle for 48 hours. Following, RNA was extracted using the Direct-zol RNA MiniPrep kit (Zymo Research) coupled with DNase treatment according to the manufacturer's instructions. The RNA quality was first evaluated using Agilent 2100 Bioanalyzer® (Agilent Technologies, USA). Transcriptomic analysis was performed using Clariom^{TM} D GeneChip array hybridization (Thermo Fisher Scientific). Single-strand cDNA preparation, labeling, and hybridization were performed in accordance with Affymetrix protocols at the iGE3 Genomics Platform, University of Geneva (Geneva, Switzerland). Data obtained (CELL files) were summarized using the RMA function in the R package oligo with background correction and quantile normalization. Gene IDs were mapped using the Chip-annotation package clariomdhumantranscriptcluster.db. The R package "limma" was used for gene differential expression analysis, followed by multiple testing correction by the Benjamini-Hochberg procedure. The cutoffs for the Dabrafenib treatment signatures (Dabrafenib CNTRL vs DMSO CNTRL and Dabrafenib AR OE vs AR OE CNTRL) were FC > 1.5, and adj-p < 0.01, yielding

360 up- and 360 down-regulated genes for CNTRL Dabrafenib-treated cells, and 199 up- and 344 down-regulated genes for AR OE Dabrafenib-treated cells. The cutoff for the AR OE signature (AR OE CNTRL vs DMSO CNTRL) was FC > 2.0, and p-value < 0.05, yielding 48 up- and 39 down-regulated genes. The data generated in this study have been deposited to the public functional genomics data repository GEO (Gene Expression Omnibus), NCBI with an accession number **GSE199405**.

Gene ontology (GO) enrichment analysis was performed on the differentially expressed genes with the fold change cutoff value of 2.0 using the Enrichr. Gene Ontology and Pathway Classification System to identify the enriched biological processes.

Gene set enrichment analysis (GSEA) for GeneChip microarray data was conducted using GSEA software using default parameters. Curated gene sets were obtained from various sources as also indicated in the legends for Figs. 3 and 4: i) the Molecular Signatures Database (MSigDB version 5.2, www.broadinstitute.org/gsea/msigdb/); ii) previously published melanoma-specific signatures [17,23,37]. A list of enriched pathways is provided in Supplementary Tables S2,3.

For *AR overexpression score,* CELL files were summarized using the RMA function in the R package oligo with background correction and quantile normalization. Gene IDs were mapped using the Chip-annotation package clariomdhumantranscriptcluster.db and differential expression analysis was performed with limma, using the formula ~treatment + cell_line. The treatment referred to the comparison DMSO versus AR OE.

Signature score analysis of single cell RNA-seq profiles was performed starting from single cell RNA-seq data (GEO # GSE116237) filtering for cells with more than 1000 gene counts and genes detected in more than 3 cells. Further filtering was omitted as it has already been done by the authors of the dataset [17]. Ensembl IDs were mapped into gene symbols using biomaRt [38] and count data were summed together when multiple IDs mapped to the same

symbol. Library normalization, log transformation and further downstream analysis were performed using Seurat v4 [39]. Signature scores were calculated using AUCell [40] and significance between scores or individual gene expressions were calculated using Welch's *t*-test [41]. Gene sets were downloaded from the Molecular Signatures Database [42].

Correlation analysis between AR, EGFR, PAI-1, and AXL expression levels was calculated on 472 melanoma samples from the TCGA project (TCGA Firehose Legacy, February 2022) with the corrplot 0.92 package, using the Spearman`s correlation method.

Survival analysis was based on the melanoma TCGA dataset and calculation of the optimal cutpoint for continuous variables (log2Expression value = 3.08) from the maximally selected rank statistics from the 'maxstat' R package.

In Vivo **Studies**

NOD *scid* gamma (NSG) mice, (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ; 6-8-week-old males), were obtained from the Jackson Laboratory. BRAFi resistant human A375 melanoma cells (A375BR) were pretreated with AZD3514 (10 μM) or DMSO control for 12 hrs prior to injection into mice. Cells (1×10^6 per injection, in Matrigel (Corning), 70 μl) were injected intradermally in parallel into the left and right flanks of mice with 29-gauge syringes. Mice were sacrificed and Matrigel nodules were retrieved for tissue analysis 10 days after injection.

C57BL/6JRj mice (6-8-week-old males) were obtained from Jackson Laboratory. BRAFi resistant murine melanoma cells (YUMM1.7BR) were pretreated with AZD3514 (10 μM), ARCC4 (1 μM), or DMSO control for 12 hrs prior to injection. 2×10^6 melanoma cells per condition were injected with Matrigel (Corning) (70 μl per injection) intradermally in parallel into the left and right side of mice with 29-gauge syringes. Mice were sacrificed and Matrigel nodules were retrieved 14 days after injection. Tumors were measured post-extraction using calipers. Tumor volumes were calculated using the formula ($LxW^2x0.5$). Throughout the

study, general humane endpoints were applied. All mice were housed in the animal facility of the University of Lausanne.

Statistical analysis

All statistical tests were performed using GraphPad Prism 9 (GraphPad Software, Inc.). Data are shown as mean± SEM or mean ± SD, as indicated in the legends. Detailed information on the statistical methods applied for each experiment can be found in the corresponding figure legends. Statistical difference between two groups was determined using Student's *t*-test unless otherwise mentioned. For comparisons among more than two groups, a one-way analysis of variance (ANOVA) followed by Bonferroni's correction was used. For longitudinal data, Spearman`s correlation was used to infer significance between the experimental treatment arms.

For tumorigenicity assays, individual animal variability issue was minimized by contralateral injections in the same animals under control versus experimental conditions. No statistical method was used to predetermine sample size in animal experiments and no exclusion criteria were adopted for studies and sample collection. No exclusion criteria were adopted for animal studies or sample collection. No randomization was used, and the researchers involved in the study were not blinded during sample obtainment or data analysis.

Study approvals

Pre- and post-treatment metastatic melanoma sections were obtained from the Live Cell Biobanks of the University Research Priority Program (URPP) "Translational Cancer Research" (Mitchell P. Levesque, University Hospital Zurich). All human samples were obtained from surplus melanoma material collected from de-identified patients who provided

written, informed consent to participate in the research (BASEC-Nr 2017—00494). No access to sensitive information has been provided.

All animal studies were carried out according to Swiss guidelines for the use of laboratory animals, with protocols approved by the University of Lausanne animal care and use committee and the veterinary office of Canton Vaud (animal license No. 1854.4f/1854.5a).

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Author contributions

AS, SG, TP, BT, and MM performed experiments and analyzed the results with GPD. MKY, PO, and GC performed the bioinformatic analysis, MPL provided the clinical samples and critical feedback. AS and GPD designed the study and wrote the manuscript.

Declaration of interests

The authors declare that there are no competing financial interests.

SUPPLEMENTAL INFORMATION (Appendix)

SUPPLEMENTARY FIGURES

Supplementary Fig. 1: BRAFi treatment of melanoma cells results in increased AR expression.

Supplementary Fig. 2: Increased AR expression in clinical post-BRAFi/MEKi treatment melanoma samples.

Supplementary Fig. 3: BRAFi treatment induces expression of positive regulators of AR expression.

Supplementary Fig. 4: Decreased AR protein expression by treatment of melanoma cells with AR inhibitors.

Supplementary Fig. 5: Pharmacological AR targeting alters MHC I surface expression and CD8 T cell infiltration.

Supplementary Fig. 6: Comparative analysis of transcriptomic profiles of melanoma cells plus/minus AR gene silencing versus overexpression.

SUPPLEMENTARY TABLES are provided in the electronic version of the paper on bioRxiv (doi.org/10.1101/2022.05.27.493720).

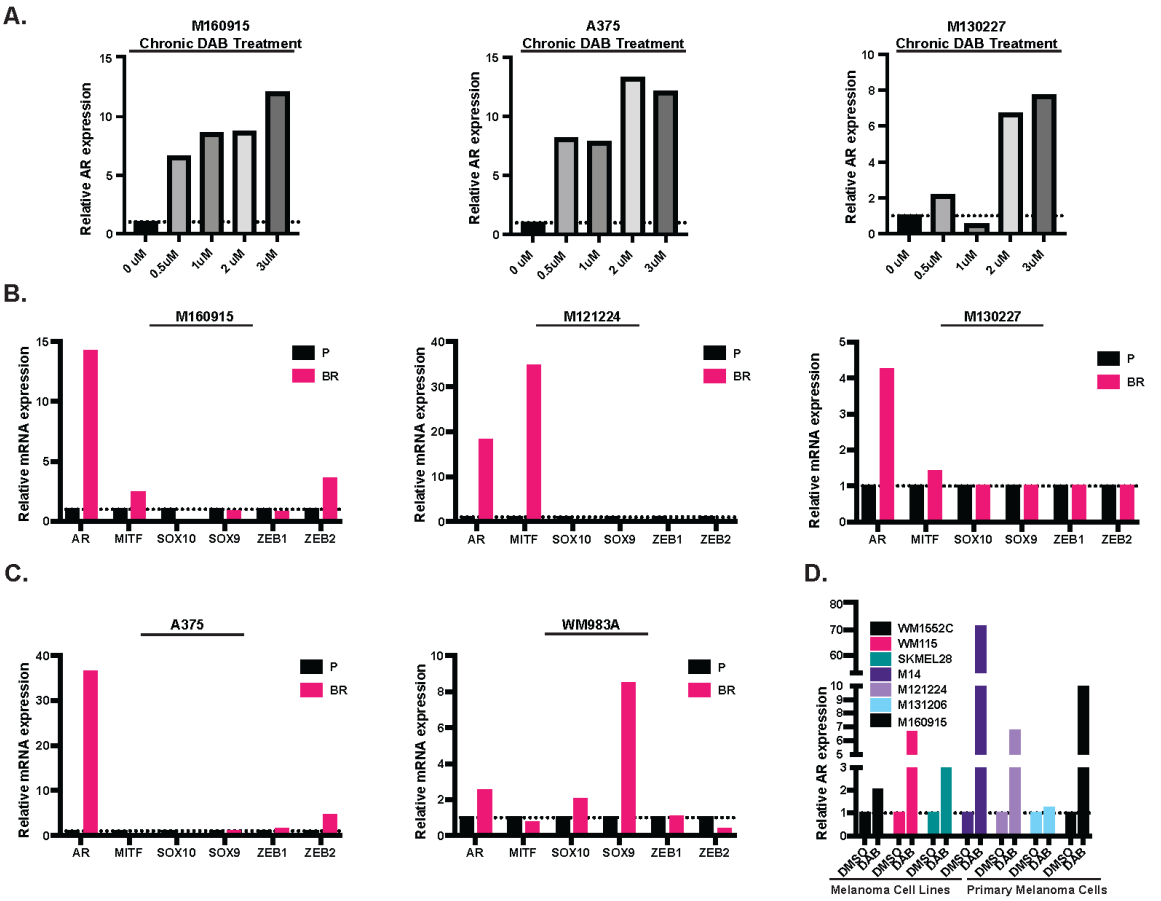
Supplementary Table 1: Gene expression levels of selected transcription factors upon Dabrafenib treatment and Spearmans`s correlation scores from the TCGA.

Supplementary Table 2: List of differentially expressed genes and gene ontology terms enriched in the control and AR overexpressing cells plus/minus Dabrafenib treatment.

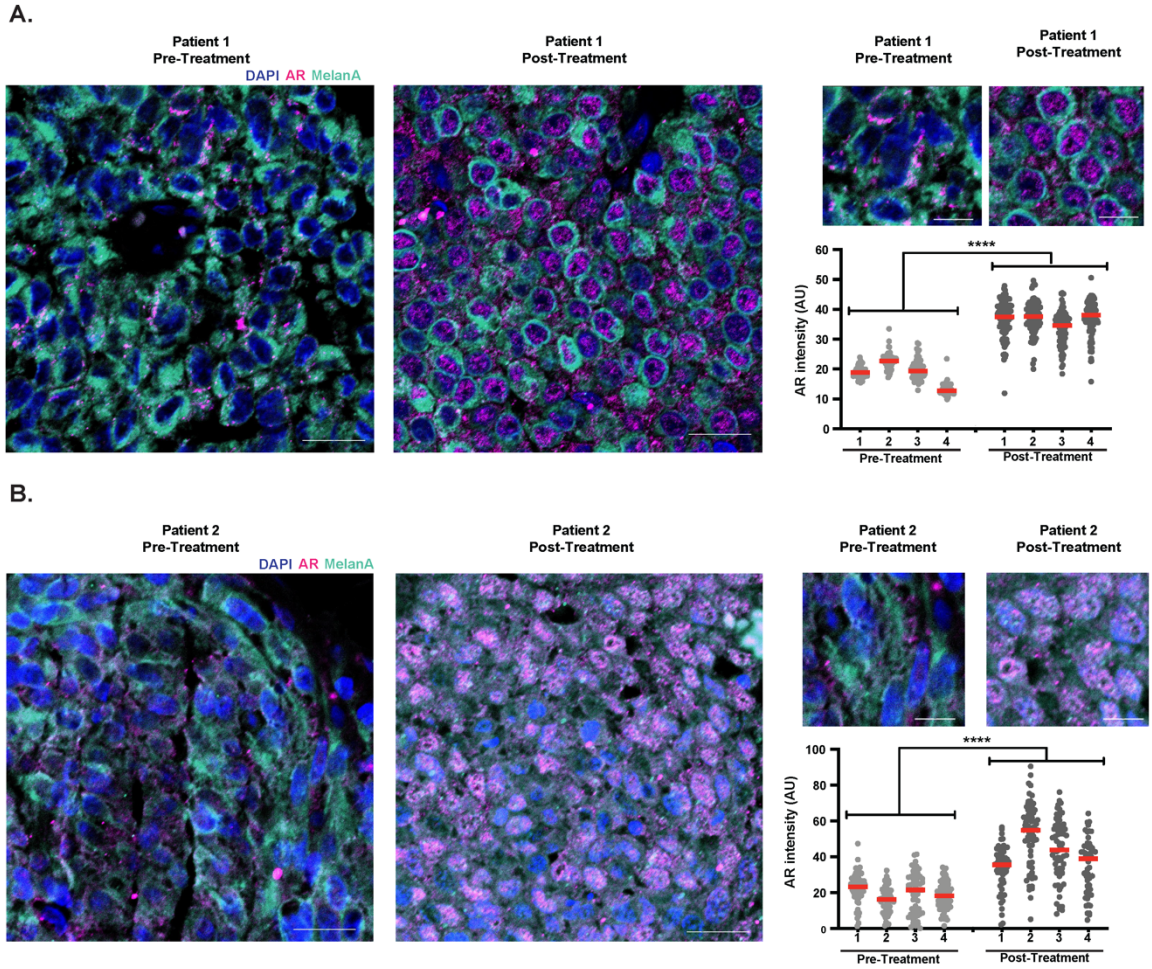
Supplementary Table 3: List of differentially expressed genes and GSEA terms enriched in AR overexpressing cells under basal conditions.

Supplementary Table 4: List of cell lines used in the study.

Supplementary Table 5: List of key reagents and resources used in the study.

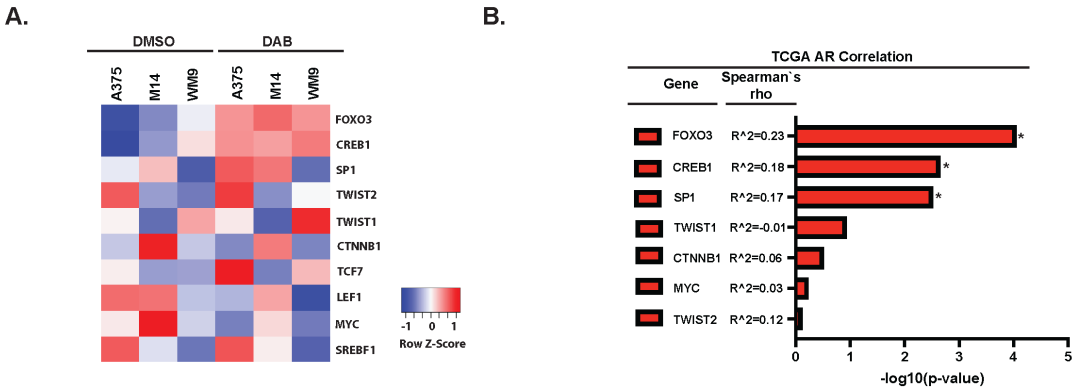


Supplementary Fig. 1: BRAFi treatment of melanoma cells results in increased AR expression.
A) *AR* expression in three additional human primary and established melanoma cell lines. Cells were cultured with multistep weekly increases of the BRAF inhibitor Dabrafenib (0.5, 1, 2, and 3 µM). Cells were collected at the end of each week of treatment and analyzed together with the untreated parental cells for levels of *AR* expression by RT-qPCR, with *RPLP0* for internal normalization. Related to Fig. 1A.
B-C) Relative expression of the indicated genes in BRAFi resistant (BR) primary (B) and established melanoma cell lines (C) versus parental cells (P). Shown are individual bar plots of the heatmap shown in Fig. 1D. RT-qPCR results are expressed as fold changes relative to untreated controls, after *RPLP0* normalization.
D) Relative *AR* expression in the indicated primary and established melanoma cells at 48 hours of treatment with BRAF inhibitor Dabrafenib (0.5 µM) versus DMSO control. RT-qPCR results are expressed as fold changes relative to untreated controls, after *RPLP0* normalization. Related to Fig. 1E.

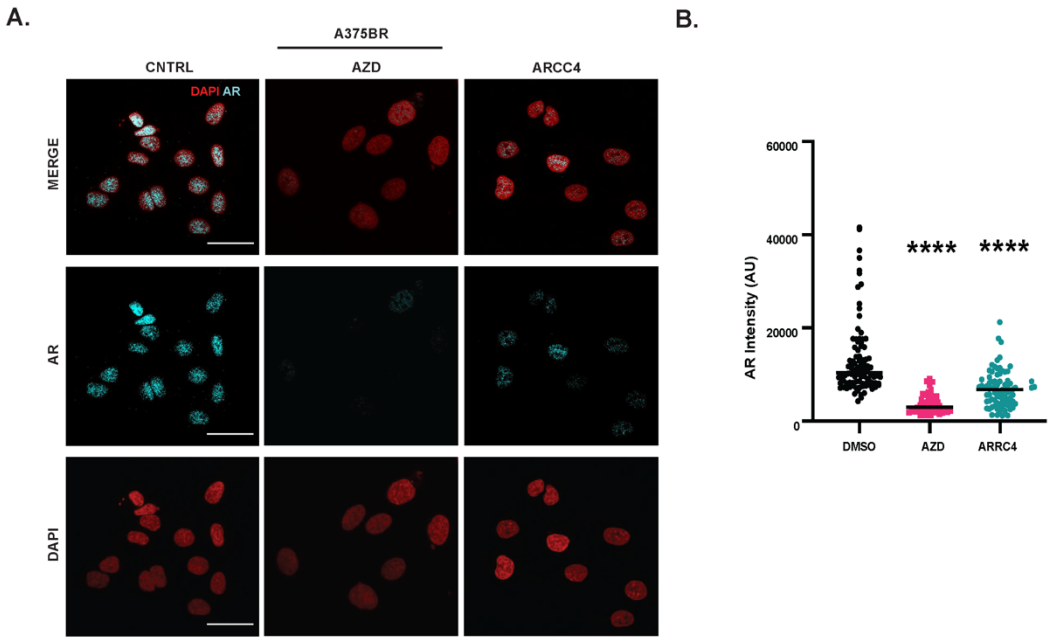


Supplementary Fig. 2: Increased AR expression in clinical post-BRAFi/MEKi treatment melanoma samples.

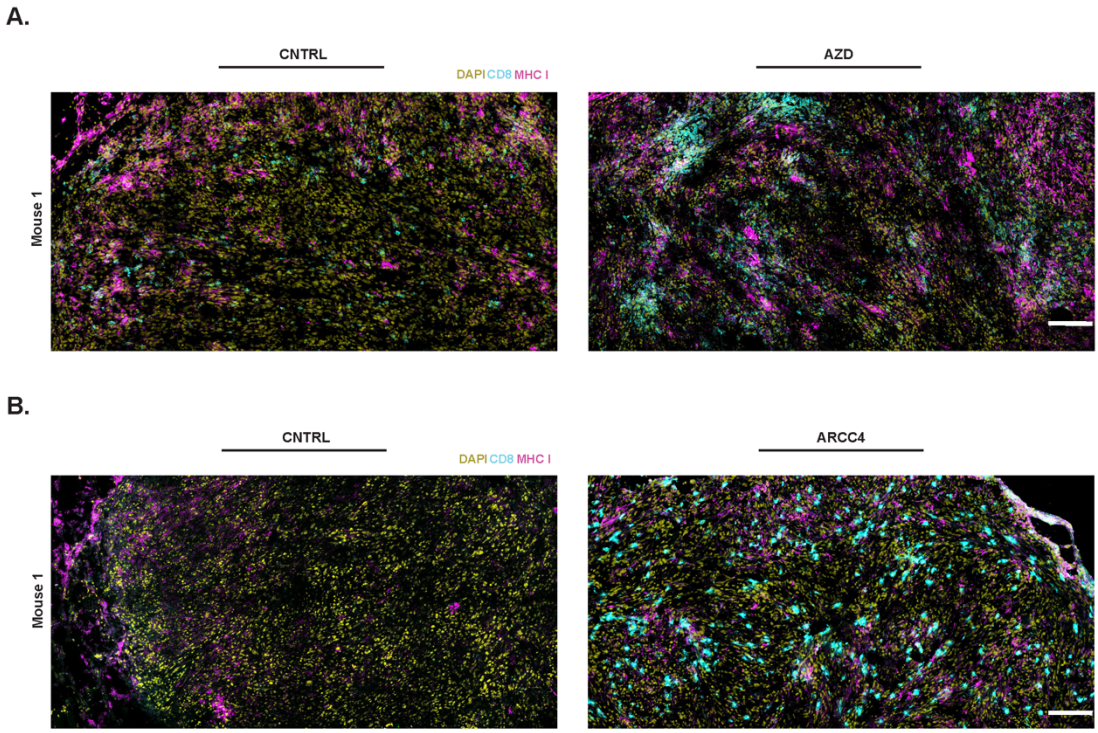
A-B) Immunofluorescence analysis of matched pre-and post- BRAFi/MEKi treatment samples (patients 1 (A) and 2 (B)). Shown are representative low- and high-magnification images of the areas used for single-cell AR expression quantification. Quantification of the AR signal intensity in arbitrary units (AU) per cell (dots) together with a mean, examining >50 cells per field (4 fields per lesion), paired *t*-test, **** $p<0.0001$. Color scale: blue, DAPI; cyan, MelanA; magenta, AR. Scale bar: 20 µm and 10 µm, respectively.



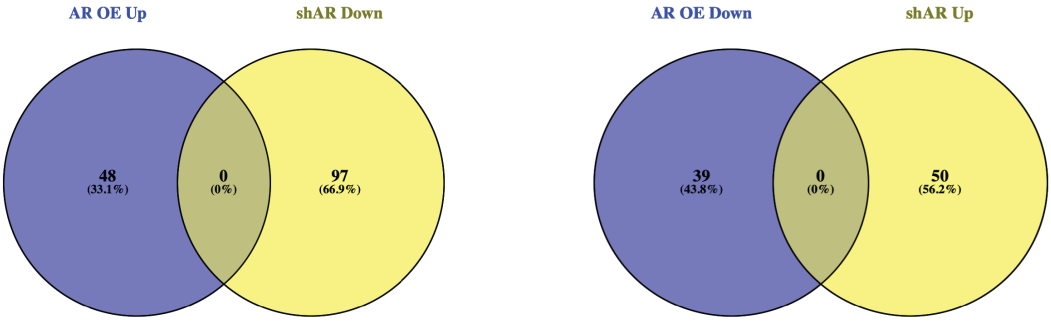
Supplementary Fig. 3: BRAFi treatment induces expression of positive regulators of AR expression. A) Heatmap of expression changes in the indicated transcription factors in three melanoma cell lines (A375, M14, and WM9) plus/minus Dabrafenib (48 hours), as obtained from global transcriptomic profiles (Suppl. Table 2). Z-scores were obtained by mean-centering log2 (expression values) and dividing them by standard deviation. Shown are z-scores for each individual experimental condition.
B) Correlation analysis between *AR* and indicated transcription factors calculated from the TCGA transcriptomic profiles (n=472 primary melanomas and melanoma metastases). Shown are the –log10 (p-value) (bar plots) and the Spearman`s rho coefficient of the correlation between AR and indicated transcription factor expression, as calculated using the corrplot v0.92 package with the Spearman`s correlation method.



Supplementary Fig. 4: Decreased AR protein expression by treatment of melanoma cells with AR inhibitors.
A-B) Immunofluorescence analysis of BRAF inhibitor-resistant melanoma cells (A375BR) treated with the AR inhibitor AZD3514 (10μM) or ARCC4 (1μM) versus DMSO control for 48 hours with anti-AR antibodies with DAPI staining for nuclei visualization. Shown are representative images (A) and quantification (B) of AR signal intensity, as obtained from digitally acquired images and ImageJ analysis, in arbitrary units (AU) per individual cell (dots) together with a mean, examining >100 cells per sample, unpaired t-test, **** $p<0.0001$. Color scale: red, DAPI; cyan, AR. Scale bar: 40 μm. Related to Fig. 5D.



Supplementary Fig. 5: Pharmacological AR targeting alters MHC I surface expression and CD8+ T cell infiltration.
A-B) Immunofluorescence analysis of the excised YUMM1.7BR tumors subjected to a single treatment with AZD3514 (10 μM) (A) or ARCC4 (1 μM) (B), as described in Fig. 6. Shown are representative low magnification images of the MHC I + area and the CD8+ T cells. Color scale: yellow, DAPI; magenta, PE-conjugated anti-MHC I antibody (MHC I-PE); cyan, anti-CD8+ antibody. Scale Bar: 100 μm. Related to Fig. 6D, F.



Supplementary Fig. 6: Comparative analysis of transcriptomic profiles of melanoma cells plus/minus AR gene silencing versus overexpression.
Venn diagrams illustrating common and differentially expressed genes in multiple melanoma cell lines plus/minus *AR* gene silencing as previously reported[9] versus those in melanoma cells plus/minus *AR* overexpression (Suppl. Table 3). Number of significantly modulated genes that were oppositely modulated in the two conditions (FC>2; p-value<0.05) is indicated.

Discussion

1. General Summary and Discussion

1.1. AR-induced Transcriptional Activation of TGF-ß and EGFR Signaling Axes Drives BRAF/MEKi Resistance in Melanoma Cells

In melanoma, the evolutionary selection pressure imposed on cancer cells by both targeted and immunotherapies presents a clinical challenge, as these cells inevitably escape via a plethora of genetic and adaptive mechanisms [136,160,161,171,173]. Drug resistance is likely a gradual multistep process involving broad epigenetic modifications, including DNA methylation, acetylation and methylation of histone tails, and incorporation of histone variants into chromatin, as well as the transcriptional reprogramming of drug-treated cells [136,171,173]. This reprogramming suggests enormous changes in the landscape of transcription factors and cofactors. Sex hormones are known modifiers of epigenetic memory and transcription, and they can have a significant impact on the chromatin binding and activity of other transcription factors through a variety of crosstalk mechanisms [5,8,200]. Surprisingly, the impact of sex hormones in targeted therapy resistance outside of reproductive cancers has been explored only to a limited extent.

A recent study has shown that AR is differentially expressed between males and females during targeted therapy treatment, with its levels being significantly increased in BRAF/MEKi resistant tumors [198]. The study, however, has not fully addressed whether AR is a “driver” or a “passenger” of the targeted resistance. The present work demonstrates that elevated AR signaling drives BRAF/MEKi resistance in cutaneous melanoma via an adaptive mechanism. We find that AR levels rise rapidly during BRAFi treatment and remain persistently elevated in BR cells. As the levels of AR increase during the course of BRAFi treatment, different sets of genes are transcriptionally turned on and off. We find that within the first 48h of BRAFi

treatment canonical AR signaling is elevated in naïve melanoma cells, along with inflammatory and apoptotic pathways. Meanwhile, AR overexpressing cells are predisposed to BRAFi resistance, as their transcriptional response to BRAFi is subverted. Phenotypically, AR overexpressing cells practically abolish BRAFi-induced growth suppression and apoptosis, while maintaining apoptosis, inflammatory, and antigen presentation transcriptional programs at low levels. These pathways are of clinical relevance, as the induction of proinflammatory and cell death programs is necessary to activate the immune system and eradicate tumors [204]. Immune "hot" tumors are more responsive to immune checkpoint blockade [205], and the tumors that relapsed on BRAF/MEKi are cross-resistant to immunotherapies via cancer-cell-instructed mechanisms [206]. These data suggest that AR may be involved in cross-resistance. In line with this idea, our *in vivo* data demonstrated that BR melanoma tumors have low expression of MHC I cell surface antigens and little CD8+ T cell infiltration. However, treatment with AR inhibitors greatly increased MHC I expression and led to the infiltration of CD8+ T cells.

Importantly, AR overexpression under basal conditions was sufficient to change transcriptional programs by inducing signatures for BRAFi resistance, including EGFR and TGF-ß. These pathways were demonstrated to contribute to the emergence of melanoma therapy resistance in multiple clinical and preclinical settings [161,164,171]. EGFR high cells represent a pre-resistant subpopulation of melanoma cells with high invasive properties [171]. Notably, these cells were shown to require transcriptional and epigenetic reprogramming during BRAF/MEKi to become stably resistant. This raises an interesting possibility that already at basal conditions enhanced AR expression increases the frequency of these drug-tolerant cells. Moreover, both EGFR and TGF-ß promote slow-cycling melanoma cells that are capable of escaping the cytotoxic effects of BRAF/MEKi and sustaining proliferation [164]. As slow-cycling cells are clinically and preclinically associated with therapy relapse [207], it is becoming increasingly important to study transcription factors and signaling axes promoting

slow-cycling phenotype. AR activity associated with EGFR and TGF-ß signaling pathways in multiple studies, including studies of BRAFi- resistant melanoma cells, preclinical models of BRAF/MEKi resistance, and in patients studies.

Lastly, AR, EGFR, and TGF-ß signaling axes operate in multiple cellular components to assure immune evasion, metastatic spread, and therapy resistance in multiple cancer types, and their significance in the context of melanoma resistance needs to be further evaluated.

1.2. Can AR Deprivation Therapy be Efficacious in Human Melanoma and Beyond?

There are well-established differences between males and females in cancer incidence, mortality, and therapy response in many cancers [3-8,133,194,200]. These differences can partly be attributed to the circulating sex hormones. The most striking difference in the sex hormone levels seen between males and females outside the menstrual cycle is in circulating androgens. The concentration of free circulating testosterone in healthy males is ten times more than what is detected in healthy females throughout their lifespans [7]. Consequently, male and female tumors develop and evolve in entirely different hormonal environments. These differences might further be enhanced by genetic sex differences, such as loss-of-function mutations in X-linked tumor suppressors, and/or the interplay of both factors.

The AR is expressed in various cell types and AR signaling has been implicated in tumorigenesis in various cancer types, including prostate, breast, bladder, kidney, lung, and liver [84,94,208-214]. Until recently, only a handful of studies have investigated the implications of AR signaling in melanoma. Rampen and colleagues were the first ones to observe male sexual bias in melanoma in 1980 [202]. They found that males are more likely to present with metastatic disease and their prognosis remained poor regardless of the tumor site, tumor thickness, histogenetic subtype, or clinical stage of the disease [201,202]. Since then, sex differences in

melanoma remained largely unexplored. Our previous findings established an important role for basal AR signaling during melanoma cell proliferation, DNA repair, and tumorigenesis in both male and female melanomas [215]. Suppression of AR activity, either genetically or pharmacologically, was shown to limit tumorigenesis of melanoma cells in mice. AR inhibition not only suppressed proliferation but also induced DNA damage. These properties render the inhibition of AR signaling as an attractive target for improved management of treatment naïve melanoma. Our present findings add a new dimension to AR activity in human melanoma, as they demonstrate that AR signaling induces transcriptional reprogramming of melanoma cells to direct induction of invasive and undifferentiated state in drug-resistant human melanoma cells via EGFR and TGF-ß signaling axes. Our data is further strengthened by clinical and preclinical data showing an association between AR activity and BRAF/MEKi resistance in males and females. As a result, BRAF/MEKi elevates AR expression in melanoma cells to promote therapeutic resistance, and AR suppression by the receptor antagonists, such as AZD3514, may be efficacious in both male and female BR patients.

It is important to note, however, that AR signaling has implications beyond metastatic melanoma treatment. Some studies have demonstrated that AR signaling can lead to T cell exhaustion, and blocking AR may improve response to anti-PD-1 immune checkpoint blockade [113,114]. Along with our findings, these studies raise an important possibility that AR signaling plays multiple different roles in the TME. In accordance with this, we have previously shown that not only elevated AR levels play a role during cancer progression but also AR loss can have detrimental consequences in the tumor stroma of various skin cancer types, including squamous cell carcinoma, basal cell carcinoma, and melanoma [216]. AR downregulation promoted the conversion of dermal fibroblasts into tumor-supporting cancer-associated fibroblasts (CAFs), which can stimulate the growth of cancer. Thus, it is important to consider these differences prior to the use of AR inhibitors in the clinic, as AR blockade may suppress

tumor growth and increase T cell activation while inadvertently promoting CAF conversion. Moreover, treatment with AR inhibitors without hypothalamic suppression may result in increased AR signaling and higher testosterone levels. It is also important to consider that cancer cells might become resistant to AR suppression by means of various mechanisms such as, nongenomic and ligand-independent signaling, expression of AR splice variants, and increased expression of sex hormone receptors. Nevertheless, AR targeting may be beneficial in the treatment regimens with BRAF/MEKi-targeted therapy and immune checkpoint inhibitors.

2. Future Perspectives

2.1. Clinical and Preclinical Perspectives

From the earliest stages of embryological development, male and female cells are different in their cell cycle, metabolism, and epigenetic profiles [1,3-5,200]. These sex differences are likely to persist in cancer and may influence multiple hallmarks of cancer. To fully understand the interplay between sex hormones, sex chromosomes, and cancer, a more in-depth analysis of clinical and preclinical cohorts is urgently needed. It is necessary to investigate changes in physiological sex hormone levels in cancer and during aging. For that, it is important to consider using age-appropriate animal models that will better recapitulate human disease. Moreover, circulating androgens, estrogens, and progesterone should be considered for disease monitoring, as their levels may serve as predictive biomarkers and their levels can be adjusted using hormone therapies. The inclusion of important parameters, such as the use of oral contraceptives, should be considered during clinical trial designs in nonreproductive cancers, as they may influence the response to therapy. Additionally, a deeper understanding of the relationship between sex hormones and the gut microbiome in men and women is needed, as it may facilitate our understanding of resistance mechanisms to immune checkpoint inhibitors.

Overall, a better understanding of the impact of sex hormones and sex chromosomes on cancer development and progression may lead to improved prevention, diagnosis, and treatment strategies for both men and women.

2.2. Biological Perspectives

Recent research has revealed that androgen receptor (AR) signaling plays a crucial role in multiple cell types present in the tumor microenvironment (TME) [113,114,198,215,216]. As AR has opposing functions in these cells, it is essential to study its signaling in different tissues and cell types. Similarly, the landscape of AR coregulators remains largely unexplored. As AR activity is already strikingly different between treatment naïve, short-term BRAFi-treated and therapy-resistant melanoma cells, it is entirely possible that AR employs different coregulators to induce its adaptive transcriptional changes. The identification of the key coregulators of AR could be clinically relevant. AR interacts with coregulators either through LBD or through the NTD, and specific blockers of AR LBD or NTD could be used. Alternatively, pharmacological inhibitors of the identified coregulator could be used, when available. Moreover, it is crucial to explore the role of estrogens and progesterone during BRAF/MEKi, as different sex hormones and sex hormone receptors could interact in this process. For instance, DBDs of AR, GR, and PR are structurally similar and both can bind similar DNA motifs [54]. Therefore, it is entirely plausible that PR and GR can induce similar transcriptional changes in melanoma. Lastly, the present study revealed that BRAFi-induced AR signaling can convert cells from a treatment-naïve state to an invasive, undifferentiated state, suggesting that elevated AR activity could promote cancer cell migration and eventual metastatic dissemination. Once metastasized to a distant site, the transcriptional program of AR high melanoma cells could be further adjusted by the circulating androgens.

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