ZIP9 is a Druggable Determinant of Sex Differences in Melanoma

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Abstract

Melanoma and most other cancers occur more frequently, and have worse prognosis, in males compared with females. Though sex steroids are thought to be involved, classical androgen and estrogen receptors are not detectable in most melanomas. Here we show that testosterone promotes melanoma proliferation by activating ZIP9 (*SLC39A9*), a zinc transporter that is not intentionally targeted by available therapeutics, but is widely expressed in human melanoma. This testosterone activity requires zinc influx, MAPK activation and YAP1 nuclear translocation. We demonstrate that FDA approved inhibitors of the classical androgen receptor also inhibit ZIP9, and thereby antagonize the pro-tumorigenic effects of testosterone in melanoma. In male mice, androgen receptor inhibitors suppressed growth of ZIP9-expressing melanomas, but had no effect on isogenic melanomas lacking ZIP9, nor on melanomas in females. These data suggest that ZIP9 might be effectively targeted in melanoma and other cancers by repurposing androgen receptor inhibitors that are currently approved only for prostate cancer.

Introduction

Cancer incidence and mortality are higher in males than in females in the U.S. and worldwide^{1,2}. In the U.S., males are 15% more likely to develop cancer, and 40% more likely to die from this disease than females¹. These sex differences were recognized as early as 1949³, are observed in the majority of cancer types from non-reproductive tissues¹, and remain even after controlling for known risk factors such as environmental and occupational exposures⁴. While recent advances in modern targeted and immune therapeutics have markedly improved survival for both female and male melanoma patients⁵, females still have more favorable outcomes^{1,4}. Defining the mechanisms underlying the broad and persistent sex differences in cancer incidence and outcomes will address a major unresolved question in cancer pathobiology.

We previously showed that the female sex hormone estradiol inhibits melanoma proliferation and tumor growth *in vivo*. This effect is independent of biologic sex of the tumor, independent of classical estrogen receptors (ER), and results from activation of a nonclassical surface estrogen receptor on melanocytes and melanoma cells called the G Protein Estrogen Receptor (GPER)^{6,7}. This work led us to consider whether male sex hormone signaling might also contribute to female vs. male differences in melanoma progression.

Testosterone is the most abundant androgen and circulates at much higher levels in males (630 ng/dl) than in females (32 ng/dl)⁸. This androgen promotes proliferation *in vitro* in non-gonadal cell types including adipocytes⁹, mouse skeletal muscle myoblasts¹⁰, glioblastoma-derived cells¹¹, lung cancer cell lines¹² and melanoma¹³. As the classical androgen receptor (AR) is not consistently detected in most of these tissues, the receptor(s) mediating the testosterone-dependent increased proliferation and the corresponding downstream mechanisms are not yet

defined. Also unknown is whether this androgen activity observed *in vitro* is relevant to cancer progression *in vivo*.

Here, we define a nonclassical testosterone signaling pathway that establishes a novel mechanistic link between male androgens and melanoma behavior, and highlights a new therapeutic opportunity using currently-available drugs.

Results

Melanoma tumors grow more quickly in male vs. female mice

To test whether preclinical melanoma models recapitulate the male vs. female survival disparity observed in humans, we first determined growth of human BRAF-driven melanoma (WM46, female origin, BRaf^{V600E}; CDK4^{R24C}). Tumors grew faster in immunodeficient SCID male mice compared with matched female mice (Fig. 1A and Fig. S1A), indicating that the differences do not depend on T and/or B cell immune responses. To test whether this phenotype extends to a genetically-engineered preclinical mouse model, we used murine YUMM1.7 cells (male origin, BRaf^{V600E/wt};Pten^{-/-}CdkN2a^{-/-})¹⁴ in syngeneic immunocompetent C57BL/6 mice. These tumors also consistently grew faster in male mice compared with matched females (Fig. S1B).

Testosterone, but not dihydrotestosterone, promotes proliferation in melanoma cells independent of the classical androgen receptor

Physiologic concentrations of testosterone promoted proliferation of both normal early passage primary human melanocytes and melanoma cells (Fig. 1B, C and Fig. S1C). The proliferative response to testosterone correlated to the dose and was saturable, suggesting a specific receptor-mediated activity. In contrast, dihydrotestosterone (DHT) had no effect on proliferation of these

cell types across a wide concentration range (Fig. 1C). Dihydrotestosterone is a more potent AR agonist than testosterone^{15,16}. The fact that the melanocytes and melanoma cells responded to testosterone, but not to DHT (Fig. 1B, 1C, Fig S1D, E), suggests the possibility that a nonclassical androgen receptor mediates testosterone effects in these cells. Supporting this idea, we were unable to detect AR mRNA transcript in any of the 7 melanoma cell lines (Table S1) and primary melanocyte (MC) cultures we tested, although AR mRNA was readily detectable in prostate cancer cells (Fig. 1D, Fig. S1F). Consistent with this lack of AR transcript, we were also unable to detect AR protein in these cells via western blotting using 2 different AR antibodies, or via immunofluorescence directly on the cultured cells, although AR protein was readily detectable in 2 prostate cancer lines used as positive controls (Fig. 1E and Fig. S1G).

To begin testing whether a nonclassical androgen receptor mediates testosterone effects in melanoma we looked to *SLC39A9*/ZIP9, a transmembrane zinc transporter recently discovered in Atlantic Croaker (fish) cells to be activated by androgen, and unlike AR, to have much higher affinity for testosterone than for dihydrotestosterone¹⁷. ZIP9 transports zinc (Zn^{++}) across cell and organelle membranes into the cytoplasm and is the only steroid receptor known to directly regulate zinc homeostasis¹⁸. The human ZIP9 homolog, encoded by the *SLC39A9* gene, is broadly expressed in most cancers (Fig. S2A), normal human melanocytes, and all melanoma lines that we tested (Fig. 2A). To test whether ZIP9 was active in melanoma we used the fluorescent Zn^{++} -specific probe FluoZin-3. Testosterone induced a rapid increase in cytosolic free Zn^{++} ($[Zn^{++}]_i$) in human melanoma cells (Fig. 2B), that was followed by a sustained elevation for at least 2 days in the presence of testosterone (Fig. 2C). We determined that Zn^{++} influx was necessary for the testosterone-dependent increase in melanoma cell proliferation, as treatment with the Zn^{++} chelator N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine

(TPEN), blocked testosterone induced proliferation at TPEN concentrations that had no significant effect on proliferation when used alone (Fig. 2D and Fig. S2B).

Although TPEN has high affinity for Zn^{++} , it also has some ability to chelate copper¹⁹. To test whether copper flux might contribute to testosterone effects on melanoma proliferation, we treated WM46 cells with $CuSO_4$, which had no significant effect on cell proliferation (Fig. S2C). Moreover, the highly specific copper chelator bathocuproinedisulfonic acid (BCS), did not affect the proliferative response to testosterone, nor did BCS affect testosterone induced Zn^{++} influx (Fig. S2D and Fig. S2E).

After determining that Zn^{++} influx was necessary for testosterone-induced proliferation, we next tested whether increased Zn^{++} was sufficient to increase melanoma proliferation. Exogenous zinc pyridine (also a Zn^{++} ionophore), and $ZnCl_2$ each increased melanoma proliferation (Fig. 2E). Other biologically-relevant divalent cations, Fe^{++} and Mn^{++} , known to be transported by other ZIP family members²⁰, had no effect on proliferation (Fig. 2E). Together, these results demonstrate that testosterone, but not DHT, promotes zinc dependent proliferation in melanoma cells that express ZIP9 and lack detectable AR.

We next used immunocytochemistry to test whether AR and/or ZIP9 protein are expressed in human melanocytic lesions (14 benign nevi, 63 primary melanomas, and 21 metastatic melanomas). AR was determined in tissue sections using a highly validated CLIA (Clinical Laboratory Improvement Amendments) certified method in the clinical pathology lab at the Hospital of the University of Pennsylvania. This procedure uses a validated antibody different from the two we used for western blotting and immunofluorescence. Each tissue was scored in a blinded fashion by a board-certified pathologist. While AR was readily detectable in prostate tissue used as positive control, AR was not detected in any of the nevi, nor in the

melanomas (Fig. 2F). In parallel, we analyzed the same samples for ZIP9. CLIA grade ZIP9 IHC is not available. However, we validated our ZIP9 antibody using parental WM46 ZIP9 positive (wtZIP9) and isogenic ZIP9 negative (Δ ZIP9) cell lines. These cells were grown on chamber slides and processed for IHC in parallel with the human samples. The antibody used labeled only the wtZIP9 cells. ZIP9 protein was observed in 100% of the nevi, 97% of primary melanomas and 100% of the metastatic samples (Fig. 2G). Further, ZIP9 relative staining intensity positively correlated with tumor stage (Fig. 2H). No significant differences in ZIP9 staining were observed between males and females (Fig. S3).

Testosterone signaling through ZIP9 promotes melanoma growth

To determine whether ZIP9 mediates testosterone effects in our models, we used CRISPR-Cas9 and gRNA targeting Slc39A9 exon1 to ablate ZIP9 in human (WM46) melanoma and in murine (YUMM1.7) melanoma. Isogenic clonal populations of parental ZIP9-expressing (wtZIP9) and ZIP9-ablated (Δ ZIP9) cells were established, and the mutations disrupting the reading frame were mapped by sequencing (Fig. S4A, S4B and S4C). ZIP9 protein was not detectable in Δ ZIP9 cells (Fig. 2F, Fig. 3A, Fig. 3B and Fig. S4D). Δ ZIP9 cells did not respond to testosterone, whereas isogenic wtZIP9 control clones responded similarly to the parental wtZIP9 cells (Fig. 3A, 3B and Fig. S4E).

The testosterone insensitive phenotype associated with CRISPR-Cas9 engineered ZIP9 loss was rescued by expression of a human ZIP9 transgene. Lentiviral mediated constitutive ZIP9 expression in the Δ ZIP9 WM46 cells restored both ZIP9 protein and the proliferative response to testosterone, verifying the on target effects of the CRISPR-Cas9 mediated ZIP9 ablation (Fig. S4F, S4G).

Consistent with the conclusion that ZIP9 is the major mediator of testosterone and exogenous zinc effects in these melanoma cells, exposure to testosterone or to extracellular zinc failed to increase $[Zn^{++}]_i$ in Δ ZIP9 cells (Fig. 3C and 3D). Therefore, testosterone induces a rapid, ZIP9-dependent, increase in $[Zn^{++}]_i$ that is required for the increased proliferation in melanoma cells.

Testosterone-induced melanoma proliferation through ZIP9 requires downstream MAPK and YAP1 activation

Although the studies detailed above show that testosterone promotes melanoma proliferation via ZIP9 dependent Zn^{++} influx, zinc is involved in myriad cellular processes, making it difficult to predict *a priori* which downstream signaling pathways are required for the testosterone activity. To start identifying these, we used a 447-element Reverse Phase Protein Array (RPPA) analysis of human WM46 melanoma cells treated with testosterone for 0, 30, 60 and 480 min. While the relative expression of most represented proteins was unaffected by testosterone, some were significantly under or overexpressed, including several with known tumor-promoting or tumor-suppressive functions (Fig. 4A and Table S2). Downregulated proteins included 14-3-3 ϵ , a tumor suppressor and negative YAP1 regulator previously implicated in liver, lung, and gastric cancers²¹, and CDKN2A (p16), a cyclin-dependent kinase (CDK) inhibitor, and one of the most studied tumor suppressors²². Upregulated proteins included key elements of tumor-promoting pathways, most notably phosphorylated ERK (T202; Y204) and YAP1 (Fig. 4A). Importantly, Δ ZIP9 cells displayed decreased basal levels of ERK phosphorylation compared with isogenic wild-type clones (Fig. S5A). MAPK activation was necessary for the testosterone-dependent proliferative response, as the specific ATP-competitive ERK1/2 inhibitor, ulixertinib²³

(RVD-523; Ei) blocked the testosterone-dependent proliferative response, at ulixertinib concentrations that had no effect on the basal proliferation rate when used alone (Fig. 4B). Testosterone-dependent ZIP9 activation appeared to render WM46 cells more sensitive to ulixertinib, as cell viability was compromised when they were treated with both testosterone and RVD-523. This vulnerability to combination treatment seems to be specific and ZIP9 dependent, as Δ ZIP9 cells treated with both testosterone and ulixertinib proliferated at rates comparable to controls (Fig. 4B).

We determined that YAP1 activation is also required for the augmented proliferative response driven by testosterone. YAP1 is a transcriptional coactivator whose activity is largely regulated by its localization²⁴. Testosterone induced rapid YAP1 translocation from cytoplasm to nucleus in human melanoma cells in a ZIP9-dependent manner (Fig. 4C, 4D and Fig. S5B). YAP1 subcellular localization is controlled largely by LATS, which phosphorylates YAP1 at Serine 127 and thereby retains YAP1 in the cytoplasm²⁵. The YAP1 inhibitor dobutamine also promotes phosphorylation of YAP at Ser127²⁶. Consistent with this, the testosterone induced YAP1 nuclear localization and increase in cell proliferation were both blocked by dobutamine, while this compound had no significant effect on its own (Fig. 4D, Fig. S5C and Fig. 4E). When ZIP9 expression was rescued via lentiviral transduction of a ZIP9 transgene to Δ ZIP9 WM46 cells, testosterone dependent YAP1 translocation into the nucleus was also restored (Fig. S5C).

Testosterone promotion of YAP1 activity was evidenced by increased expression of several well-known YAP1 target genes including CDC6, CTGF, CYR61 and THBS1. As expected, these testosterone-induced expression changes were also blocked by dobutamine (Fig. 4F).

Pharmacologic ZIP9 blockade inhibits testosterone-driven melanoma proliferation and melanoma tumor growth *in vivo*.

We next tested whether FDA-approved compounds could be repurposed to effectively target ZIP9. Although specific ZIP9 inhibitors are not yet developed, molecular modeling and competition assays with fluorescent-tagged testosterone suggest that the androgen receptor inhibitor bicalutamide (BIC) competes with testosterone for ZIP9 binding to the same extracellular pocket and thereby acts as a competitive ZIP9 antagonist²⁷. Bicalutamide was developed and approved for prostate cancer and has now been largely replaced by enzalutamide (ENZ) and apalutamide (APA), which are structurally related analogs with higher affinity for the androgen receptor and greater clinical efficacy against advanced prostate cancer^{28,29}.

While ZIP9 was not known to be an androgen receptor at the time these drugs were developed, we show here that they are nonetheless effective inhibitors of testosterone effects in melanoma cells that express ZIP9, but lack detectable AR. Each compound completely blocked testosterone-induced proliferation and MAPK activation in several melanoma cell lines (Fig. 5A, 5B). Importantly, these agents alone (without testosterone) had no effect on cell proliferation (Fig. S6A). The testosterone dependent increase of intracellular zinc was also efficiently blocked by bicalutamide (Fig. S6B). Moreover, Δ ZIP9 cells transduced to re-express ZIP9, responded to testosterone, and this effect was again blocked by bicalutamide (Fig. S6C).

To further confirm that these pharmacologic agents work through ZIP9, we next used cyproterone acetate (CPA), an anti-androgen that blocks the testosterone interaction with AR, but that does not bind to ZIP9³⁰. In our melanoma cells, up to a 20-fold molar excess of CPA did not significantly inhibit testosterone effects on proliferation, consistent with the conclusion that AR is not the major mediator of testosterone effects in these melanoma models (Fig. S6D).

To test whether these pharmacologic flutamide class AR inhibitors block the physical interaction between testosterone and ZIP9 in melanoma, we performed direct binding assays using a membrane-impermeable testosterone analogue (T-BSA-FITC). This reagent labels the plasma membrane surface of wild-type ZIP9 expressing WM46 melanoma cells (Fig. 5C). This membrane bound testosterone was displaced by apalutamide, demonstrating the specificity of the interaction (Fig. 5C). Further demonstrating that the binding is ZIP9 specific, testosterone localization at the plasma membrane was markedly reduced in Δ ZIP9 cells (Fig. 5C) that did not respond to either testosterone, or to APA in vitro (Fig. 5D).

Next, we tested whether apalutamide and related analogs inhibit melanoma *in vivo*. For this, we introduced wtZIP9 or isogenic Δ ZIP9 human WM46 melanoma into SCID mice. Once-daily systemically administered apalutamide (20 mg/kg/day via oral gavage) significantly suppressed growth of wtZIP9 tumors in male mice and extended its survival (doubling time for tumor growth was 11.1 days for vehicle treated males and 25.2 days for APA treated males) (Fig. 5E, Fig. S7A). Similar results were obtained when males were treated with bicalutamide (30mg/kg/day) (Fig. S7B). Importantly, bicalutamide had no effect on wtZIP9 tumors in female mice, nor on Δ ZIP9 tumors (Fig. S7C and S7C). Together, these data show that ZIP9 promotes melanoma progression specifically in males.

Discussion

Although many have speculated that sex steroids contribute to sex differences in cancer pathobiology, definitive functional studies are lacking, and the mechanisms by which they contribute to the male-female cancer sex gap are largely unknown. We previously described the tumor suppressor activity of the nonclassical estrogen receptor GPER in melanoma⁶. This

finding led us to question whether circulating androgens in males might also contribute to the melanoma sex gap.

Testosterone promoted proliferation of melanoma cells in a saturable-dose-dependent manner. Curiously, the same cell lines did not respond to DHT, which is generally considered the more biologically active androgen. DHT displays 4-fold increased affinity than testosterone for AR, and it dissociates from AR three time slower than testosterone¹⁶. Lack of response to DHT in melanoma is consistent with the absence of detectable AR expression in the models used for this study, and is highly consistent with our genetic data showing that ZIP9, which has higher affinity for testosterone vs. DHT, is an important mediator of testosterone effects.

We show that ZIP9 activation via testosterone binding promotes an increase in cytosolic zinc in melanoma cells. The mammalian family of zinc transporters SLC39A comprises 14 members (ZIP1–14)³¹ grouped into four subfamilies that were established according to their amino acid sequence similarities. There may be roles for these other family members in some cancers, as ZIP1 (SLC39A1, from ZIPII subfamily) that has been associated with the regulation of zinc uptake in prostate cancer cells³². However, this rapid increase of intracellular zinc through ZIP1 appears to be AR dependent, as PC-3 cells only respond to testosterone after they are transfected with exogenous AR. ZIP9 amino acid residues predicted to be most critical for testosterone and bicalutamide binding include Ala167, Val241, Met248, and Ala167, Leu302, Ser171, respectively²⁷, however only Ala167 is present in ZIP1, suggesting that this receptor may not be similarly regulated by testosterone. Regarding other members of the SLC39A family, ZIP9 is unique member of ZIPI subfamily³³, and the only one known to directly interact with testosterone.

A recent report³⁴ also considered a possible role for testosterone in melanoma and concluded that the androgen promotes melanoma. Authors attributed this effect to the classical AR. However, that report did not consider ZIP9, did not show that AR was necessary for melanoma response to pharmacologic AR inhibitors, and did not show that AR was a determinant of sex differences in melanoma tumor growth. Here we studied 98 human melanocytic lesions (nevus, primary and metastatic melanoma) and did not detect AR in any of them, while ZIP9 protein was detectable in 97% and 100% of the primary and metastatic melanomas respectively. We recognize the possibility that some melanoma cell lines and perhaps even some human tumors, may express AR that also contributes to melanoma growth.

There may be many drivers of the cancer sex gap in humans, including differences in immune surveillance³⁵. However, differences in immune surveillance do not appear to be a major driver of the differences in the melanoma models used for this study, as tumors progressed faster not only in male vs. female immunocompetent mice, but also in human melanomas grown in male vs. female SCID mice. Therefore, the testosterone effects on melanoma in these models are dependent on ZIP9, but independent of B and T cell mediated anti-tumor activity. Consistent with this, ZIP9 expressing tumors respond to bicalutamide and apalutamide in SCID mice.

As ZIP9 is widely expressed in nearly all tissues, it may be a major determinant of the sex disparity in outcomes not just for melanoma, but also for many other cancer types. Consistent with this, we observed that testosterone promotes proliferation in many diverse cancer cell types, and that this effect is blocked by bicalutamide (Fig. S8).

The demonstration here that ZIP9 is pharmacologically accessible, suggests that ZIP9 may be a new eminently druggable therapeutic target (Fig. 5F), and that currently approved androgen receptor inhibitors might be useful alone, or in combination with current standard of

care therapeutics for a wide range of cancers, especially those that disproportionately affect males.

Materials and Methods

CRISPR-Cas9 mediated ablation of Slc39A9

We used lentiviral transduction to deliver dox-inducible Cas9 and gRNA targeting exon 1 of Slc39A9 in human WM46 and murine YUMM1.7 melanoma cells. Transduced cells were selected with puromycin, and single cells subsequently isolated, expanded and examined for ZIP9 protein expression, compared to clones isolated in parallel with no doxycycline treatment.

The following gRNA sequences were used (5'-3'):

hZIP9_gRNA_Fw caccgTTGGTGGGATGTTACGTGGC

hZIP9_gRNA_Rv aaacGCCACGTAACATCCCACCAAC

hZIP9_gRNA_Fw caccgCGTGGCCGGAATCATTCC

hZIP9_gRNA_Rv aaacGGAATGATTCCGGCCACG

To map the targeted sequencing, the region surrounding the gRNA target sequence was amplified in both WM46 (282bp) and YUMM1.7 (259bp) isogenic clones. The following

primers were used (5'-3'): hZIP9_CRISPRmut_Fw:

TAAGCAGAATTCATGGATGATTTTCATCTCC

hZIP9_CRISPRmut_Rv:TAAGTAAGTCCAAGCTTCTGCTGCTTTGTCTGATGCA.

mZIP9_CRISPRmut_F:TAAGCAGAATTCATGGATGACTTTCTCTC.

mZIP9_CRISPRmut_Rv TAAGTAAGTCCAAGCTTGATATTTCTGCTGCTTTGT.

Once amplified, DNA fragments were cloned into pUC19 vector using EcoRI and HindDIII sites, and sequenced (Sanger) by the DNA Sequencing Facility (University of Pennsylvania). Sequences were analyzed using the free software CRISP-ID (V1.1) and ICE Analysis by Synthego were the knock-out scores were obtained.

Cell culture and proliferation assays

YUMM1.7, SH-4 and SK-MEL-2 cells were purchased from ATCC (YUMM1.7 ATCC® CRL-3362™; SH-4 ATCC® CRL-7724™; SK-MEL-2 ATCC® HTB-68™) and cultured in DMEM (Mediatech, Manassas, VA, USA) with 5% FBS (Invitrogen, Carlsbad, CA, USA) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific. # 15140122). SK-MEL-3 cells were purchased from ATCC (ATCC® HTB-69™ and cultured in McCoy's 5A (Modified) Medium with 10% FBS (Invitrogen, Carlsbad, CA, USA) and 1% Penicillin-Streptomycin. SK-MEL-24 cells were purchased from ATCC (ATCC® HTB-71™) and cultured in Eagle's Minimum Essential Medium with 15% FBS and 1% Penicillin-Streptomycin. WM46 melanoma cells were a gift from Meenhard Herlyn (Wistar Institute, Philadelphia, PA, USA) and were cultured in TU2% media. Tumor cells were regularly tested using MycoAlert Mycoplasma Detection Kit from Lonza (Allendale, NJ, USA). For monitoring cell proliferation 10×10^5 YUMM1.7 or 12×10^5 WM46 cells were seed per well in 12-well Cell culture Plates. All the experiments performed in this work utilized charcoal stripped serum (Fetal Bovine Serum, charcoal stripped, USDA-approved regions, One Shot™ format. Catalogue number #A3382101; Thermofisher Scientific). Cells were treated every second day and manually counted in triplicate using a hemocytometer. All the experiments were performed in cell populations that were in culture during a maximum of 3 weeks (5 passages in average) since thaw from the corresponding stock.

Zinc influx analysis

WM46 cells were loaded with 5 μ M FluoZinTM-3, AM cell permeantTM (Thermo Fisher Scientific, #F24195), for 20 minutes then incubated in 2 Ca Tyrode's solution (in mM: 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose and 5 (Na) Pyruvate – pH 7.4) for 5-10 minutes at room temperature prior to imaging. Cells were imaged on a Nikon Ti microscope using a 20x/0.75 NA objective for fluorescence at 340 nm and 380nm excitation/515 nm emission (Ca²⁺-free Fura2 or FluoZinTM-3) and 380 nm excitation/515 nm emission (Ca²⁺-free Fura2 FluoZinTM-3). Coverslips were perfused at 1-3 mL/min following this protocol: Ca²⁺ Tyrode's (0-30 secs); Ca²⁺ Tyrode's + DMSO & 5 μ M Zinc (30-90 secs); Ca²⁺ Tyrode's + Testosterone & 5 μ M Zinc (90-360 secs); Ca²⁺ Tyrode's + Testosterone + BIC & 5 μ M Zn (360-470 secs); Ca²⁺ Tyrode's washout (470-500 secs). 100 milliseconds exposure images for each wavelength were collected every 2 seconds. For analyzing the long-term consequences of testosterone treatment, cells were treated with the androgen for 96 hours and then loaded FluoZinTM-3. Cells were incubated with FluoZinTM-3 following manufacturer recommendation and exposed to 400nM Zn pyrithione or ZnCl₂. Images were acquired with EVOS BLA. Fluorescence was quantitated using ImageJ (National Institutes of Health, Bethesda, MD, USA), and statistical analyses were performed using Graphpad Prism software.

Reverse Phase Protein Array (RPPA)

We used the Functional Proteomics Core at MD Anderson (<https://www.mdanderson.org/research/research-resources/core-facilities/functional-proteomics-rppa-core.html>) to perform a 447-element Reverse Phase Protein Array (RPPA) analysis of human melanoma cells grown in medium with stripped serum and treated with testosterone

(100nM) for 0, 30', 60' and 8 hours. Cells were trypsinized and washed with PBS. After centrifugation (5 minutes; 1200rpm), supernatant was discarded, and cells were frozen at -80°C prior to be sent to the Functional Proteomics Core at MD Anderson.

Western blot, immunofluorescence and antibodies

Adherent cells were washed once with PBS and lysed with 8M urea containing 50mM NaCl and 50mM Tris-HCl, pH 8.3, 10mM dithiothreitol, 50mM iodoacetamide. Lysates were quantified (Bradford assay), normalized, reduced, and resolved by SDS gel electrophoresis on 4–15% Tris/Glycine gels (Bio-Rad, Hercules, CA, USA). Resolved protein was transferred to PVDF membranes (Millipore, Billerica, MA, USA) using a Semi-Dry Transfer Cell (Bio-Rad), blocked in 5% BSA in TBS-T and probed with primary antibodies recognizing β -actin (Cell Signaling Technology, #3700, 1:4000, Danvers, MA, USA), ZIP9 (Abcam, #137205, 1:500), P-ERK (Cell Signaling Technology, Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370, 1:1000), ERK (Cell Signaling Technology, p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb #4695, 1:1000), Androgen Receptor [(D6F11) XP® Rabbit mAb #5153] and Recombinant Anti-Androgen Receptor antibody [EPR1535(2)] (ab133273. Antibody already validated by the Human Protein Atlas). After incubation with the appropriate secondary antibody [(Rabbit Anti-Mouse IgG H&L (Biotin) preabsorbed (ab7074); Anti-mouse IgG, HRP-linked Antibody #7076, 1:2000)] proteins were detected using Clarity™ Western ECL Substrate (Bio-Rad, #170-5060). All western blots were repeated at least 3 times. To monitor YAP1 nuclear translocation by western blot analysis, nuclear fractionation was performed using protein lysis buffer containing 10mM HEPES, 1mM KCl, 1.5mM MgCl₂ and 10% glycerol (Buffer A). After washing the adherent cells with DPBS, samples were resuspended in Buffer A and incubated for

5 minutes on ice in the presence of 0.1% Triton-X-100. After centrifugation, the nuclear fraction remained as a pellet while the supernatant corresponding to the cytosolic fraction. Nuclear fraction was washed with Buffer A. After centrifugation, the nuclear fraction was resuspended in RIPA buffer and samples were boiled for 5 min prior to sample loading. The cytosolic fraction was centrifuged for 15 min at 13500 rpm. Only the supernatant was kept after centrifugation. Western blot was performed as described before and the following antibodies were used for protein detection: β -tubulin (Cell Signaling Technology, β -Tubulin (9F3) Rabbit mAb #2128. 1:1000), PARP (Cell Signaling Technology, Rabbit mAb #9542. 1:1000), YAP1 (Cell Signaling Technology, YAP (D8H1X) XP® Rabbit mAb #14074. 1:1000). For immunofluorescence in adherent cells, samples were fixed with 4% paraformaldehyde (Affymetrix; #19943) for 7 min at room temperature and permeabilized with iced-cold methanol. After blocking with 10% FBS:0.03%Triton-X100, primary antibodies were incubated overnight in blocking solution (β -actin and YAP1 antibodies detailed above). After three washes in DPBS:0.03% Triton X-100) cells were incubated with secondary antibodies for 45 minutes at room temperature [(Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594. Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488)]. Cells were rinsed with PBS-0.03%-Triton three times and coverslips were mounted with ProLong Gold antifade reagent with DAPI (#P36935; Thermo Fisher Scientific). Images were captured using a Leica DM IL microscope and registered using LAS software. For fluorescence intensity quantification ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used, and statistical analyses were performed with GraphPad Prism software.

Immunohistochemistry and quantification

FFPE tissue microarrays (ME1004h: Malignant melanoma, metastatic malignant melanoma and nevus tissue array) were obtained from US Biomax, Inc. (Derwood, MD). For the staining with anti-ZIP9 antibody [(SLC39A9 Antibody (PA5-52485), Thermofisher Scientific, Waltham, MA)], slides were deparaffinized and rehydrated following the standard immunohistochemistry protocol [(xylenes 5 minutes x 3, 100% alcohol (5 min. x 3), 95% alcohol (5 min.), 80% alcohol (5 min.), 70% alcohol (5 min.), and 50% alcohol (5 min.) and finished with distilled water)]. The antigen retrieval was done by loading the slides into a retriever (Electron Microscopy Sciences EMS) with R-Buffer A. After 20 minutes, samples were allowed to cool for 30 minutes inside the retriever and for 20 minutes at room temperature. Samples were washed twice with PBS and blocked with Dako Dual Endogenous Enzyme Block (Code S2003. Agilent Santa Clara, CA) for 20 minutes at R.T. Samples were washed twice with PBS and blocked for 20 minutes with 2 drops of Vector Avidin Block. After washing twice with PBS, slides were blocked for 20 minutes with two drops of Vector Biotin Block (Avidin/Biotin Blocking kit. SP-2001. Vector Laboratories, Inc. Burlingame, CA). Samples were washed twice with PBS with Protein Block Serum-Free Ready-To-Use for 30 minutes at R.T. (Code X0909. Agilent Santa Clara, CA). Primary antibody was prepared 1:500 in PBST (100µl per slide) and samples were incubated overnight at 4°C. Samples were washed three times with PBS and incubated with Biotinylated Secondary antibody (Vectastain Kit, Peroxidase Rabbit IgG, PK-4001) for one hour at R.T. After three washes with PBS, ABC reagent (prepared 30 minutes in advance) was added and samples were incubated for 30 minutes at R.T. Samples were washed twice with PBS and incubated for 3 minutes with ImmPACT® DAB Substrate, Peroxidase (HRP) (SK-4105. Vector laboratories, Burlingame, CA). Tissues were counterstained with hematoxylin (30 seconds, R.T.) (GHS316.

Sigma-Aldrich) dehydrated, and mounted with SecureMount (Fisher HealthCare™ PROTOCOL™ Mounting Media. #022-208. Fisher Scientific. Thermofisher Scientific). John T. Seykora M.D., Ph.D, performed scoring of the stained tissue microarray, and scoring index was determined by scoring the percentage of positive cells on a scale of 0 to 3 as well as the intensity of ZIP9 staining on a scale of 0 to 4 (1=1-25%, 2=26-50%, 3=51-75%, 4=76-100%).

The staining of the tissue microarrays (ME1004h) for AR detection was performed by University of Pennsylvania Pathology Clinical Service Center—Anatomic Pathology Division, using the highest grade, CLIA (Clinical Laboratory Improvement Amendments) certified and validated test available. Briefly, five-micron sections of formalin-fixed paraffin-embedded tissue were stained using antibody against Androgen Receptor [(Leica AR-318-L-CE, clone AR27 (clone AR27, 1:25)]. Staining was done on a Leica Bond-III™ instrument using the Bond Polymer Refine Detection System (Leica Microsystems DS9800). Heat-induced epitope retrieval was done for 20 minutes with ER2 solution (Leica Microsystems AR9640). All the experiment was done at room temperature. Slides are washed three times between each step with bond wash buffer or water. The slides were reviewed and scored in blinded fashion by a board-certified U. Penn pathologist. Prostate tissue was used as the positive control.

Immunocytochemistry

To detect ZIP9 protein in WM46 isogenic clones, the protocol described in ProSciΨ™ for immunocytochemistry. Briefly, cells were fixed in 4% PFA for 7 minutes. After two washes with PBS (5min), cells were permeabilized with PBS/0.1% Triton X-100 for 1 minute at R.T. Cells were washed twice with PBS on a shaker. Once treated with 1.5% H₂O₂/PBS solution for 15 minutes (R.T.), cells were washed again and blocked with 5%BSA for one hour at R.T. For

primary antibody incubation, α -ZIP9 antibody [(SLC39A9 Antibody (PA5-52485), Thermofisher Scientific, Waltham, MA)] was diluted 1:500 in 1% BSA and cells were incubated overnight at 4°C. After washing three times with PBS on a shaker, the slide was incubated with Biotinylated Secondary antibody (Vectastain Kit, Peroxidase Rabbit IgG, PK-4001) for one hour at R.T. Cells were washed three times with PBS, ABC reagent (prepared 30 minutes in advance) was added and samples were incubated for 30 minutes at R.T. After three washes with PBS, samples were incubated for 1.5 minutes with Vector Laboratories DAB Peroxidase (HRP) Substrate Kit (NC9276270. Vector laboratories, Burlingame, CA). Cells were counterstain with hematoxylin (10 seconds, R.T.) (GHS316. Sigma-Aldrich) and mounted with SecureMount (Fisher HealthCare™ PROTOCOL™ Mounting Media. #022-208. Fisher Scientific. Thermofisher Scientific).

Cell membrane labeling with testosterone-BSA-FITC

WM46 were seeded over coverslips in p12-well culture plate, after 24 hours cells TU2% media was removed, and cells were grown in Serum-free TU media for 24 hours. Cells were treated with APA (8mM) or DMSO as vehicle control. After one hour, cells were treated with 0.25 μ M testosterone 3-(O-carboxymethyl) oxime:BSA-fluorescein isothiocyanate (T-BSA-FITC) conjugate (T5771, Sigma-Aldrich, Munich, Germany) diluted in Tris-Buffer (pH 7.2) for 20 min at room temperature. Negative control cells were incubated with 0.25 μ M BSA-FITC (A9771, Sigma-Aldrich, Munich, Germany) diluted in Tris-Buffer (pH 7.2) for 20 min at room temperature. The medium was then aspirated, and cells were fixed for 7 minutes with 4%PFA. Cells were washed three times with PBS (5 minutes each) and mounted with ProLong Gold

antifade reagent with DAPI (#P36935; Thermo Fisher Scientific). Images were captured using a Leica DM IL microscope and registered using LAS software.

Quantitative RT-PCR

RNA was extracted using RNeasy kit (Qiagen, #74104) following the manufacturer's instructions. cDNA was obtained using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4368814). For quantitative real-time PCR, PowerUP™ SYBR™ Green Master Mix (Applied Biosystems #A25741) was used. ViiA 7 Real-Time PCR System was used to perform the reaction (Applied Biosystems). Values were corrected by β -actin expression. The $2^{-\Delta\Delta C_t}$ method was applied to calculate the relative gene expression. Primers used for the amplification are included in Table S2.

Mice, subcutaneous tumors and pharmacologic treatments

All mice were purchased from Taconic Biosciences, Inc. (Rensselaer, NY, USA). 8- to 10-week old male and female C57BL/6NTac or IcrTac:ICR-Prkdcscid mice were allowed to reach sexually mature ages or to acclimatize for one week prior to being used in experiments. These studies were performed without inclusion/exclusion criteria or blinding but included randomization. Based on a two-fold anticipated effect, we performed experiments with at least 5 biological replicates. All procedures were performed in accordance with International Animal Care and Use Committee (IACUC)-approved protocols at the University of Pennsylvania. Subcutaneous tumors were initiated by injecting tumor cells in 50% Matrigel (Corning, Bedford, MA, USA) into the subcutaneous space on the right flanks of mice. 10×10^6 human WM46 or 10×10^5 murine YUMM1.7 cells were used for each tumor. Oral administration of vehicle,

bicalutamide (30mg/kg/day) or apalutamide (20mg/kg/day) was performed daily. 100µl of drug was administrated by oral gavage [10%DMSO:90%Vehicle (15% ethanol:85% sesame oil)]. Weight and health status of the mice as well as tumor growth were monitored daily. As subcutaneous tumors grew in mice, perpendicular tumor diameters were measured using calipers. Volume was calculated using the formula $L \times W^2 \times 0.52$, where L is the longest dimension and W is the perpendicular dimension. Animals were euthanized when tumors exceeded a protocol-specified size of 15 mm in the longest dimension. Secondary endpoints include severe ulceration, death, and any other condition that falls within the IACUC guidelines for Rodent Tumor and Cancer Models at the University of Pennsylvania.

Statistical analysis

For experiments comparing two groups (treated vs control), significance was calculated using the Mann-Whitney test. For experiments with more than two groups, one-way ANOVA with Tukey's honest significance difference test was performed. For the tumor growth studies *in vivo*, non-linear regression analysis was performed to get the Exponential Growth Equation and doubling times for tumor growth. To analyze the slopes of the curves and to compare them between groups, linear regression analysis was performed. All the statistical analyses in this work were performed using Graphpad Prism Software. Error bars represent standard error of the mean (SEM). **** p value≤0.0001; *** p value≤0.001; ** p value≤0.01; * p value≤0.05; n.s>0.05.

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Author Contributions

C.A.P., R.P., A.T. performed the experiments. C.A.P., R.P. and J.K.F designed and performed the experiments to study zinc influx. J.K.F provided experimental material and reviewed the manuscript. J.T.S, scored the tissue microarray and reviewed the manuscript. C.A.P, C.A.N. and T.W.R designed experiments and wrote the article. **Competing interests:** C.A.P., C.A.N., and T.W.R., and are inventors on a provisional patent held by the University of Pennsylvania related to this work.

Conflict of interest

T.W.R., C.A.N and C.A.P. are inventors on a provisional patent held by the University of Pennsylvania related to this work.

The Paper Explained

The Problem: Melanoma incidence and mortality are higher in males than females. This sex difference remains, even after controlling for known melanoma risk factors such as environmental and occupational exposures. While modern immune and targeted therapeutics have markedly improved outcomes for many melanoma patients, most with metastatic tumors still die from their disease. There is acute need for identification of new therapeutic targets, and effective agents to safely engage them.

Results: Here, we define a nonclassical testosterone signaling pathway that establishes a novel mechanistic link between male androgens and melanoma pathobiology. Using medically relevant preclinical in vivo models, we first demonstrate that melanomas progress more rapidly in males vs. females. We then show that 97% of primary melanomas and 100% of metastatic lesions from people express the nonclassical membrane androgen receptor and zinc transporter, ZIP9, and that ZIP9 activation promotes melanoma proliferation. Using complementary pharmacologic and genetic approaches, we show that ZIP9 is a major determinant of the sex dependent difference in tumor growth in vivo, and that ZIP9 may be an eminently druggable new therapeutic target for melanoma.

Impact: ZIP9 is a zinc transporter that is not intentionally targeted by any available therapeutics but is widely expressed in nearly all tissues. The demonstration that this receptor is pharmacologically accessible suggests that inhibiting ZIP9 by repurposing currently approved inhibitors designed to target the classical androgen receptor (AR) may be useful alone or in combination with current standard of care therapeutics for a wide range of cancers, especially those that disproportionately affect males.

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Figure legends

Fig. 1: Biologic sex and testosterone promote proliferation of melanoma models lacking

detectable AR. Tumor growth of human WM46 melanoma in male and female immunodeficient

SCID mice. Tumor doubling times are 16.2 and 18.6 days for males and females respectively

(Non-linear regression analysis/Exponential fit. See also Sup. Fig. 1 for expanded statistical

analysis). **B.** Cell proliferation (cell number) determined after 6 days of treatment with vehicle

(DMSO), 100nM testosterone (T) or 100nM dihydrotestosterone (DHT). Human primary

melanocytes (H.Mel.) and human melanoma WM46 cells are shown. Graphs represent the

average of three independent experiments. **C.** Cell proliferation of human WM46 and murine

YUMM1.7 melanoma cells exposed to increasing concentrations of testosterone (T) (left panels)

or dihydrotestosterone (DHT) (right panel). **D.** AR mRNA expression in primary melanocytes

(L: low melanin content; D: High melanin content), a battery of human melanoma lines, and

murine YUMM1.7 determined by qPCR. VCaP cell line (prostate cancer) was used as a positive

control. **E.** AR protein expression determined by Western blot with two different antibodies.

Upper line = Androgen Receptor [(D6F11) XP® Rabbit mAb #5153. Lower line = Recombinant

Anti-Androgen Receptor antibody [EPR1535(2)] (ab133273). Two human prostate cancer lines

were used as a positive control (VCaP and LNCaP). β -actin was used as loading control.

** p value ≤ 0.01 ; * p value ≤ 0.05 .

Fig 2: ZIP9 is active in human melanoma cells, and is broadly expressed in human

melanocytic tumors. **A.** ZIP9 protein expression determined by western blot in primary

melanocytes and a battery of human melanoma cell lines. **B.** Time-lapse *in vivo* analysis of Zn^{++}

influx in WM46 cells upon testosterone addition (100nM). FluoZin-3 was used as Zn^{++} reporter.

C. Intracellular levels of Zn^{++} after long-term testosterone treatment (96 hours; 100nM

testosterone). Zinc levels were measured as fluorescence intensity per cell. FluoZin-3 was used as Zn^{++} reporter. Representative images are shown on the right at indicated time-points. **D.** WM46 relative proliferation (cell number) after 6 days in the presence of 100 nM testosterone (T) +/- 200 nM zinc chelator (TPEN). **E.** WM46 proliferation in the presence exogenous divalent cations Zn^{++} Fe^{++} and Mn^{++} . Cells were grown for 6 days and treated as indicated in the legend. Error bars represent standard error of the mean (SEM). **F.** Validation of ZIP9 [(SLC39A9 Antibody (PA5-52485)) and androgen receptor [(Leica AR-318-L-CE, clone AR27 (clone AR27, 1:25))] antibodies for immunohistochemistry. ZIP9 staining performed in wild-type and ZIP9 knock-out cells. Prostate gland tissue and human prostate cancer samples were used as positive controls for AR. Representative images of human melanoma samples stained for ZIP9 and AR. Tumors expressing low, medium and high levels of ZIP9 are shown. Replicates from the same samples stained for AR are shown. 20X magnification (1.6X zoom). Scale bar=60 μ M. **G.** Graphic representation of the % of tumors that express ZIP9. Data from nevus, primary melanomas and metastatic melanoma are displayed. **H.** Graphic representation of the percentage of nevi, primary lesions and metastatic tumors classified according to ZIP9 intensity (Score 1=1-25%, 2=26-50%, 3=51-75%, 4=76-100%).

**** p value $\leq$ 0.0001; ** p value $\leq$ 0.01; * p value $\leq$ 0.05; n.s>0.05.

Fig. 3: ZIP9 mediates testosterone effects in melanoma. **A.** Proliferation (cell number) of isogenic clonal populations of WM46 wtZIP9 and Δ ZIP9 (gRNA #3) cells exposed to increasing concentrations of testosterone (T) and dihydrotestosterone (DHT). **B.** Proliferation (cell number) of isogenic clonal populations of WM46 wtZIP9 and Δ ZIP9 (gRNA #4) cells exposed to increasing concentrations of testosterone (T) and dihydrotestosterone (DHT). The graph represents the average of three independent experiments. **C.** Intracellular levels of zinc in human

melanoma Δ ZIP9 cells measured as relative fluorescence intensity of FluoZin-3. Graphs represent the average of three independent experiments. **D.** Relative cell proliferation in the presence of 100nM testosterone (T), zinc chelator (200nM TPEN) and $ZnCl_2$ (400nM). Cells were cultured for 6 days. The graph represents the average of three independent experiments.

**** p value $\leq$ 0.0001; ** p value $\leq$ 0.01; * p value $\leq$ 0.05; n.s>0.05.

Fig. 4: Testosterone driven increase in melanoma proliferation requires ZIP9 and activation of MAPK and YAP1. **A.** RPPA analysis displaying changes in protein expression in WM46 human melanoma cells following exposure to 100 nM testosterone (T) for increasing amounts of time. Down-regulated proteins are shown in green; red color corresponds to up-regulated proteins and control proteins showing no fold-change when compared to vehicle-treated cells are shown in blue. **B.** Relative proliferation (cell number) after exposure to pharmacologic ERK1/2 inhibition via 50nM RVD-523 (Ei) alone or in combination with testosterone. wtZIP9 and Δ ZIP9 WM46 cells are shown. The western blot shows levels of phosphorylation of the ERK target RSK in wtZIP9 and Δ ZIP9 WM46 cells (Ei represents RVD-523). **C.** Western blot for YAP1 in fractionated WM46 lysates. Cells were treated with 100 nM testosterone for the indicated times. β -Actin is used as cytoplasmic fraction positive control. PARP is used as nuclear fraction positive control. **D.** Quantification of YAP-1 nuclear immunodetection after 30 minutes of exposure to 100 nM testosterone (T) and/or 8 μ M dobutamine (Dob) in wtZIP9 and Δ ZIP9 cells. **E.** Proliferation of wtZIP9 and Δ ZIP9 WM46 cells after treatment with 100nM testosterone (T) and/or the YAP inhibitor dobutamine (Dob). **F.** Relative mRNA expression of YAP1 target genes after 30 minutes in the presence of 100 nM testosterone and/or 8 μ M dobutamine. Error bars represent standard error of the mean (SEM).

*** p value $\leq$ 0.001; ** p value $\leq$ 0.01; * p value $\leq$ 0.05; n.s>0.05.

Fig. 5: Pharmacologic ZIP9 blockade inhibits melanoma *in vivo*. **A.** Proliferation of human melanoma cells (WM46) in the presence of 100 nM testosterone (T) +/- 2 μ M AR inhibitors (BIC:Bicalutamide; ENZ:Enzalutamide; APA:Apalutamide). Western blot showing ERK and p-ERK proteins in WM46 cells treated with 100nM testosterone +/- 2 μ M BIC +/- 200 nM zinc chelator (TPEN). **B.** Cell proliferation in human and murine derived melanoma cells (SK-MEL-3, SK-MEL-2 and YUMM1.7) treated with 100 nM testosterone (T) in combination with apalutamide (2 μ M) (APA). **C.** Cell membrane labeling with cell impermeable testosterone-BSA conjugated with FITC (0.25 μ M). BSA-FITC (0.25 μ M) was used as a negative control for unspecific binding. Quantification of membrane labeling with T-BSA or the control BSA. The graph represents the fluorescence intensity relative to the total area of each cell. **D.** Proliferation of WM46 wtZIP9 and Δ ZIP9 treated with 100 nM testosterone (T) in combination with apalutamide (2 μ M) (APA). **E.** Tumor growth and survival analysis in SCID male mice bearing WM46 derived subcutaneous tumors (APA treatment: 20 mg/kg/day via oral gavage). ** p-value<0.005 by ANOVA. Doubling time (non-linear regression analysis): Vehicle=11.11 days; APA=25.22 days. **F.** Working model: ZIP9 activation promotes ERK phosphorylation and induces YAP1 nuclear translocation. AR inhibitors [represented in the figure by apalutamide (APA)] block testosterone effects through ZIP9 inactivation.

**** p value $\leq$ 0.0001; *** p value $\leq$ 0.001; ** p value $\leq$ 0.01; * p value $\leq$ 0.05; n.s>0.05.

740 **Figures**

Figure 1

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Figure 2

Figure 3

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Figure 4

Supplementary Figures

Supplementary Figure 1

Fig. S1. Non-linear regression analysis (Exponential fit) for males and females. Doubling time for tumor growth: males=16.99 days and females=19.95 days. (Linear-regression analysis for slope comparison: **** p-value<0.0001). **B.** Tumor growth in C57BL/6 male and female mice bearing syngeneic murine melanoma (YUMM1.7). Doubling time for tumor growth: males = 3.7 days, females = 4.6 days (Linear-regression analysis for slope comparison: **** p-value<0.0001). **C.** YUMM1.7 cell number determined after 6 days of treatment with vehicle (DMSO), 100 nM testosterone (T) or 100 nM dihydrotestosterone (DHT). Graphs represent the average of three independent experiments. Error bars represent standard error of the mean (SEM). **D.** Relative cell proliferation of human melanoma cell lines treated for 6 days with increasing concentrations of testosterone (T). **E.** Relative cell proliferation of human melanoma cells treated for 6 days with dihydrotestosterone (DHT 100 nM). **F.** AR protein expression determined by Western blot in YUMM1.7 cells. **G.** Immunofluorescence detecting AR (D6F11) in a battery of human-derived melanoma cells. AR is shown in green; DNA was stained with DAPI and it is shown in blue. Scale bar 25μM. Error bars represent standard error of the mean (SEM). **** p value≤0.0001; *** p value≤0.001.

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Supplementary Figure 2

Fig. S2. A. RNA-seq data from 17 cancer types reported as median FPKM (number Fragments Per Kilobase of exon per Million reads), generated by the The Cancer Genome Atlas (TCGA). RNA cancer tissue category calculated based on mRNA expression levels across all 17 cancer tissues and include: cancer tissue enriched, cancer group enriched, cancer tissue enhanced, expressed in all, mixed and not detected. Normal distribution across the dataset is visualized with box plots, shown as median and 25th and 75th percentiles. Points are displayed as if they are above or below 1.5 times the interquartile range. **B.** WM46 relative proliferation (cell number) in presence of 100 nM testosterone (T) +/- 200 nM zinc chelator TPEN. **C.** WM46 proliferation treated with 100 nM testosterone (T), +/- copper sulfate (CuSO₄) and copper chelator (25μM BCS). **D.** Relative cell proliferation of WM46 cells treated with testosterone (100nM) +/- zinc chelator TPEN (200nM) and the copper chelator BCS (25μM). **E.** Relative intracellular Zn²⁺ in

788 cells treated with testosterone and/or copper chelator (BCS). The graph represents relative
789 fluorescence intensity. Error bars represent standard error of the mean (SEM).

790 **** p value $\leq$ 0.0001; ** p value $\leq$ 0.01; * p value $\leq$ 0.05; n.s>0.05.

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Supplementary Figure 3

Fig. S3. Graphic representation of ZIP9 staining intensity in human samples from nevus, primary melanoma and metastatic melanoma in males vs. females (Score 1=1-25%, 2=26-50%, 3=51-75%, 4=76-100%). n.s>0.05.

Supplementary Figure 4

Fig. S4. A. Table shows the knock-out scores for WM46 isogenic clones obtained using ICE Analysis software (Synthego). **B.** Blast alignment of the DNA sequences surrounding the gRNA target sequence and SLC39A9 (Ensembl. Homo Sapiens_SLC39A9_202) are shown for each isogenic clone. As control for the sequencing, WM46 parental cells were sequenced and aligned

with human SLC39A9 (Ensembl. Homo Sapiens_SLC39A9_202). Query = SLC39A9_202; Subject = isogenic clone sequences **C.** Target sequence of YUMM1.7 isogenic clone was aligned with *mus musculus* Slc30a9 sequence. **D.** Western blot showing ZIP9 expression in wild type and knock-out YUMM1.7 isogenic clones. **E.** Relative proliferation (cell number) of YUMM1.7 wtZIP9 and Δ ZIP9 in the presence of 100 nM testosterone (T) for 6 days. The graphs represent the average of three independent experiments. Error bars represent standard error of the mean (SEM). * p value ≤ 0.05 . **F.** Immunocytochemistry in WM46 isogenic clones transduced with control pRRL lentivector (E.V.) or hZIP9 expressing lentivector. Western blots below show the proteins in each sample 72 hours after transduction. **G.** Relative cell proliferation (cell number) with testosterone treatment (100 nM) for 7 days in WM46 Δ ZIP9 isogenic clones transduced with empty pRRL lentivector (E.V.) (left panel) or pRRL_hZIP9 expressing vector (right panel). Error bars represent standard error of the mean (SEM). **** p value ≤ 0.0001 ; n.s. > 0.05 .

Supplementary Figure 5

Fig. S5. A. Western blot showing baseline levels of ERK phosphorylation in wtZIP9 and Δ ZIP9 WM46 cells. **B.** Representative images of YAP-1 immunodetection after 30 minutes of exposure to 100 nM testosterone (T) +/- 8 μ M dobutamine (Dob) in wtZIP9 and Δ ZIP9 cells. The percentage of cells with nuclear YAP-1 (per field) are shown in the upper-left side of each panel. YAP1 is shown in green; β -actin is shown in red; DAPI highlights nuclear DNA in blue. Thin

arrows indicate cells where YAP1 is not localized inside the nucleus. Opaque arrows indicate cells with nuclear YAP-1. Scale bar=15μm. C. Percentage of WM46 wtZIP9 and ΔZIP9 cells transduced with pRRL empty vector (E.V.) or pRRL_hZIP9 expressing vector with nuclear localization of YAP1. Cells were treated with vehicle and 100 nM testosterone.

*** p value≤0.001; n.s>0.05.

Supplementary Figure 6

Fig. S6. A. Relative cell proliferation (cell number) in human-derived melanoma cells (WM46) treated with 2μM AR inhibitors (BIC:Bicalutamide; ENZ:Enzalutamide; APA:Apalutamide). **B.** Intracellular Zn⁺⁺ in WM46 cells treated with 100 nM testosterone and/or 2 μM bicalutamide (BIC). **C.** Relative cell proliferation (cell number) of WM46 wtZIP9 and ΔZIP9 transduced with pRRL empty vector (E.V.) or pRRL_hZIP9 expressing vector. Cells were treated with vehicle (V) or 100 nM testosterone +/- bicalutamide (2μM) **D.** Relative cell proliferation of WM46 cells treated with 100nM testosterone and/or cyproterone acetate (CPA) for 6 days.

**** p value≤0.0001; *** p value≤0.001; ** p value≤0.01; * p value≤0.05; n.s>0.05.

Supplementary Figure 7

Fig. S7. A. Non-linear regression analysis (Exponential fit) for males treated with vehicle vs. APA. Doubling time for tumor growth: Vehicle treated males=16.0 days and APA treated males=36.4 days. Linear regression analysis for slope comparison demonstrate significant differences between vehicle treated and apalutamide treated males (p-value<0.0001). **B.** Tumor growth in mice bearing WM46 derived subcutaneous tumors. Daily treatment with bicalutamide (30 mg/kg/day, via oral gavage) or vehicle are shown for both male and female mice. Linear regression analysis of slopes demonstrates significant differences between vehicle treated and

890 Bic treated males (p-value<0.0001). C. Tumor growth in SCID male mice bearing Δ ZIP9 WM46
 891 melanoma. Mice were treated daily with bicalutamide (30 mg/kg/day) or vehicle via oral gavage.
 892 Exponential fit analyses are included bellow each tumor growth graphical representation.
 893 **** p value $\leq$ 0.0001; n.s>0.05.

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Supplementary Figure 8

Fig. S8. Relative cell proliferation of a battery of cell lines derived from diverse tumor types in response to 100nM testosterone +/- bicalutamide treatment (2μM). *** p value≤0.001; ** p value≤0.01; * p value≤0.05; n.s>0.05.