AWARD NUMBER: W81XWH-21-1-0860

TITLE: Preventing Melanoma Progression by Targeting Nonclassical Androgen Signaling

PRINCIPAL INVESTIGATOR: Dr. Todd Ridky

CONTRACTING ORGANIZATION: University of Pennsylvania, Philadelphia, PA

REPORT DATE: October 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2022	Annual	01Sep2021-31Aug2022

4. TITLE AND SUBTITLE

Preventing Melanoma Progression by Targeting Nonclassical Androgen Signaling

5a. CONTRACT NUMBER
W81XWH-21-1-0860

5b. GRANT NUMBER
ME200089

5c. PROGRAM ELEMENT NUMBER
W81XWH-20-MRP-MCAA

6. AUTHOR(S)

Dr. Todd Ridky

EMail: ridky@pennmedicine.upenn.edu

5d. PROJECT NUMBER
0011641996

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA
3451 WALNUT ST STE 440A
PHILADELPHIA PA 19104-6205

8. PERFORMING ORGANIZATION REPORT NUMBER
583121
10080232

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
DOD

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Melanoma is associated with exposure to solar UV radiation, and disproportionally affects males. Military personnel often spend significant time outdoors, are 80% male across all branches, and are therefore at especially high melanoma risk. We have strong preliminary data showing that increased melanoma burden in males results from far more than just differences in environmental exposure to sunlight. In laboratory mice housed inside, melanomas grow much more aggressively in male mice than in female mice. Our findings suggest that the male hormone testosterone is responsible for much of this difference. Our pilot studies show that the testosterone effects in melanoma result from testosterone binding to a protein in melanoma cells called ZIP9, which was first discovered as a transport protein for zinc. There are no drugs that were designed to target ZIP9, however, we discovered that drugs approved for prostate cancer do inhibit ZIP9 in melanoma, and inhibit the growth of melanomas in male mice. Because apalutamide is routinely used in people, and is well tolerated, this proposal has the potential to rapidly translate to new melanoma trials.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	17	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

Melanoma is associated with exposure to solar ultraviolet radiation, and disproportionally affects males, with incidence and mortality in the U.S. 1.4X and 2.1X higher, respectively, in males vs. females. Military personnel often spend significant time outdoors, are 80% male across all branches, and are therefore at especially high melanoma risk. The consistently higher melanoma burden in males has been appreciated every year since at least 1969, is consistent across all stages of melanoma progression, parallels sex-associated differences in many other cancer types, and seems to result from far more than just differences in environmental exposures between males and females. Defining the mechanisms underlying this disparity will address a major unresolved question in melanoma pathobiology and will likely highlight new therapeutic opportunities. We posit that melanoma in males is accelerated by pro-tumorigenic effects of testosterone (T), and critically, that this activity results from activation of a newly discovered zinc transporter and nonclassical androgen receptor, ZIP9, rather than the classic AR, which is not detectable in most melanomas. Preliminary data suggest that the AR inhibitor apalutamide (APA, approved only for prostate cancer) also blocks the pro-tumorigenic effects of T in melanoma. These aims are designed to define a novel mechanistic link between endogenous male androgens, a nonclassical newly appreciated androgen receptor (ZIP9) and melanoma pathobiology. We propose preclinical efficacy studies that would likely support a FDA application for a new trial testing the utility of repurposing a well-tolerated and already approved prostate cancer drug for melanoma. This work has the potential to rapidly translate to the clinic.

2. KEYWORDS:

melanoma, ZIP9 (Zrt- and Irt-like Protein 9), zinc, YAP (Yes-Associated Protein 1) , testosterone, sex differences, apalutamide, enzalutamide, bicalutamide, zinc

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Test whether apalutamide blocks testosterone effects in melanoma by competing with androgen binding to ZIP9

Major Task 1: Use targeted genetic depletion to test whether ZIP9 mediates testosterone effects in melanoma.

Major Task 2: Test if Testosterone effects on melanoma cells depend on activation of MAPK and YAP pathways downstream of ZIP9.

Major Task 3: Use fluorescently labeled testosterone to test if testosterone and bicalutamide class AR inhibitors directly bind to ZIP9 at compete with each other for ZIP9 binding.

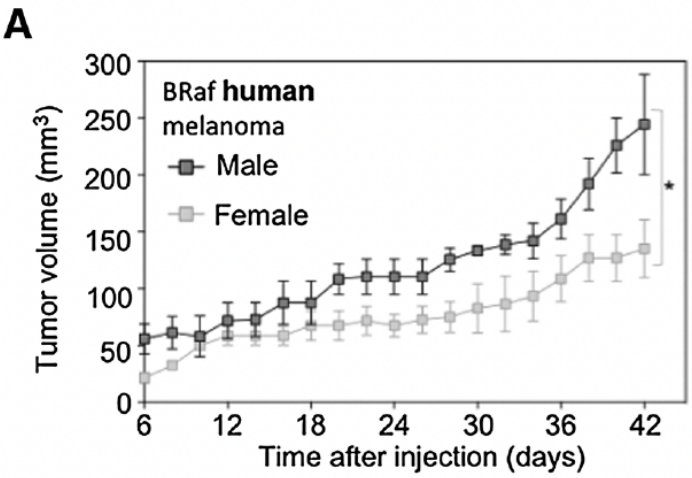
Specific Aim 2: Define the role of ZIP9 in melanoma progression

Major Task 1: Use preclinical mouse models (YUMM1.7. WM46, WM2664, A375) to test if ZIP9 contributes to male vs female sex differences in melanoma in vivo.

Major Task 2: Use preclinical mouse models to test if apalutamide inhibits melanoma in vivo.

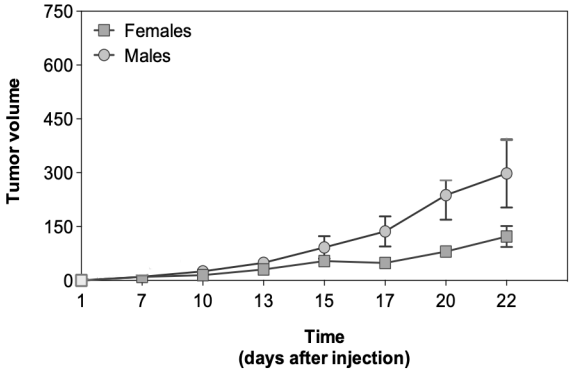
What was accomplished under these goals?

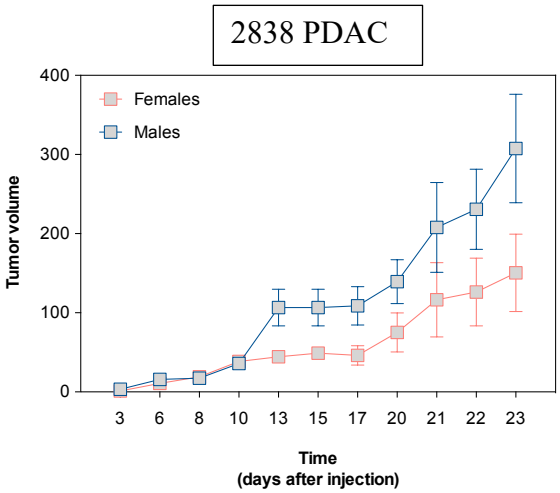
We made major progress on both Aim 1 and Aim 2, and results so far have been entirely consistent with our initial hypotheses. We validated preliminary data that melanomas progress faster in male vs. female mice:



WM46 BRaf driven melanomas (subcutaneous) consistently grow faster in male SCID vs. female SCID mice (above).

We then questioned whether this male vs. female difference was unique to the WM46 line, or whether this was a more general phenomenon that extended to other lines and to perhaps even to other cancers. To test that we performed similar tumor experiments using BRaf driven mouse melanoma (YUMM1.7) and in KRas driven pancreatic cancer (2838). Pancreatic cancer is frequently encountrered in some melanoma prone familes.

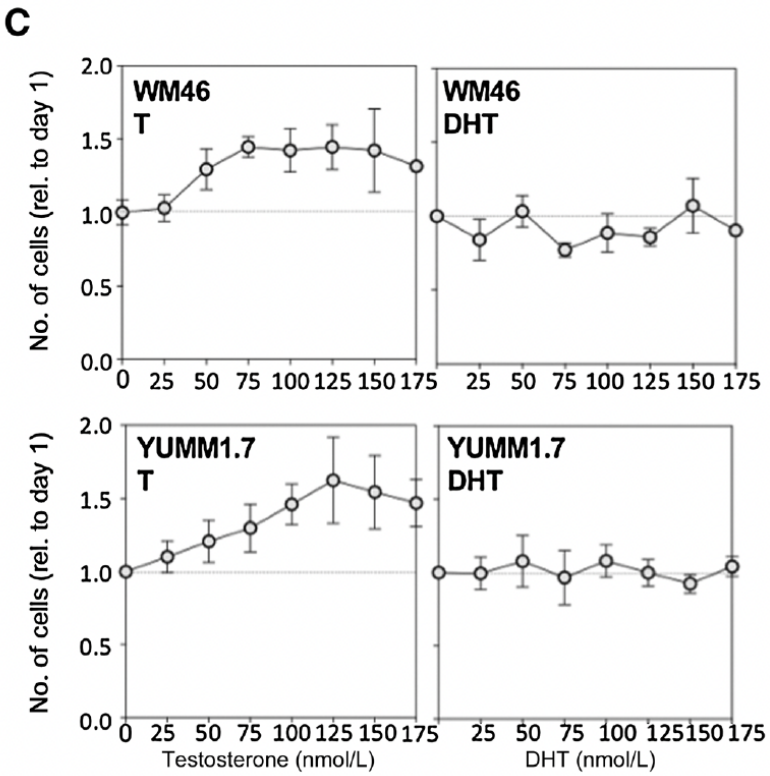




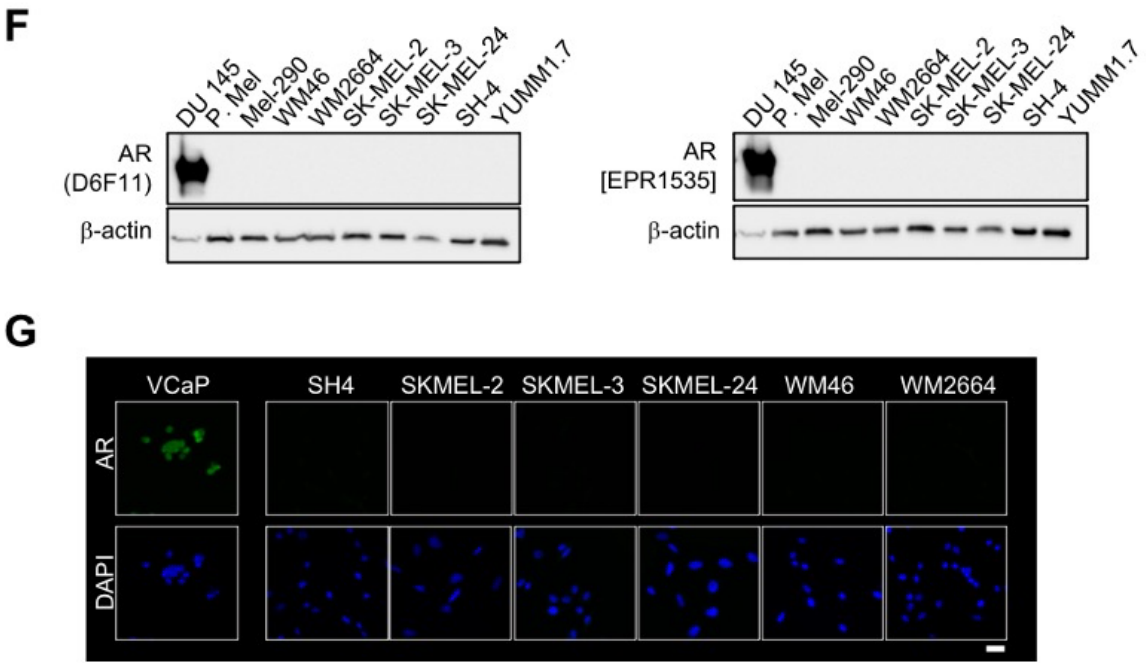
In both YUMM melanama (above left) and 2838 pancreatic cancer (above right), tumors grew faster in male mice. n=5 mice per group.

This shows that the drivers of male vs. female differences in melanoma likely extand to other cancer settings and may not result from a melanocyte specific feature.

We then established that testosterone, but not dihydrotestosterone promotes melanoma proliferation:

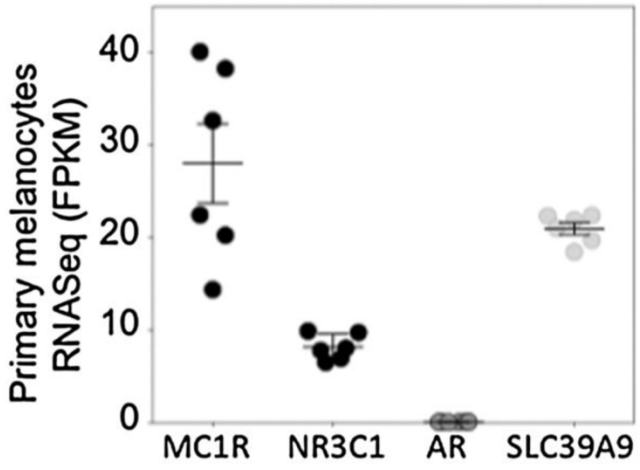


In a dose responsive and saturable manner, testosterone (T) promotes proliferation of both human melanoma (WM456) and mouse melaoma (YUMM1.7). Interestingly, dihydrotestosterone (DHT) does not promote proliferation. DHT is a much better agonist of the classical androgen receptor (AR) than is T, suggesting that testosterone effecst are not mediated by AR. Consistent with this, AR protein is not detectable in melanoma cells, nor in primay human melanocytes. We validated this on western blot on immunofluorescense using multiple AR antibodies.



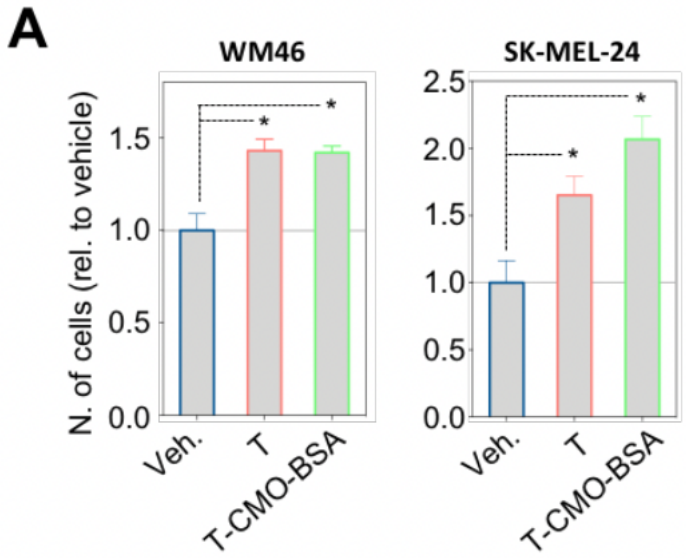
Western blot and IF (above) using multiple different AR antibodies readily detects protein in fixed cells and lysates from prostate cancer cells (DU145, VCaP), but no protein is observed in primary melanocytes (P. Mel) or in any melanoma cell lines.

To further test if AR was expressed in melanoma, and to validate the results of the IHC and IF we next determined levels of AR and ZIP9 mRNA transcript by RNASeq:



These fata show that the AR was not detectable in melanocytes. However, ZIP9 (SLC39A9), the glucocorticoid receptor (NR3C1) and the melanocortin receptor (MC1R) were each readily detectable, as expected. Combined with the protein level expression data, this clearly establishes that AR is not detectable in melanocytes, while ZIP9 is readily observed at both the protein ansd transcript level.

Consistent with the idea that classical AR is not the mediator of testosterone effects in melanoma, membrane impermeable testosterone also promotes melanoma prioliferation, and does it as well as free testorterone:



Membrane impermeable testosterone (T-CMO-BSA) promoted proliferation of human melaoma cells (WM46 and SK-MEL-24). Classical AR is a nuclear receptor and can not be activated by T-CMO-BSA. This is additional strong evidence that testosterone effects are mediated by something other than AR.

Next, we used CRISPR-Cas9 to ablate ZIP9 in melanoma cells. These bulk cultures were reduced to single cells and expanded to establish several isogenic clonal lines derived from the same parental tumor line (WM46). This was major effort that required significant technical skill, and also quite a bit of time to establish the the lines from single cells. We then sequenced the genomic DNA in the region targed by the guide RNA to establish the genetic basis for the protein loss and used a validate dpredictive algorithm to quantitate efficiency of gene knockout:

ICE Analysis_Synthego	Indel %	Model Fit (R2)	Knock-out Score
WM46 wtZIP9 gRNA 3. Clone 3.3	0	1	0
WM46 koZIP9 gRNA 3. Clone 3.2	92	0.92	92
WM46 wtZIP9 gRNA 3. Clone 4.6	0	1	0
WM46 koZIP9 gRNA 4. Clone 4.b	90	0.92	90
YUMM1.7 koZIP9 gRNA 3. Clone 3.3	90	0.9	90

The clonal lines with the best scores showed clear CRISPR-induced mutations while lines with score of "0" maintained the WT ZIP9 sequence, which validates our method, and the efficacy of the genetic knockdown (which also correlated with loss of ZIP9 protein on western blot).

WM46 vs hZIP9 sequence (ENSMBL)

```
Query  18   TTCCCTTGGCTGTTAATTTCTCAGAGGAACGACTGAAGCTGGTGACTGTTTT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  71   TTCCCTTGGCTGTTAATTTCTCAGAGGAACGACTGAAGCTGGTGACTGTTTT

Query  78   GCCTTCTCTGTGGAACTGCTCTGGCAGTCATCGTGCCTGAAGGAGTACATGC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  131  GCCTTCTCTGTGGAACTGCTCTGGCAGTCATCGTGCCTGAAGGAGTACATGC
```

WM46 wtZIP9 Clone 4.6

```
Query  18   TTCCCTTGGCTGTTAATTTCTCAGAGGAACGACTGAAGCTGGTGACTGTTTT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  71   TTCCCTTGGCTGTTAATTTCTCAGAGGAACGACTGAAGCTGGTGACTGTTTT

Query  78   GCCTTCTCTGTGGAACTGCTCTGGCAGTCATCGTGCCTGAAGGAGTACATGC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  131  GCCTTCTCTGTGGAACTGCTCTGGCAGTCATCGTGCCTGAAGGAGTACATGC
```

WM46 wtZIP9 Clone 3.3

```
Query  3    GGCCGGAATCTTTCCCTTGGCTGTTAATTTCTCAGAGGAACGACTGAAGCTG
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  60   GGCCGGAATCATTCCCTTGGCTGTTAATTTCTCAGAGGAACGACTGAAGCTG

Query  63   TTTGGGTGCTGGCCTTCTCTGTGGAACTGCTCTGGCAGTCATCGTGCCTGAA
            |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  120  TTTGGGTGCTGGCCTTCTCTGTGGAACTGCTCTGGCAGTCATCGTGCCTGAA
```

WM46 koZIP9 Clone 4.b

```
Query  7    TCTCTCACTAAGCCTGCTGTCTCTGGCTATGTTGGTGGGATGTTACGTGGCCGCCCTCTT
            |||| || | ||||||||||||||||||||||||||||||||||||||||| || |
Sbjct  9    TCTC-CA-TIAGCCTGCTGTCTCTGGCTATGTTGGTGGGATGTTACGTGGCCGGAATCAT

Query  67   TCCCTTGGCTGTTAATTTCTCAGAGGAACGACTGAAGCTGGTGACTGTTTTGGGTGCTGG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67   TCCCTTGGCTGTTAATTTCTCAGAGGAACGACTGAAGCTGGTGACTGTTTTGGGTGCTGG
```

WM46 koZIP9 Clone 3.2

```
Query  64   TTGGCTGTT-ATTTCTCAGAGGA-CGGCTGACCCTGGTGACGGTGCTGGGTGCTGGTCTT
            ||||||||| |||||||||||||| |||||| |||||||||||||||||||||||||||
Sbjct  63   TTGGCTGTTAATTTCTCAGAGGAGCGGCTGAACCTGGTGACGGTGCTGGGTGCTGGTCTT

Query  122  CTCTGTGGAACTGCACTGGCGGTCATCGTCCCGGAAGGAGTGCACGCACTTTATGAAGAG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123  CTCTGTGGAACTGCACTGGCGGTCATCGTCCCGGAAGGAGTGCACGCACTTTATGAAGAG
```

YUMM1.7 koZIP9 Clone 3.2.9

```
Query  1    TAGCCTGCTGTCTCTGGTTATGTTGGTGGGATGTTACGTTGGCCCGCCTCATTCCCTTGG
            |||||||||||||||| |||||||||||||||||| ||||| | ||||||||||||
Sbjct  38   TAGCCTGCTGTCTCTGGCTATGTTGGTGGGATGTTACG-TGGCCGGAATCATTCCCTTGG

Query  61   CTGTTAATTTCTCAGAGGAACGACTGAAGCTGGTGACTGTTTTGGGTGCTGGCCTTCTCT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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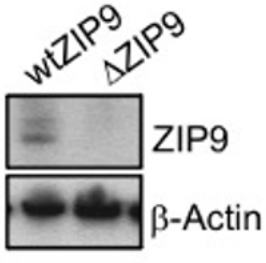
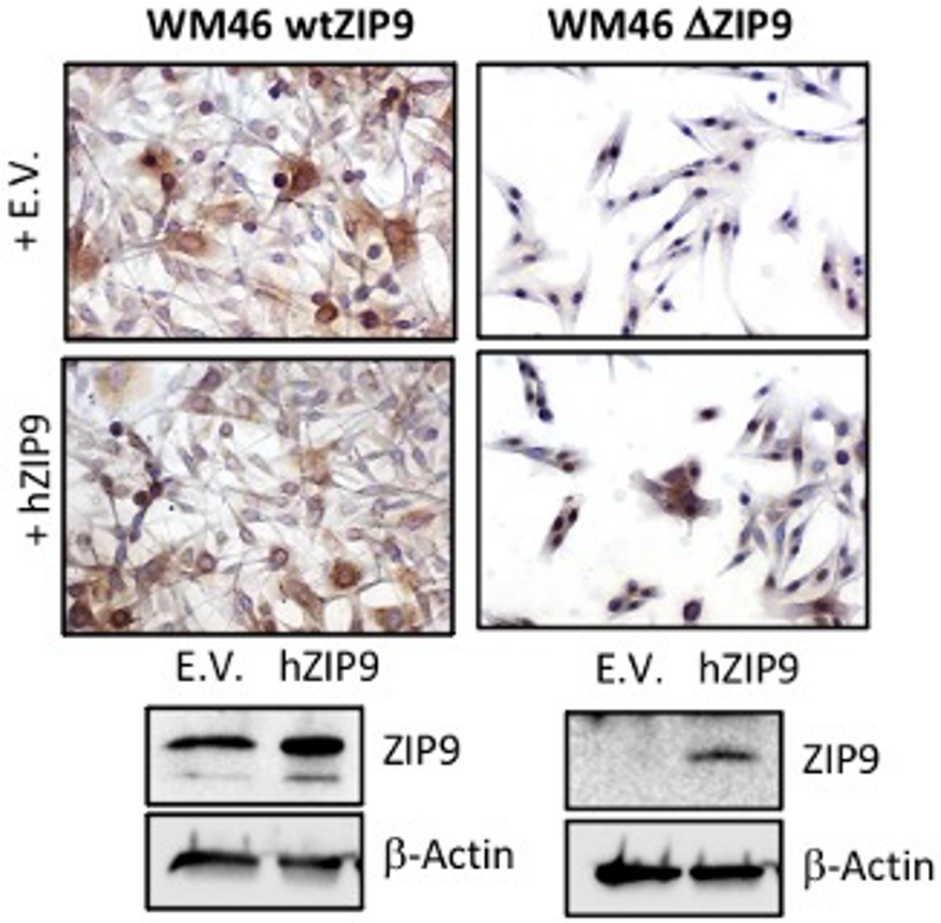
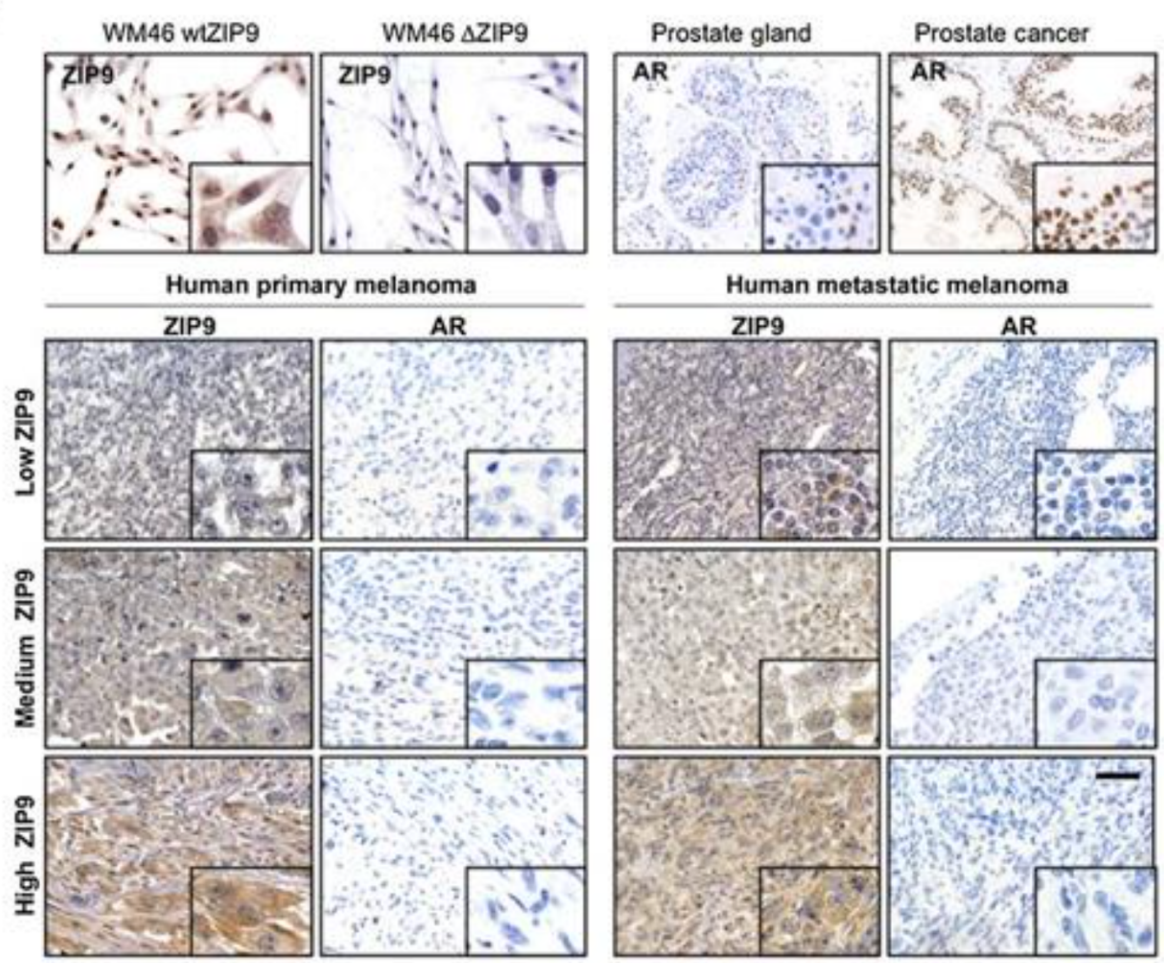


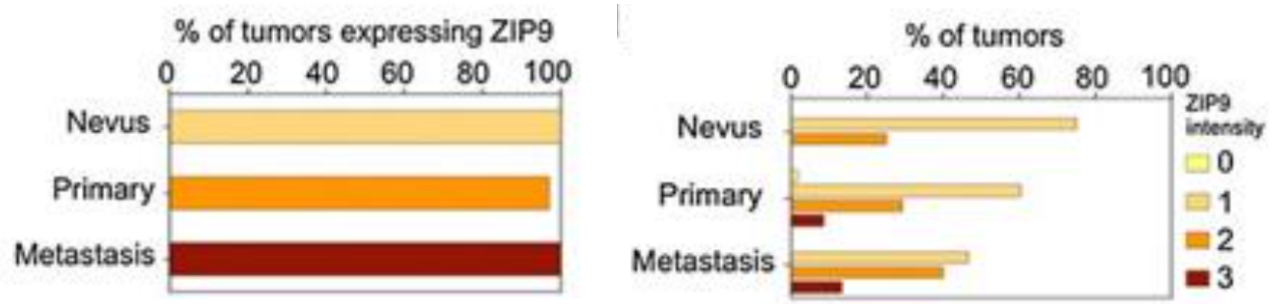
Hacving thoroughly validated the complete CRISPR-indiced loss of ZIP9 in the clonal lines, we used these to validate the ZIP9 antibody for use on IHC of FFPE samples. To do this we grew **1)** parental ZIP9 containing control WM46 melanoma cells **2)** WM46 ZIP9 knockout cells **3)** WM46 ZIP9 knockout cells into which we reexpressed ZIP9 via lentiviral transduction, and **4)** parental WT WM46 transduced with the ZIP9 lentivirus on chamber slides and then fixed them in formalin as is routinely done for human tissues. We then optimized an antigen retrieval and chromogen detection method that we could also use on human FFPE sections.



This clearly shows that the ZIP9 antibody specifically detects only ZIP9 on formalin fixed samples.

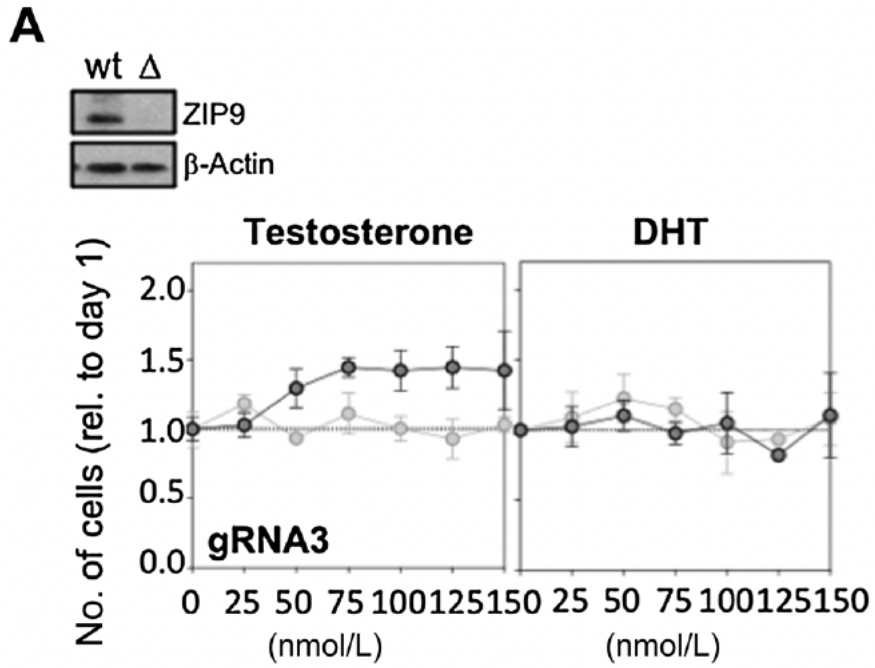
We then used this antobody and an expanded TMA set of 100 human melanocytic neoplasms to test whether ZIP9 and/or AR was detectable. This showed that that melanoma tumors from people do not express detectable AR, but that nearly all of them express ZIP9. Normal and malignant prostate tissue was used for positive control for AR.





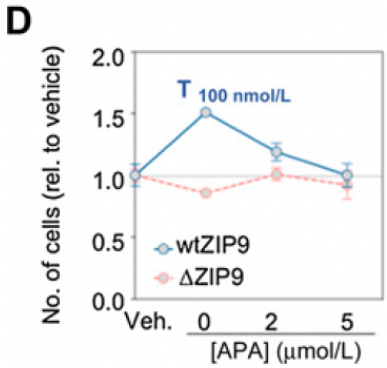
We did note some variation in ZIP9 staining intensity between samples, but this did not correlate with stage.

We next determined that testosterone effects are mediated by ZIP9:



CRISPR-Cas9 was used to deplete ZIP9 in WM46 melanoma cells (above). This rendered cells unresponsive to testosterone. Response in ZIP9 depleted cells is shown in light grey, cells with repose in WT ZIP9 shown in dark grey.

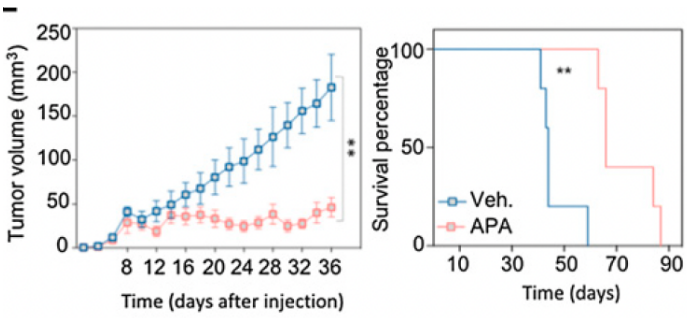
We next determined that FDA approved AR receptor inhibitors block ZIP9, and thereby the pro-proliferative testosterone effects in melanoma cells:



The FDA approved AR inhibitor apalutamide (APA) blocks the testosterone effects in melanoma cells, but has no effect on cells lacking ZIP9 (above).

Using fluorescenty labeled membrane impermeable testosterone, we were able to show that testosterone binds to the surface of melanomas cells, and that this can be competed off by APA.

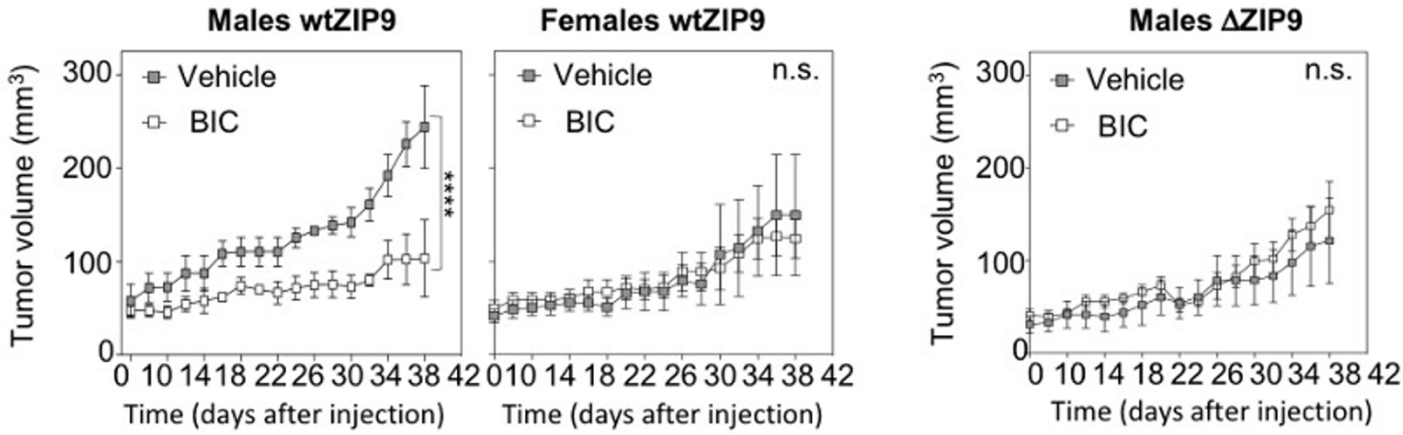
We then tested the ability of APA to inhibit melanomas in vivo:



Systemically delivered apalutamide significantly inhibited BRAF driven human melanoma growth in male SCID mice and extended survival (above).

We next tested whether ZIP9 in melamoma tumors was necessary for the tumors to respond to the AR inhibitor in vivo. This is a major and critical experiment that validates the on-target effects of the drug, and thereby our paradigm shifting discovery that 1) a nonclassical testosterone receptor (ZIP9) is important for melanoma, 2) that testosterone activation of ZIP9 contributes to male vs. female differences in melanoma pathobiology, and 3) that ZIP9 is imminently druggable by repurposing an already approved AR inhibitor that was not designed, nor approved for its ability to inhibit ZIP9. However, our work clearly shows that this bicalutamide class of drugs is effective at inhibiting ZIP9 in vivo.

This type of complex in vivo experiment is not often done (for any drug) and was only possible for us because of the exhaustive and time consuming validation steps (summarized above) that we had taken to get to this point.



Human BRaf driven WM46 melanomas +/- ZIP9 (validated above) were used to establish tumors in male and female mice. Half of the moce in each group were treated with the AR inhibitor bicalutamide, which were established in vitro also inhibits ZIP9. In male mice with WT ZIP9 expressing tumors, bicalutamide inhibits melanoma growth (left). In female mice with the same WT WM46 tumors established at the same time in parallel, tumors grow more slowly in the males, and critically, bicalutamide has no effect. In male mice with WM46 tumors in which ZIP9 was ablated, the tumors grow at the female rate, and critically, no longer responded to bicalutamide. This is a striking result that definitively proves that our initial hypothesis inspired by perplexing but strong preliminary data was correct.

What opportunities for training and professional development has the project provided?

COVID-19 limited the opportunities to formally present this work at meetings. Nonetheless, this work was presented at the Society for Investigative Dermatology meeting, and also in our department seminars at the

University of Pennsylvania. This work was also discussed during invited (Zoom) talks by Dr. Ridky at Moffitt Cancer Center and at Michigan State University.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
We will continue to work on the efforts proposed in the primary aims. In particular, we will focus on the potential therapeutic utility of combining ZIP9 pharmacologic blockade with anti-PD-1 immune therapy, and with targeted BRAF/MEK inhbitors as detailed in Aim 2.

Additionally, over the past 1-2 years we have discovered that there are cell intrinsic differences between human melanocytes from dark vs. light skin that are independent of both UV and of melanin pigment itself that affect melanoma susceptibility and response to oncodrivers such as BRaf (Doepner et al. *Science Advances,* 2022). We do not yet know if these differences that correlate with baseline pigment type also affect response to testosterone, but this is a newly appreciated biologic variable that we will consider in these studies, especially those involving human engineered melanoma surface grafts. This new consideration could impact future translational studies.

4. **IMPACT:**
These studies have clearly established that testosterone promotes melanoma, and contributes to worse outcomes in males. Therefore this work is strong rationale for clinical studies to test whether targeting testosterone signaling is efficacious in males with melanoma. This work is also the first to highlight the functional importance of the nonclassical androgen receptor ZIP9 in cancer, and the potential utility of targeting ZIP9 in vivo. These discoveries will have major impact on the consideration of sex as a consider sex as a biologic variable in future melanoma and other cancer research, and may eventually lead to improvements in clinical standards of care.

What was the impact on other disciplines?
ZIP9 is widely expressed, and most cancers are worse in males than females. Therefore, the discoveries made regarding ZIP9 in melanoma may also apply to many other cancers.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals
The Ridky lab IACUC protocol #803381 underwent the mandated 3 year renewal and was renewed 9/15/22.

Significant changes in use of biohazards and/or select agents
Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  ZIP9 is a Druggable Determinant of Sex Differences in Melanoma. Aguirre-Portolés C, Payne R, Trautz A, Foskett JK, Natale CA, Seykora JT, Ridky TW. Cancer Res. 2021 Oct 27:canres.0982.2021. doi: 10.1158/0008-5472.CAN-21-0982. PMID: 34706862. *Federal support was acknowledged.*

-
- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to report.

- **Other Products**

  Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name: Todd Ridky
Project Role: PI
Research Identifier: ORCID: 0000-0001-8482-1284
Nearest person month worked: 2
Contribution to Project: Dr. Ridky is responsible for the overall design, implementation, and interpretation of all experiments. He provides instruction to the researchers in the study and supervises their progress. He also directly conducts research experiments. He also helps prepare all abstracts, manuscripts, and grant proposals for extramural funding. He presents relevant findings at scientific meetings.
Funding Support: n/a

Name: Roderick Brathwaite
Project Role: Predoc
Research Identifier: ORCID: 0000-0002-1304-856
Nearest person month worked: 6
Contribution to Project: Works alongside the postdoc and is responsible for performing experiments within the Ridky lab.
Funding Support: n/a

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *N/A*

QUAD CHARTS: *N/A*

9. APPENDICES: *N/A*