Loss of *prdm1a* accelerates melanoma onset and progression

Running title: Prdm1a functions as a tumor suppressor in melanoma by inhibition of Sox10

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ABSTRACT

Melanoma is an aggressive and deadly skin cancer that develops from melanocytes, a neural crest cell derivative. Melanoma cells and neural crest cells share similar gene expression, behaviors, and cellular mechanisms. Using cross-species oncogenomics, we identified genes recurrently deleted in both human and zebrafish melanomas, which includes *PRDM1/prdm1a,* a neural crest developmental regulator. We investigated its role in melanoma tumor formation using datasets, human tissue arrays, and transgenic zebrafish lines. High *PRDM1* expression in melanoma patients is correlated with better patient survival. We used a stable transgenic zebrafish line, Tg(*mitfa*:BRAF^{V600E});p53-/-, and found that when one copy of *prdm1a* was mutated, melanoma onset occurred more quickly and the resulting tumors were more invasive. We then analyzed expression of a downstream target and well-established melanoma marker, *SOX10,* and found in the human and zebrafish data, when *PRDM1* expression is low, *SOX10* is

correspondingly high. Thus, the mechanism by which PRDM1 functions as a tumor suppressor in melanoma is likely through regulation of *SOX10* expression.

Keywords: melanoma, zebrafish, PRDM1, SOX10, neural crest cells

SIGNIFICANCE (100 Words)

Melanoma cells behave similarly to neural crest cells in that they express many of the same genes, share a common regulatory network, and activate similar genes for self-renewal and migration. Here we show that PRDM1/Prdm1a, a neural crest developmental regulator, also acts as a tumor suppressor in melanoma. Low PRDM1 expression is correlated with worse patient survival in humans and leads to quicker melanoma onset and more invasive tumors in zebrafish. Moreover, in both species, low PRDM1 corresponds to high expression of *SOX10,* another neural crest regulator and clinical melanoma marker, suggesting that PRDM1 regulates *SOX10* expression.

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INTRODUCTION

Melanoma is an aggressive cancer that accounts for the majority of human skin cancer deaths. The 5-year survival rate of melanoma is only 23% ("Cancer Facts & Figures," 2019). Early stage melanoma can be cured by surgical excision; however, only a few treatment options exist once the melanoma has metastasized. More than 50% of melanoma patients have a somatic BRAF mutation (Ascierto et al., 2012), and up to 90% of these are an amino acid change from valine (V) to glutamic acid (E) at position 600 (V600E) (Bradish & Cheng, 2014). Targeted therapies and immunotherapy were developed with oncogene addiction in mind, and they typically target the BRAF/MAPK pathway (Mackiewicz & Mackiewicz, 2018). Although some patients respond well to these treatments, most eventually relapse (Ribas et al., 2016; Tronnier & Mitteldorf, 2014). Therefore, it is important to identify additional genes and pathways involved in melanoma initiation, as it can lead to the development of future therapies and treatment options.

The cell of origin for melanoma is widely debated. Recent publications suggest that melanoma cells share many of the same characteristics as neural crest cells (Civenni et al., 2011; Mort, Jackson, & Patton, 2015; Shakhova, 2014; White & Zon, 2008). Neural crest cells are a stem cell like population that form between the neural and non-neural ectoderm that give rise to a variety of derivatives, including melanocytes. Melanoma and neural crest cells express many of the same markers, share a common gene regulatory network, and activate similar genes that allow them to self-renew, undergo an epithelial to mesenchymal transition (EMT), and migrate. *EDNRB, MITF,* and *SOX10* have already been described as genes common to both melanoma and neural crest cells (White & Zon, 2008). Identifying additional parallel genes and pathways will help us better understand the mechanisms driving melanoma and then develop new therapies.

We used cross-species oncogenomics to identify potential drivers of melanoma in both humans and zebrafish. Zebrafish have emerged as useful models for studying cancer, including melanoma (Patton et al., 2005), as they can be maintained at a lower cost, develop rapidly, are relatively easy to genetically manipulate, and can be imaged *in vivo.* One candidate gene was *PRDM1* (also known as *Blimp1*), a key regulator of neural crest cells during embryonic development (Artinger, Chitnis, Mercola, & Driever, 1999; Hernandez-Lagunas et al., 2005). PRDM1 and other members of the PRDM family function as tumor suppressors for many cancers, including lymphoma, colorectal and lung cancer (Boi, Zucca, Inghirami, & Bertoni, 2015; Hayashi, Yoshida, & Matsui, 2005; Kucuk et al., 2011; Mzoughi, Tan, Low, & Guccione, 2016), but to our knowledge, it has not yet been studied in melanoma. Given PRDM1's known roles in other cancer and neural crest cell regulation, we hypothesize that loss of PRDM1 promotes melanoma.

Using data mining of public melanoma omics datasets, human tumors, melanoma cell lines, and transgenic zebrafish, we identify PRDM1 as a novel tumor suppressor in melanoma. We show that loss of PRDM1 results in an acceleration in melanoma onset and more aggressive tumors. We also looked at the relationship between PRDM1 and *SOX10* in melanomas and during zebrafish embryonic development, as *SOX10* is a known downstream target of PRDM1 and its role in melanoma has been well-established (Cronin et al., 2013; Olesnicky, Hernandez-Lagunas, & Artinger, 2010; Powell, Hernandez-Lagunas, LaMonica, & Artinger, 2013). Our work suggests that PRDM1 acts as a tumor suppressor through its regulation of *SOX10*.

MATERIALS AND METHODS

Zebrafish

Zebrafish were maintained as described previously (Westerfield, 2007). The University of Colorado Anschutz Institutional Animal Care and Use Committee (IACUC) reviewed and approved all animal procedures. Wildtype strains include AB and EKK lines. Transgenic lines include *prdm1a*^{m805} *(nrd)*, referred to as *prdm1a-/-*, *tp53*$^{M214/M214}$ from now on referred to as p53-/-, and Tg(*mitfa*:BRAF^{V600E});p53-/- (Patton et al., 2005; White et al., 2011). The zebrafish were scored weekly for the presence of visible tumors. A log-rank test of $p<0.05$ was considered statistically significant.

Data mining of public melanoma data sets

We queried the cBioPortal website (http://www.cbioportal.org/) to investigate the mutational status of PRDM1 in melanoma patients. Melanoma data sets with mutation profiles were investigated for examining the distribution of the PRDM1 mutation.

Survival analysis of The Cancer Genome Atlas (TCGA) cutaneous melanoma data

Clinical and mutational data of 329 TCGA melanoma patients were obtained from the supplementary tables of the TCGA publication (http://cancergenome.nih.gov/) . Normalized RNA-seq gene expression profiles (Level 3, RSEM value) for these patients were downloaded from the cBioPortal website. Patients were assigned to high (hi) and low (lo) groups based on above or below mean gene expression of PRDM1 and SOX10. Kaplan-Meier survival analysis was performed comparing hi and lo groups. A log-rank test of $p<0.05$ was considered statistically significant.

Mining public gene expression data sets

Gene expression profiles of the Riker data set (GSE7553) (Riker et al., 2008) were downloaded from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/gds). This data set contains 87

melanoma patients and was profiled by the Affymetrix Human Genome U133 Plus 2.0 microarray. Raw data was downloaded and normalized with the Robust Multiarray Average (RMA) method using the Affymetrix Power Tools (http://www.affymetrix.com/partners_programs/programs/developer/tools/powertools.affx). From this data set, there were 4 normal skin, 14 primary melanoma and 40 metastatic melanoma patients. Gene expressions for PRDM1A and SOX10 were compared across these patients, and a one-way ANOVA test was performed to determine the different expressions across these patients. A Pearson correlation test was used to determine if there was a correlation in PRDM1A and SOX10. Melanoma patient-derived xenografts (PDX) gene expressions and mutational profiles were obtained from previously published data (Micel et al., 2014; Turner et al., 2019). In brief, gene expressions were profiled by RNA-seq. Alignment and FPKM (Fragments Per Kilobase of transcript per Million mapped reads) quantification were performed as previously described (Keysar et al., 2013).

Immunohistochemistry staining

Zebrafish specimens were fixed in 4% paraformaldehyde and paraffin embedded, sectioned at a 5μM thickness and fixed to glass slides. All of the staining, including H&E staining were performed by the Histology Core in the Department of Pathology at the University of Colorado Anschutz School of Medicine. Histologic images were captured on an Olympus BX51 microscope equipped with a four megapixel Macrofire digital camera (Optronics; Goleta, CA) using the Picture Frame Application 2.3 (Optronics). Primary antibody against PRDM1 (Novus, Cat# NB 600-235SS) and S100 (Agilent, Cat# Z0311) were used. Human melanoma tissue arrays, ME804b and ME1004f, were obtained from Biomax (Derwood MD, USA). The PRDM1 expression was scored blindly by a collaborating pathologist (D. Orlicky).

RNA extraction and real-time PCR (qRT-PCR)

Total RNA was isolated from zebrafish embryos and the melanoma cell lines using the Trizol reagent (Invitrogen Corp., Cat#15596018, Carlsbad, CA). Twenty embryos were used in each reaction and the reaction was performed three times. Total RNA was purified with Direct-zol (Zymo Research Irvine, CA). For each sample, cDNA was produced from 1 μg of purified RNA using the Verso cDNA Synthesis Kit according to the manufacturer's protocol (AB1453 Thermo Scientific). All real-time PCR reactions (qRT-PCR) was conducted in the Step One Plus Real-time PCR System (https://www.thermofisher.com/order/catalog/product/4376600). Taqman primers for *prdm1a*, *sox10*, *dct, mitfa*, *mc1r,* and *tyr* primers were obtained from

Applied Biosystems. *rpl13a* was used as the zebrafish internal control: probe 5'-/56-FAM/atgaagtTggCtggaagtacca/3lABlk_FQ/-3', forward 5'-CACACGCAAATTTGCCCTGCTTGG-3', and reverse 5'-GCCTGCTTAGTCAGCTTCATC-3'. GAPDH was used as the internal control in human: forward 5'-TGCACCACCAACTGCTTAGC-3' and reverse 5'-GGCATGGACTGTGGTCATGAG-3'. Relative expression was compared using a two-tailed, independent t-test. Tumors from the transgenic zebrafish were compared using a Mann-Whitney U test. Results were statistically significant when $p<0.05$.

Western blotting

50 to 100 zebrafish embryos per stage were dechorionated and harvested. The proteins were isolated using lysis buffer (0.1% glycerol, 0.01% SDS, 0.1M Tris-HCL [pH6.8]). Primary antibodies against PRDM1 (1:750, dilution generous gift from Dr. Philip W. Ingham) (von Hofsten et al., 2008) and Histone H3 (1:5000 dilution, Cell Signaling Technology, Cat# 9715) were used. The blots were incubated with secondary antibody, HRP-conjugated goat anti-rabbit (1:5000 dilution, Sigma-Aldrich, Cat# A0545) for 2 hours. Bio-Rad imaging system (http://www.bio-rad.com/en-us/sku/1709690-image-lab-software) was used to detect and quantify the protein levels.

Tyrosinase assay

The tyrosinase assay was performed as previously described (Cha, Ko, Kim, & Jeon, 2011; Choi et al., 2007) with a few modifications. About 100 embryos at two days post fertilization (dpf) were dechorionated and sonicated in Pro-Prep protein extraction solution (iNtRON Biotechnology, Cat#17081). 200 µg of total protein in 75 uL of lysis buffer was added to 75 µL of 1.0 mM of L-3,4-dihydroxyphenylalanine (L-DOPA) in a 96-well plate. The control well contained 75 µL of lysis buffer and 75 µL of 1.0 mM L-DOPA. After incubation at 37°C for 60 minutes, absorbance was read at 425 nm. The assay was performed twice. The data were analyzed using a one-way ANOVA test where $p<0.05$ was statistically significant.

RESULTS

PRDM1 is recurrently deleted in human and zebrafish melanoma

We used a cross-species oncogenomics approach to identify tumor suppressor genes common in both human and zebrafish melanomas. 2,617 genes were recurrently deleted in humans, 1,515 genes for zebrafish, and 72 of these were deleted in both (**Figure 1A).** Of the shared genes, 34 were significantly downregulated in melanomas compared to melanocytes, including *PRDM1/prdm1a* (**Figure 1B**), the human ortholog. *prdm1b* and *prdm1c* are also reduced, but are ubiquitously expressed and not specific to

melanocytes (Sun et al., 2008). *PRDM1* insufficiency has been observed in many tumor types, such as hematopoietic malignancies (Calado et al., 2010; Mandelbaum et al., 2010) and non-hematopoietic tumors like colorectal cancer, lung cancer and glioma (Liu et al., 2018; Wang et al., 2013; Zhu et al., 2017). However, little is known about the role of *PRDM1* in melanoma. We queried several publicly available melanoma data sets and found *PRDM1* was mutated in up to 12% of patients (Gao et al., 2013) (**Figure 1C**). Patient mutations were widely distributed across the gene, suggesting that no hot spot mutations were observed in *PRDM1*.

We then examined transcriptomics data previously published by White et al. (2011), where they compared gene expression in normal melanocytes to melanomas in a transgenic zebrafish melanoma model (Tg[*mitfa*:BRAF^{V600E}];p53-/-). *Prdm1a* was significantly downregulated by -3.84 in the melanoma compared to normal melanocytes (p=4.68E-20) (see table in **Figure 5C**).

PRDM1 expression correlates with better survival in melanoma patients.

From the analysis of the TCGA cutaneous melanoma data set, we found that higher PRDM1 expression is correlated with a better overall survival rate in human melanoma ($p<0.001$) (**Figure 1D)**, suggesting that PRDM1 downregulation is associated with poor clinical outcomes and metastatic melanoma incidence. In the Riker Melanoma data set (GSE7553 Moffitt data), PRDM1 expression was lower in primary melanoma samples and significantly lower in metastatic melanoma compared to normal skin ($p<0.001$) (**Figure 1E).** There is also a significant difference between primary melanoma and metastatic melanoma in this data set ($p<0.05$). These data demonstrate that the expression of PRDM1 negatively correlates with metastatic status. We next analyzed PRDM1 expression in melanoma patient derived xenograft (PDX) models and found that PRDM1 was lower in patients with the BRAF^{V600E} mutation compared to wildtype individuals (**Figure 1F**). We confirmed this using qPCR with different human melanoma cell lines and found that *PRDM1* was significantly lower in lines A375, MeWo, M14, WM266.4, and WM3727 compared to normal primary melanocytes ($p<0.001$) (**Figure 1G)**.

In order to investigate PRDM1 protein levels, we performed immunohistochemical staining against human PRDM1 using 3-Amino-9-Ethylcarbazole (AEC) to visualize expression on two melanoma human tissue arrays (Biomax). AEC is used to more easily visualize PRDM1 in the black pigmented melanoma samples. We evaluated PRDM1 expression exclusively in the cancer cells (**Figure 2**). Representative images are shown for benign, stage 2 and stage 3/metastatic tumors (**Figure 2A**). The data suggest that PRDM1 is more highly expressed in the earlier stages and is lost in later metastatic stages. Interestingly, we observed PRDM1 protein expression in both the nuclear and cytoplasmic cellular compartments in the cancer cells on the tissue arrays. We quantified this by examining if PRDM1 immunopositivity and immunointensity associates with melanoma stages (**Figure 2B, 2C**). However, in

this small sample size, we did not observe the statistical difference between the stages in nuclear or cytoplasmic PRDM1 expression. We predict that this is partly due to tumor heterogeneity and the fact that analysis of the same tumor section shows different immunointensity. Intriguingly, of the 62 Stage I and II sample tumors, only two were PRDM1 negative (2.7%), three out of the 21 Stage III and IV tumors were PRDM1 negative (14.3%), and seven of the 58 metastatic sites were PRDM1 negative (12.1%) (**Figure 2D, 2E**). These data together suggest that PRDM1 is negativity associated with advanced stages of melanoma and metastatic sites, PRDM1 normally reduces melanoma growth, and inhibits melanoma initiation and progression.

Heterozygous loss of *prdm1a* in zebrafish increases melanoma onset and progression

We then determined whether PRDM1 functions as a tumor suppressor for melanoma *in vivo*. We injected an oncogenic *mitfa*:BRAF^{V600E} construct into p53-/- and p53-/-; *prdm1a*+/- single cell staged zebrafish embryos by pressure injection. With the p53 mutant background, this construct causes melanoma that is histologically similar to humans (Patton et al., 2005; White et al., 2011). We then monitored tumor formation, which includes the presence of hyperpigmentation that grow into tumors, over time. The p53-/-;*prdm1a*+/- zebrafish (n=8) developed tumors significantly faster than p53-/- zebrafish (n=12) ($p<0.05$) (**Figure 3A)**. Unfortunately, p53-/- zebrafish often grew malignant peripheral nerve sheath tumors (MPNST) (Berghmans et al., 2005) before melanoma formation (about 10 months of age, data not shown), and for ethical reasons, we euthanized these zebrafish at an earlier time point.

To circumvent this issue, we then established stable Tg(*mitfa*:BRAF^{V600E})p53-/- and Tg(*mitfa*:BRAF^{V600E})p53-/-;*prdm1a*+/- transgenic models. The overall time to onset of melanoma occurred at a faster rate (about 4 months faster) compared to the injection model. As expected, similar to the *mitfa*:BRAF^{V600E} injection model, Tg(*mitfa*:BRAF^{V600E});p53-/-; *prdm1a*+/- zebrafish (n=33) developed melanoma statistically more rapidly than p53-/- zebrafish (n=18) ($p<0.05$) (**Figure 3B**). To visualize the effect of heterozygous loss of *prdm1a*, we performed both whole mount and sectional analysis of the resulting melanomas. The p53-/-;*prdm1a*+/- tumors at 7 months of age were much larger at the same age compared to the p53-/- tumors (7 months and 11 months; **Figure 3C**). To evaluate the pathophysiological differences, we performed H&E staining, which showed that the melanoma in Tg(*mitfa*:BRAF^{V600E});p53-/-;*prdm1a*+/- zebrafish was more aggressive. When we compared same size tumors (7 months for p53-/-;*prdm1a*+/- and 11 months for p53-/-), the melanoma in p53-/-;*prdm1a*+/- had spread into the skeletal muscle and vessels, but only when one copy of *prdm1a* was mutated (**Figure 3D**).

Intriguingly, the H&E staining revealed two different cell types in the tumors – cells with a melanocyte morphology and less melanin as well as cells that resemble macrophages. These "melanophages" have a more rounded morphology and more melanin (Lister et al., 2014; F. Zhang,

Zhang, Neng, & Shi, 2013; W. Zhang et al., 2012). Both cell types were highly invasive. Indeed, the melanophages were observed in the fins, liver, kidney, upper gastrointestinal, and between adipose and bones, suggesting that when one copy of *prdm1a* is mutated, the fish exhibit a more metastatic phenotype (**Figure 3D**). Next, we performed immunohistochemistry with S100, a clinically used melanoma marker (**Figure 3E**). In the zebrafish melanoma, S100 positive cells were rare in the p53-/- tumor. Only one S100 positive cell was observed in the entire tumor. In contrast, in the p53-/-;*prdm1a*+/- tumors, there were more S100 positive cells present (**Figure 3E**). S100 family member expression has been linked with aggressive tumor phenotype, which is consistent with our data here (Bresnick, Weber, & Zimmer, 2015).

Loss of PRDM1 increases neural crest stem cell signature and decreases differentiated melanocytes expression and activity.

Melanoma cells and neural crest cells behave similarly and express many of the same genes. Therefore, in order to better understand melanoma cells, it is useful to study the development and differentiation melanocytes, a neural crest cell derivative, as it allows us to make inferences about melanoma initiation (Civenni et al., 2011; Mort et al., 2015; Shakhova, 2014; White & Zon, 2008). First, we analyzed the expression of *prdm1a* by qRT-PCR in wildtype whole embryos. We found that expression was highest at 2 dpf and gradually decreases by 7 dpf when the yolk is completely absorbed (**Figure 4A**). Analysis of protein levels by western blot showed that Prdm1a protein expression peaks at 2 dpf (**Figure 4B, 4C)**. From previous studies, we know that *prdm1a* is expressed during development, particularly in neural crest progenitors, but is downregulated in neural crest melanocyte precursors (Artinger et al., 1999). Thus, Prdm1a function is likely most active at 2 dpf when pigment cells are differentiating and forming mature melanocytes.

To determine the effects on differentiated melanocytes in *prdm1a* mutants, we also analyzed the expression of late melanocyte markers. We found that the expression of dopachrome tautomerase (*dct*) and microphthalmia associated transcription factor (*mitfa*) decreased over time in both wildtype and mutant embryos, *with* prdm1a mutant embryos expression significantly less overall ($p<0.05$ and $p<0.01$, respectively) (**Figure 4D, 4E)**. As neural crest cells further develop to embryonic melanoblasts, the melanocortin 1 receptor (*mc1r*) is expressed strongly beginning at 3 dpf and increases over time. Similarly, the expression of *mc1r* was slightly lower in the *prdm1a* mutant embryos as compared to wildtype at 5 and 6 dpf, but the trends were similar ($p<0.0001$) (**Figure 4F)**.

We also examined the expression of tyrosinase (*tyr*), the enzyme responsible for producing melanin pigment in melanocytes. Overall, mutants expressed lower mRNA levels compared to the wildtype ($p<0.01$) (**Figure 4G**). To further test the activity of tyrosinase in melanocytes, we performed a functional assay to determine if pigment production was affected by Prdm1a reduction. At 2 dpf, *prdm1a*

mutant, wildtype/heterozygous, and wildtype embryos were lysed then incubated with the tyrosinase substrate, L-DOPA, to activate the enzyme. Using spectrometry to quantify melanin synthesis, we found that pigment production was significantly reduced in homozygous *prdm1a* mutants ($p<0.0001$) as compared to wildtype embryos. We were unable to distinguish between wildtype and heterozygous embryos by phenotype and performed the assay on the mixed embryos. The wildtype/heterozygous mix had significantly reduced pigment production compared to wildtype ($p<0.05$) (**Figure 4H)**. Thus, Prdm1a is required for fully differentiated melanocyte formation and function.

PRDM1 likely acts as a tumor suppressor through regulation of *SOX10*

To determine the mechanism by which PRDM1 was acting as a tumor suppressor in both human and zebrafish melanomas, we examined the expression of *sox10*. We have already shown that Prdm1a is an indirect regulator of neural crest specification gene *sox10* (Olesnicky et al., 2010; Powell et al., 2013). *SOX10* is also a well-established melanoma marker (Mohamed, Gonzalez, Lawson, Wang, & Cohen, 2013). Therefore, we looked at its expression during normal zebrafish development, in human data sets, *in vitro,* and in zebrafish tumors. We predict to observe high *SOX10* expression in both the human and zebrafish melanomas, particularly when *PRDM1/prdm1a* was mutated.

In the Riker Melanoma data set, *PRDM1* and *SOX10* are negatively correlated ($r=-0.73$, $p<0.0001$) (**Figure 5A).** When *PRDM1* expression is low, *SOX10* expression is high. This suggests that the interaction between PRDM1 and *SOX10* persists after embryonic development and plays a role in human melanoma. Moreover, in the TCGA data set, we found that lower *SOX10* expression is correlated with a better overall survival rate in metastatic melanoma patients ($p<0.05$) (**Figure 5B)**, suggesting that *SOX10* expression is linked with poor clinical outcomes and metastatic melanoma incidences. The association between SOX10 and melanoma has been published and suggests that SOX10 as a tumor associated antigen (Khong & Rosenberg, 2002)

We next examined *sox10* expression in whole zebrafish embryos using qRT-PCR and compared wildtype and *prdm1a* mutants. In the wildtype, *sox10* expression decreases over time and levels off as the neural crest cells differentiate. In contrast, in the mutant, *sox10* expression is significantly higher than the wildtype, particularly at 2 dpf and 4 dpf ($p<0.0001$) (**Figure 5D**). This suggests that there is a relationship between Prdm1a and *sox10,* in which expression of *sox10* increases when Prdm1a function is reduced.

We reanalyzed the transcriptomics data published by White et al. (2011) and also found that *sox10* was significantly upregulated in zebrafish melanoma by 4.97 ($p=0.24E-15$) (**Figure 5C**). This data suggests that *prdm1a* normally represses *sox10* in context dependent manner. Next, we harvested the melanoma tumors and extracted RNA to examine the expression of *sox10*. Tumors in Tg(*mitfa*:BRAF^{V600E});p53-/-;*prdm1a*+/+ zebrafish had negligible *sox10* expression. In contrast, expression

in Tg(*mitfa*:BRAF^{V600E});p53-/-;*prdm1a*+/- tumors was generally higher, and while trending towards significance, the difference was not statistically significant (p>0.05) (**Figure 5E)**. This is likely due to variability in the differentiation states of the p53-/-;*prdm1a+/-* tumors since tumor heterogeneity is a common theme in melanoma (Grzywa, Paskal, & Wlodarski, 2017).

Together, the zebrafish development data shows that in the absence of *prdm1a,* neural crest cells express higher levels of stem cell markers, particularly *sox10* and lower levels of melanocyte differentiation markers *mitfa, dct, mc1r,* and *tyr.* This suggests that in *prdm1a* mutants, neural crest cells are not able to fully differentiate into mature melanocytes, and likely remain in the embryo as melanocyte stem cells that then can be activated by oncogenic insult. Therefore, *prdm1a* is important for regulating melanocyte stem cells and promoting differentiation. We speculate then that PRDM1 suppresses melanoma tumor formation in humans and zebrafish by regulating the melanocyte stem cell population. Reduction of PRDM1 results in tumor cells that have more stem cell-like characteristics and are less differentiated, permitting faster tumor progression.

DISCUSSION

These studies demonstrate how PRDM1 acts as a novel tumor suppressor in both human and zebrafish melanoma formation. In melanoma, patients that have lower *PRDM1* expression have worse outcomes compared to patients with higher expression. Human tissue arrays reveal that PRDM1 is downregulated in advanced stages of melanoma and metastatic sites. We also show that in both the zebrafish injection models and stable transgenic lines, loss of one allele of *prdm1a* leads to significantly faster onset and progression of melanoma. The tumors in *prdm1a* heterozygote zebrafish were also more aggressive and invasive. PRDM1's function as a tumor suppressor has been previously studied (Boi et al., 2015; Kucuk et al., 2011), but this is the first time it has been shown in melanoma.

PRDM1 is also a key regulator of embryonic development, particularly for neural crest cells. It contains an N-terminal PR/SET domain and five DNA-binding zinc fingers, rendering it capable of regulating gene expression both transcriptionally and epigenetically. During vertebrate development, PRDM1 is required for determining neural crest stem cell progenitor fate. We have shown in zebrafish that Prdm1a regulates neural crest genes *sox10* and *crestin* (Olesnicky et al., 2010) and directly binds to and activates *foxd3* and *tfap2a* (Powell et al., 2013). Moreover, null mutants exhibit a partial loss of neural crest cell derivatives, such as cranial and dorsal ganglion and most importantly, pigment cells (Artinger et al., 1999; Hernandez-Lagunas et al., 2005). Postnatally, PRDM1 remains expressed in adult stem/progenitor cell compartments where it is thought to be critical for stem cell homeostasis, particularly in the mammary gland (Elias, Morgan, Bikoff, & Robertson, 2017) and hair follicles (Horsley et al.,

2006). Loss of *PRDM1* in the hair follicle leads to enhanced numbers of stem cells in the bulge region with increased proliferation.

Our data here suggest that PRDM1 suppresses melanoma tumor formation through regulation of *SOX10,* which is a well-established melanoma marker (Mohamed et al., 2013). Melanoma patients with low *PRDM1* tend to have high *SOX10* expression. When we extract RNA from zebrafish tumors, *sox10* expression is high, particularly when one allele of *prdm1a* is lost. SOX10's role in melanoma disease progression has been well-studied, as it is highly expressed in human melanoma tumors (Mohamed et al., 2013). Clinicians use Sox10 as a highly sensitive and specific marker for detecting primary and metastatic melanomas. It is required for cell proliferation in melanoma cell lines, and haploinsuffiency has been shown to significantly reduce tumorigenesis in a transgenic mouse model (Cronin et al., 2013). Furthermore, forced overexpression of *sox10* in zebrafish melanocytes accelerates tumorigenesis (Kaufman et al., 2016). In development, SOX10 is a master transcription factor of neural crest cells. It is expressed in migratory cells, and it is necessary for the melanocyte differentiation (Harris, Baxter, Loftus, & Pavan, 2010). SOX10 directly activates *MITF,* which in turn induces the expression DCT and TYR to promote the melanocyte cell fate. Harris et al. (2013) have shown that SOX10 is important for regulating melanocytes postnatally as well. Using conditional transgenics, *Sox10* knockout mice were used for hair pluck assay and observed for hair regeneration. The new hair was nonpigmented, and the hair bulbs had significantly reduced melanocyte stem cells, differentiated melanocytes, and *MITF* expression. In addition, overexpression of *Sox10* led to a loss of melanocyte stem cells and premature hair graying in plucked mice. This suggests that postnatally, *Sox10* is necessary for cultivating the stem cell population and for generating mature, pigment-producing melanocytes.

To better understand the interaction between PRDM1 and *SOX10* in melanoma, we took a developmental biology approach to examine the differentiation of melanocytes from neural crest cells in zebrafish embryos. *sox10* expression in wildtype embryos was steady over time, but homozygous *prdm1a* zebrafish mutants have an increase in *sox10* expression at 2 and 4 dpf. These peaks likely correspond to the timing of the first and second waves of melanocyte migration (Milos & Dingle, 1978). This high expression implies that the neural crest cells remain in the embryo as melanocyte stem cells as opposed to mature melanocytes. Indeed, expression of melanocyte differentiation markers, *mitfa, dct, mc1r,* and *tyr,* are significantly decreased in *prdm1a* mutants compared to the wildtype. In addition, pigment production is also reduced, suggesting that when Prdm1a is decreased and *sox10* is increased, the melanocyte stem cell population is imbalanced, and cells cannot properly differentiate to mature, pigment producing melanocytes. In turn, the cells assume a stem cell-like state, in which they can proliferate uncontrollably. This data in zebrafish embryos supplements the melanoma results and suggest a mechanism by which PRDM1 is acting as a tumor suppressor. When PRDM1 is decreased, this results in an increase in SOX10

in the melanocyte stem/progenitor cells, which are then prone to rapid self-renewal and migration. Melanoma tumors are known for being hyperpigmented, but our embryo data contrastingly show a decrease in pigment production in *prdm1a* mutants. It is likely that the tumor cells are hijacking pigment genes at a later time to overproduce melanin. Unfortunately, we cannot test this later pigment production in the *prdm1a* mutant embryos as they die in early embryogenesis (Hernandez-Lagunas et al., 2005).

PRDM1 and *SOX10* are both critical regulators of neural crest cell development, and here we propose that they play an important role in the initiation and progression of melanoma as well. Parallels between neural crest cells and melanoma have been shown before. Using a similar model as we describe here, Kaufman et al. (2016) saw activation of an early neural crest gene, *crestin,* during melanoma initiation, just before the appearance of pigmented lesions. Isolation of *crestin:EGFP*$^{+}$ scales revealed increased expression of *sox10* compared to surrounding *crestin:EGFP*$^{-}$ scales. They argue that during melanoma initiation, a neural crest progenitor state is reactivated, suggesting that melanoma has a neural crest origin. However, this concept is widely debated. Nevertheless, studying neural crest cells will be useful for trying to better understand the mechanisms driving melanoma. It may lead to the discovery of future therapeutics and treatments for patients.

Author Contributions: R.I. and B.T.T. contributed equally to this work, performed, analyzed, and interpreted data, and performed the statistical analysis on the zebrafish; R.V. and C.C. performed the analysis on human cell lines; A-C.T. performed bioinformatic analysis; D.O. performed histological analysis and imaging. K.B.A. conceptualized the project and obtained funding, collaborating with Y.S.; R.I., B.T.T., and K.B.A. wrote the manuscript with help of Y.S. ; and C.C. and A-C.T. edited the manuscript.

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Conflicts of Interest: We have no conflicts to declare.

FIGURE LEGENDS

Figure 1. PRDM1 is recurrently deleted in human and zebrafish melanoma

A. A Venn diagram of recurrently deleted genes in human and zebrafish melanomas. PRDM1 is deleted in both and is a potential tumor suppressor.
B. *prdm1a* ortholog is recurrently deleted in zebrafish melanoma.
C. Distribution of mutations in publicly available datasets.
D. Expression of PRDM1 inversely correlates with clinical outcomes (TCGA). ***$p>0.001$ using a log-rank test.
E. Riker melanoma dataset shows PRDM1 is lower in metastatic melanoma (***$p<0.001$) and primary melanoma (*$p<0.05$) compared to normal skin with a one-way ANOVA test.
F. PRDM1 expression in melanoma in patient derived xenograft (PDX) model. PRDM1 expression is low except for MB1688.
G. Melanoma cell lines express lower PRDM1 RNA expression compared to normal primary melanocytes. ***$p<0.001$ with a two-way ANOVA test.

Figure 2 Expression of PRDM1 is negatively correlated with melanoma stages.

A. Representative images of tumors. PRDM1 expression are shown in red.
    a. 23 year old female, benign. Melanin and PRDM1 are strongly positive
    b. 2 year old female, benign. PRDM1 is strongly positive
    c. 5 month old male, benign. Melanin and PRDM1 are strongly positive
    d. 67 year old female, stage II, nuclear and cytoplasmic are positive
    e. 45 year old female, stage II, cytoplasmic positive
    f. 46 year old female, stage IIb, cytoplasmic positive, cells with round nuclei and scant cytoplasm stained very positive (red arrow)
    g. 52 year old male, stage III, negative
    h. 70 year old male, metastasis, PRDM1 score 0.005
    i. 43 year old female, metastasis, negative
B. Nuclear PRDM1 positive tumors in the tissue array, ME804B and ME1004F. Stage I and II, III and IV, and metastasis are shown.
C. Cytoplasmic PRDM1 positive tumors in the tissue array, ME804B and ME1004F. Stage I and II, III and IV, and metastasis are shown.
D. Summary of the number of positive and negative PRDM1 tumors, and area of the expression

E. Table shows the number and percentage of positive and negative PRDM1 tumors, and area of the expression

Figure 3 Loss of *prdm1a* in zebrafish increases tumor onset

A. $BRAF^{V600E}$ microinjection in zebrafish model induces melanoma and shows heterozygous loss of *prdm1a* promotes melanoma faster. p53-/- (mut): n=12, p53-/-(mut);*prdm1a*+/- (ht): n=8. *p<0.05 with a log-rank test.
B. Stable transgenic $BRAF^{V600E}$ model confirmed heterozygous loss of *prdm1a* promotes melanomas. p53-/- (mut): n=18, p53-/-(mut);*prdm1a*+/- (ht): n=33. *p<0.05 with a log-rank test.
C. Representative images of melanoma in p53-/- (mut) and p53-/-(mut);*prdm1a*+/- (ht) are shown (7 months and 11 months).
D. H&E staining of tumors (tumor size matched) show p53-/- (mut) ;*prdm1a*+/- (ht) tumors (7 months) have a higher metastasis rate as compared to p53-/- tumors (9 months). Red arrows indicate tumor cell invasion. Melanophages (rounded brown cells) are observed (blue arrows).
E. S100 staining in zebrafish melanoma. Red arrows indicate S100 positive cells.

Figure 4 Expression of melanocyte differentiation markers and pigment production is reduced in *prdm1a* mutants during embryonic development

A. qRT-PCR shows that *prdm1a* gene expression is highest at 2 days post fertilization (dpf).
B. Western blot shows Prdm1a expression was highest at 2 dpf and decreased over time.
C. Quantification of western blot of Prdm1a.

Expression of *dct* (D), *mitfa* (E), *mc1r* (F), and *tyr* (G) are significantly reduced in *prdm1a* mutants compared to the wildtype. Error bars indicate S.E.M. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 with a two-tailed, independent t-test.

H. Pigment production was reduced in mutants compared to wildtype embryos and in the wildtype/heterozygote mix compared to wildtype and mutant embryos. *p<0.05, **p<0.01, ****p<0.0001 using a one-way ANOVA.

Figure 5 PRDM1 likely acts as a tumor suppressor through regulation of *SOX10.*

A. PRDM1 and SOX10 are negatively correlated in human melanoma tumor samples (TCGA) as the higher the PRDM1 expression the lower the SOX10 expression. r=-0.73, ***p<0.001 with a Pearson correlation coefficient.
B. Expression of SOX10 positively correlates with clinical outcomes (TCGA). *p<0.05 with a log-rank test.

C. Microarray data comparing normal skin and melanoma in zebrafish in human samples.

D. qRT-PCR of *sox10* expression in wildtype and *prdm1a* mutant embryos. *sox10* expression is increased in mutants at 2 dpf and 4 dpf. Error bars indicate S.E.M. *$p<0.05$, ***$p<0.001$, ****$p<0.0001$ with a two-tailed, independent t-test.

E. qRT-PCR of *sox10* expression in transgenic zebrafish tumors. *sox10* is higher in *prdm1a* mutant tumors but this is not statistically significant. Error bars indicate S.E.M. $p>0.10$ with a Mann-Whitney U test.

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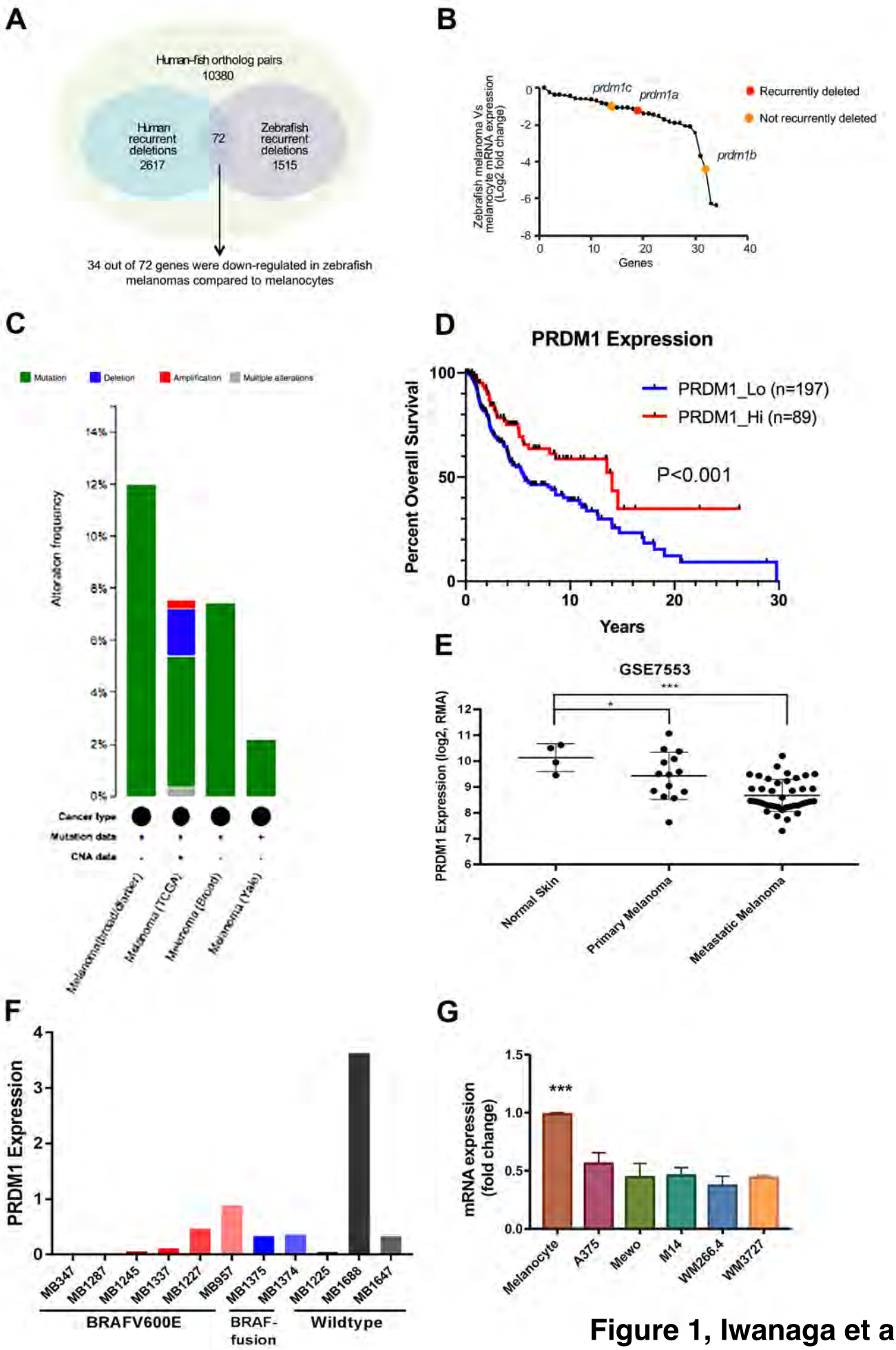
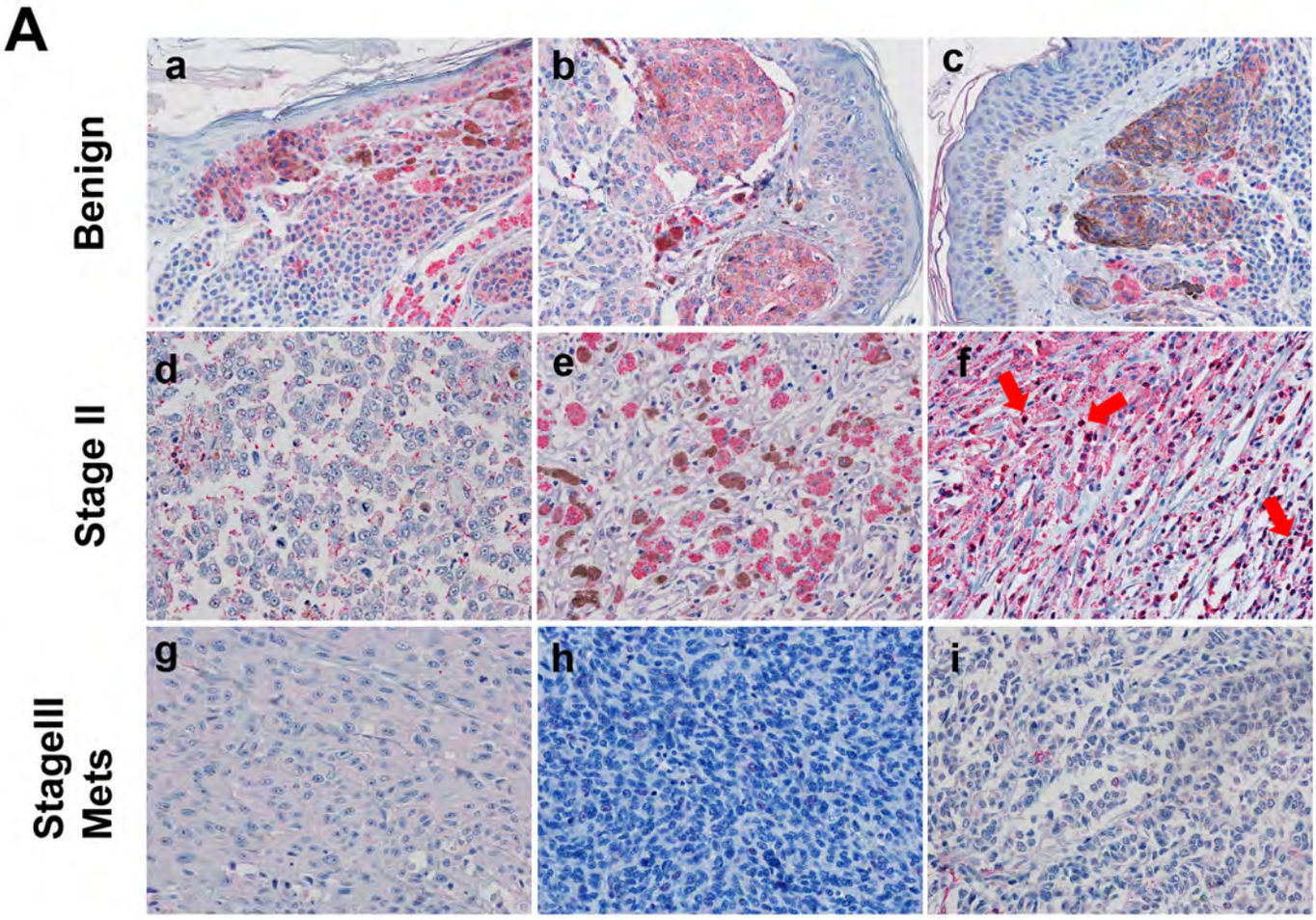
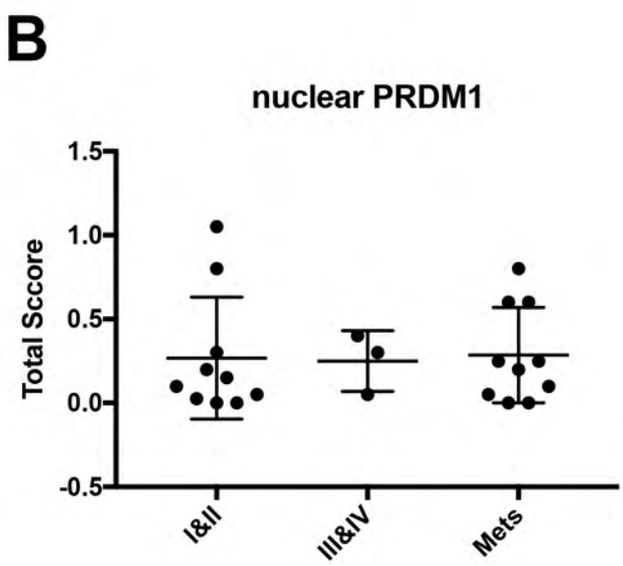
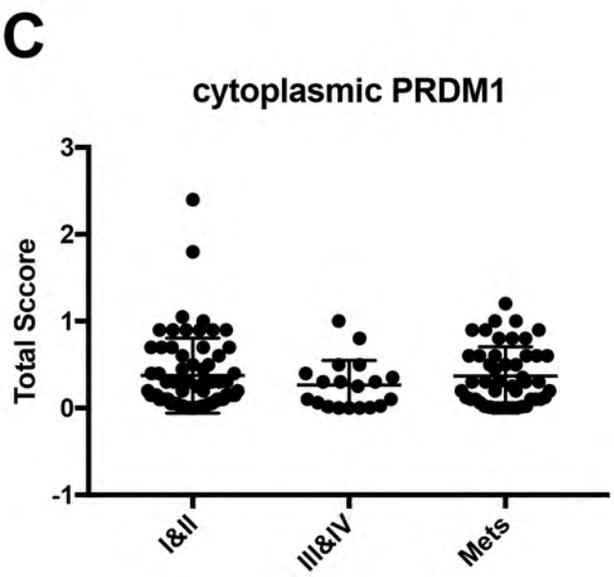
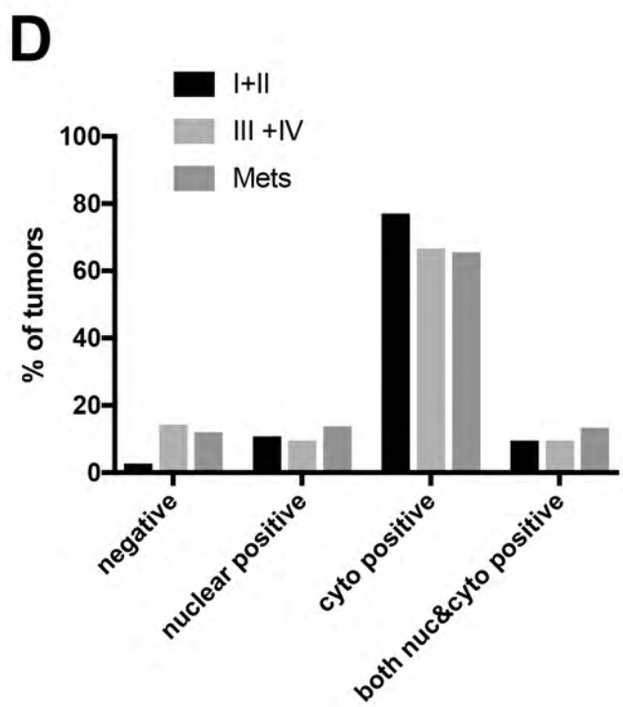


Figure 1, Iwanaga et al









E

	I+II	III +IV	Mets
Total	74	21	58
negative	**2 (2.7%)**	**3 (14.3%)**	**7 (12.1%)**
cyto positive	57 (77%)	14 (66.7%)	38 (65.5)
both nuclear and cyto positive	7 (9.5%)	2 (9.5%)	5 (8.6%)
nuclear positive	8 (10.8%)	2 (9.5%)	8 (13.8%)

Figure 2, Iwanaga et al

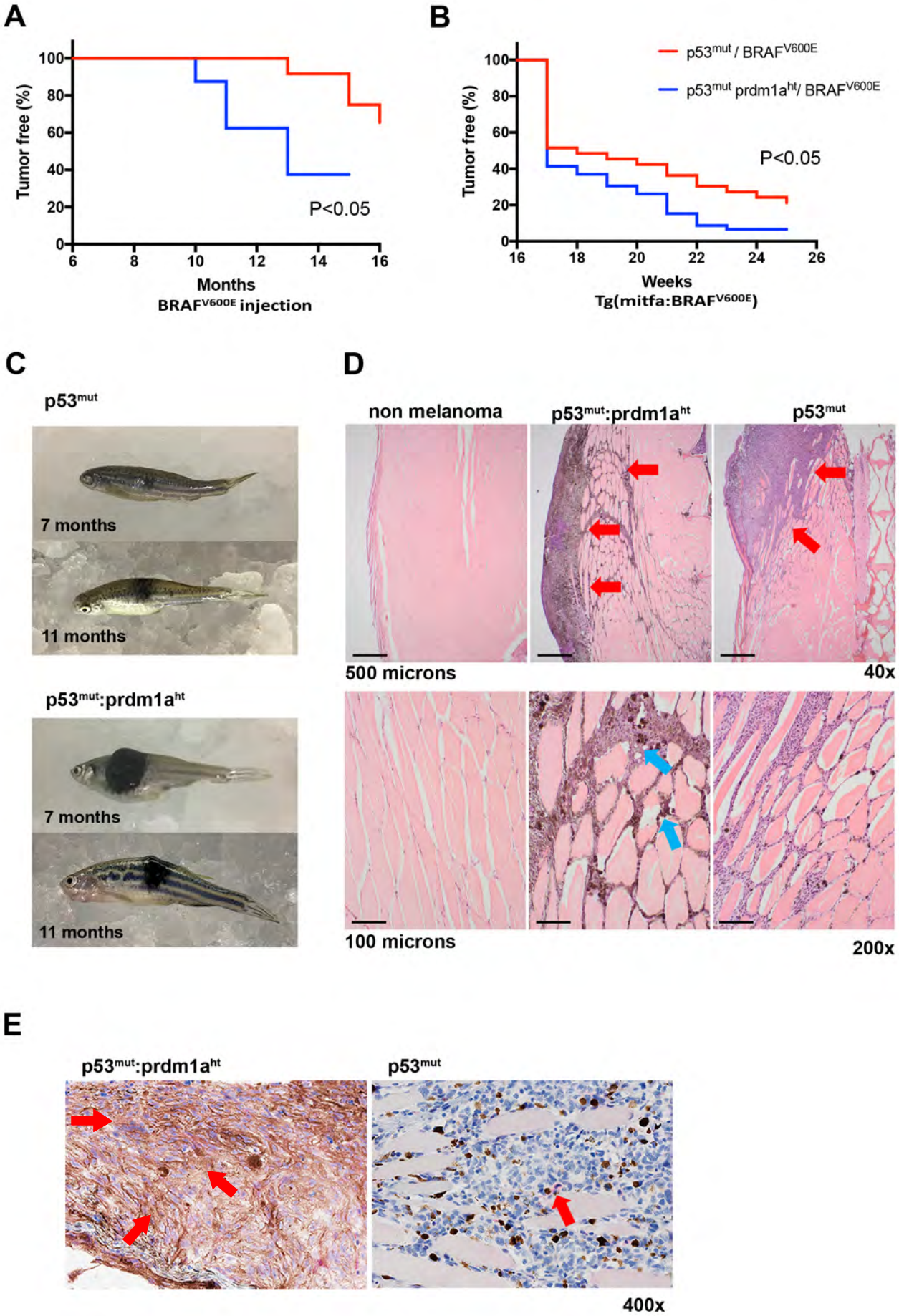


Figure 3, Iwanaga et al

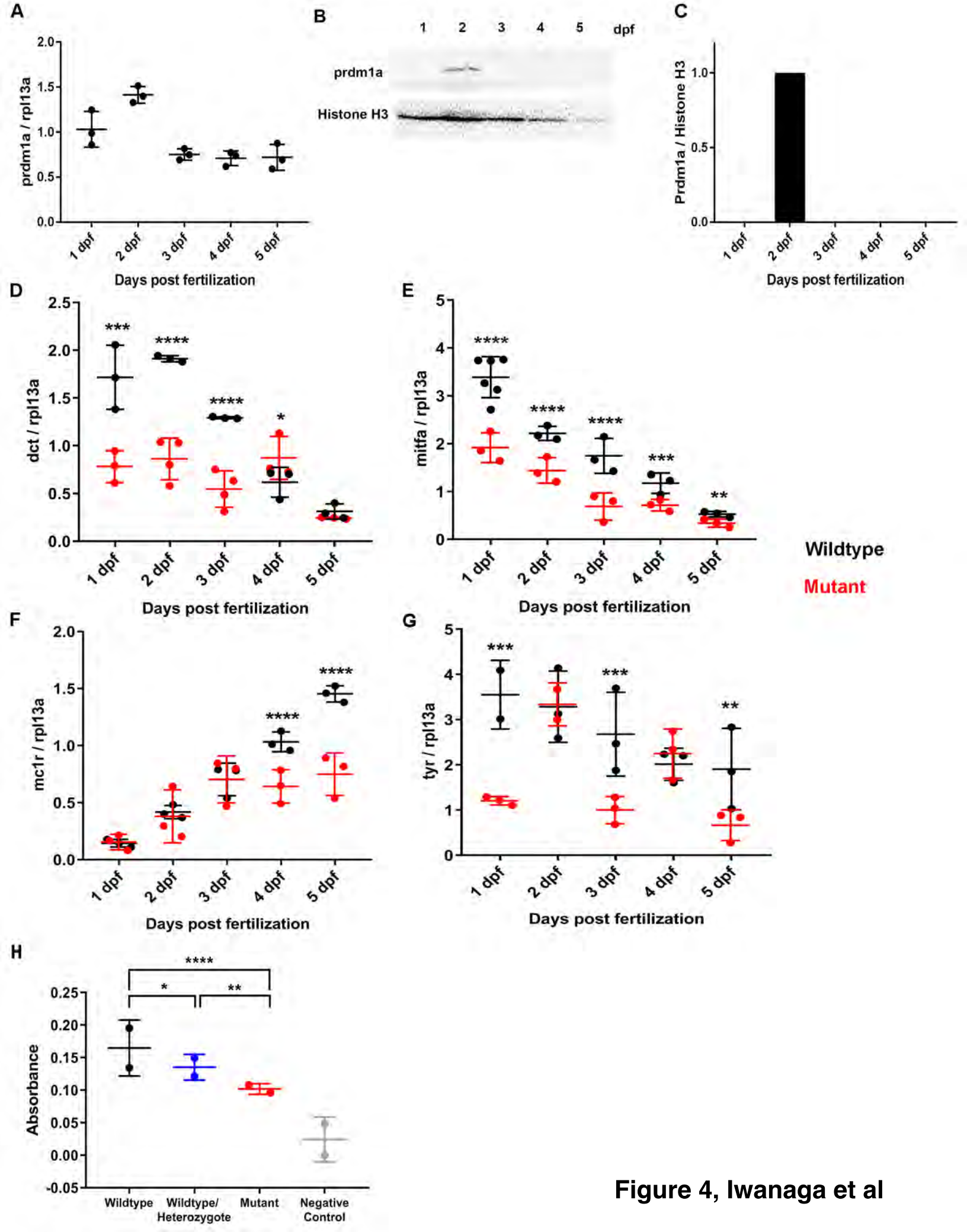
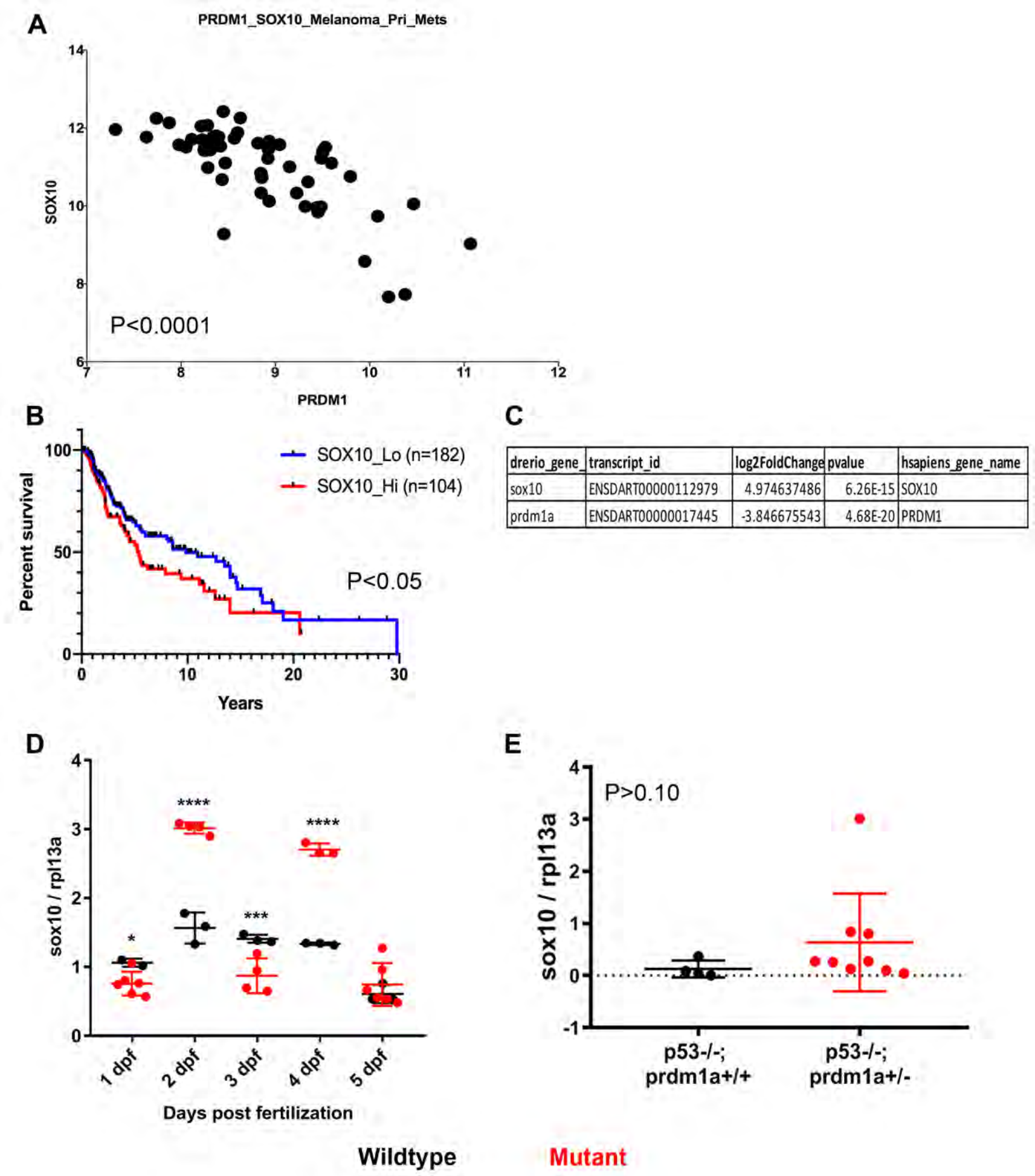


Figure 4, Iwanaga et al



drerio_gene_	transcript_id	log2FoldChange	pvalue	hsapiens_gene_name
sox10	ENSDART00000112979	4.974637486	6.26E-15	SOX10
prdm1a	ENSDART00000017445	-3.846675543	4.68E-20	PRDM1

Figure 5, Iwanaga et al