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Clinical and biological significance of EZH2 in non-sun-exposed melanoma

Elucidating the effects of modifications to the epigenetic regulator EZH2 in acral and mucosal melanoma

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Master Thesis

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Project description

Targeting a novel epigenetic regulator in Acral and Mucosal Melanoma. Elucidating the effects of modifications to this epigenetic regulator.

Master thesis project of Marlies Berger

The goal of this thesis is to address the limited understanding of the molecular pathogenesis of acral and mucosal melanoma, to figure out the underlying biology of these tumors and to identify and develop novel targets for treatment of these two non-sun-exposed histologic subtypes. I focus my work on EZH2, a key epigenetic regulator that tends to be upregulated in multiple cancers and is a driver of melanoma progression. Our central hypothesis is that increased EZH2 expression underpins ALM development and the course of the disease and may confer selective sensitivity to EZH2 inhibition. Since EZH2 inhibitors are already in clinical development for other cancer types, EZH2 represents an actionable therapeutic target in ALM and MM that can be rapidly translated to the clinic.

Relevance:
Surgical treatment after the occurrence of acral and mucosal melanoma is difficult, owing to the complexity and functional importance of the hands and feet and the location in the mucosa. Especially for acral melanoma, reconstruction after resection is usually needed. Newer treatment modalities such as immunotherapies and targeted agents are being tested in patients with advanced ALM and MM with some promising preliminary results. The progress in better understanding EZH2 and its inhibition can be used as a therapeutic approach for these tumors. In addition, the test of a novel compound that degrades EZH2 in ALM cell lines in vitro (cell proliferation, apoptosis) may help the cure of ALM.

Benefits and expected results:
In contrast to superficial spreading melanoma (SSM), patients with MM and ALM tend to have fewer atypical nevi and a lower incidence of sun burning as well as a higher personal and family history of non-cutaneous malignancies. As with other types of melanoma, ulceration and a high mitotic rate are considered poor prognostic signs. The diagnosis of ALM and MM, especially subungual lesions, can be difficult, and it is important to obtain an adequate biopsy specimen. Patients with ALM appear to have a worse prognosis overall than patients with superficial spreading and nodular melanomas Hence an improvement of the treatment and a better knowledge about these histological subtypes can help many patients in the future.

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Abbreviations

Ac	Acetylation
AM / ALM	Acral melanoma / Acral lentiginous melanoma
BRAF	B-Raf and v-Raf murine sarcoma viral oncogene homolog B
BrdU	Bromdesoxyuridin
CHX	Cycloheximide
CM	Cutaneous Melanoma
CNAs	copy number abnormalities
CNV	Copy number variation
DAB	3,3'-Diaminobenzidine
EZH2	Enhancer of zeste homolog 2
FFPE	formalin-fixed paraffin-embedded tissue
GBM	glioblastoma
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HMT	Histone methyl transferase
HMTi	Histone methyl transferase inhibitor
IHC	Immunohistochemistry
IP	Intraperitoneal injection
Me	Methylation
MM	Mucosal Melanoma
MSC	Mesenchymal Stem Cells
MTS	tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)
NAM	Non-acral melanoma
NM	nodular melanoma
NRAS	Neuroblastoma RAS viral oncogene homolog
OS	Overall Survival
P	Phosphorylation
PARP cleavage	Poly (ADP-ribose) polymerase
PCR2	Polycomb Repressive Complex 2
PES	electron coupling reagent (phenazine ethosulfate)
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PTEN	Phosphatase and Tensin homolog
PTMs	Post-translational modifications
RNAi	RNA interference
SCNA	somatic copy number alterations
shRNA	small hairpin RNA / short hairpin RNA
SSM	superficial spreading melanoma
SSM	superficial spreading melanoma
Su	Sumoylation
Ub	Ubiquitination

Glossary

Apoptosis	Cell death
Biomarker	A biological marker relates to a measurable indicator of some (biological) condition.
H3K27me3	Trimethylation of Lysine 27 on Histone 3 = repressive mark on gene expression
Housekeeping gene	Generally, housekeeping genes are constitutive genes that code for enzymes and proteins that are required for basic cellular functions, e.g. glucose metabolism. A housekeeping gene is expressed in all cells of an organism so it is independent of cell type and external factors.
Immunogenic	Substances able to produce an immune response.
Immunohistochemistry	IHC involves the process of selectively imaging antigens by antibodies specifically binding to antigens in biological tissues.
Locally recurrent melanoma skin cancer	If the tumour comes back in the same place or close to where the cancer first started, it is named locally recurrent.
Metastatic melanoma skin cancer / distant recurrence	If the cancer recurs in another part of the body farther from where it started.
Non-canonical function	Another function, other than the well-known Histone-methyl-transferase function, a so-called Histone-methyl-transferase independent function
Recurrent melanoma skin cancer	Recurrent melanoma means that the cancer has come back after treatment.
Stage 0 - IV	progression of the disease
Transduction	The transfer of genetic material from one cell to another, especially a bacterial cell, through the use of a bacteriophage.
Tumour ulceration	A lesion that is disintegrating and eroding away the skin or mucous membrane.
Westernblot	Immunoblotting enables the identification of specific proteins from a complex mixture obtained from cells.

1. Abstract

The general aim of the study was to increase the understanding about the key epigenetic regulator EZH2 gene. The histone-lysine N-methyltransferase enzyme encoded by EZH2 gene is in charge of DNA methylation and transcriptional repression.

Interestingly enough, mutation or over-expression of EZH2 has been linked to numerous forms of cancer. Interrupting EZH2 activity may slow tumor growth. Furthermore, mutations in the EZH2 gene are also associated with Weaver syndrome and involved in causing neurodegenerative symptoms in the nervous system disorder.

There is a lack of MM and ALM-specific targeted therapies which is due to incomplete understanding of the molecular alterations that drive these diseases. Furthermore, delays in diagnosis often results in more advanced diseases at presentation. EZH2 inhibitors have entered clinical trials for several cancer types, rendering EZH2 an actionable target that can be rapidly translated to the clinic.

2. Background

1.1 Melanoma

Among all cancers, skin cancer represents one-third of all tumors that are diagnosed every year worldwide. Even though being the least frequent among this group of malignant cancers, cutaneous melanoma is one of the most aggressive malignancies in human, considering its invasiveness and metastatic tumor. (R. Barra-Martínez, 2015) Melanoma develops if there is uncontrolled growth of melanocytes, the cells responsible for pigmentation. (Bandarchi, 2010)

The majority of melanomas are cutaneous, i.e. they grow within the skin, especially in areas exposed to the sun. In women, the legs are affected most frequently. In contrast, in men, the most common sites for melanoma are the back or chest. However, melanomas are also frequently found on the neck or face. Melanoma may also be found in areas of the body not usually exposed to the sun. (AmericanCancerSociety, 2016)

Table 1 Melanoma Histological Subtypes - Features

Melanoma Histological Subtypes - Features			
Location	**Subtypes**	**Frequency**	**Caused by sun exposure**
Cutaneous	Superficial spreading	70%	yes
Cutaneous	Nodular	15%	yes
Cutaneous	Acral Lentiginous	5%	no
Non-Cutaneous	Mucosal	1%	no

In general, melanoma is highly heterogeneous resulting in different ways how to classify melanoma. One is by histology, which can be seen in Figure 1. The 4 major histologic subtypes of melanoma or lentigo maligna are superficial spreading melanoma (SSM), nodular melanoma (NM), acral lentiginous (ALM)

and mucosal melanoma (MM). (R. Barra-Martínez, 2015) The majority of the melanomas are cutaneous, meaning they grow within the skin. (MelanomaResearchFoundation, 2017)

Figure 1 Classification of melanoma by histology

Another way of classifying it is by genetics (mutations), see Figure 2. It is known that cutaneous and Uveal melanomas have mainly BRAF and NRAS mutations in high percentages. Acral and mucosal, both have a lower mutational burden than other cutaneous melanomas. This lack of somatic driver mutations could be explained by lower level of sun exposure. (Cell, 2015), (Harbour JW, 2010), (Lovly C, 2016)

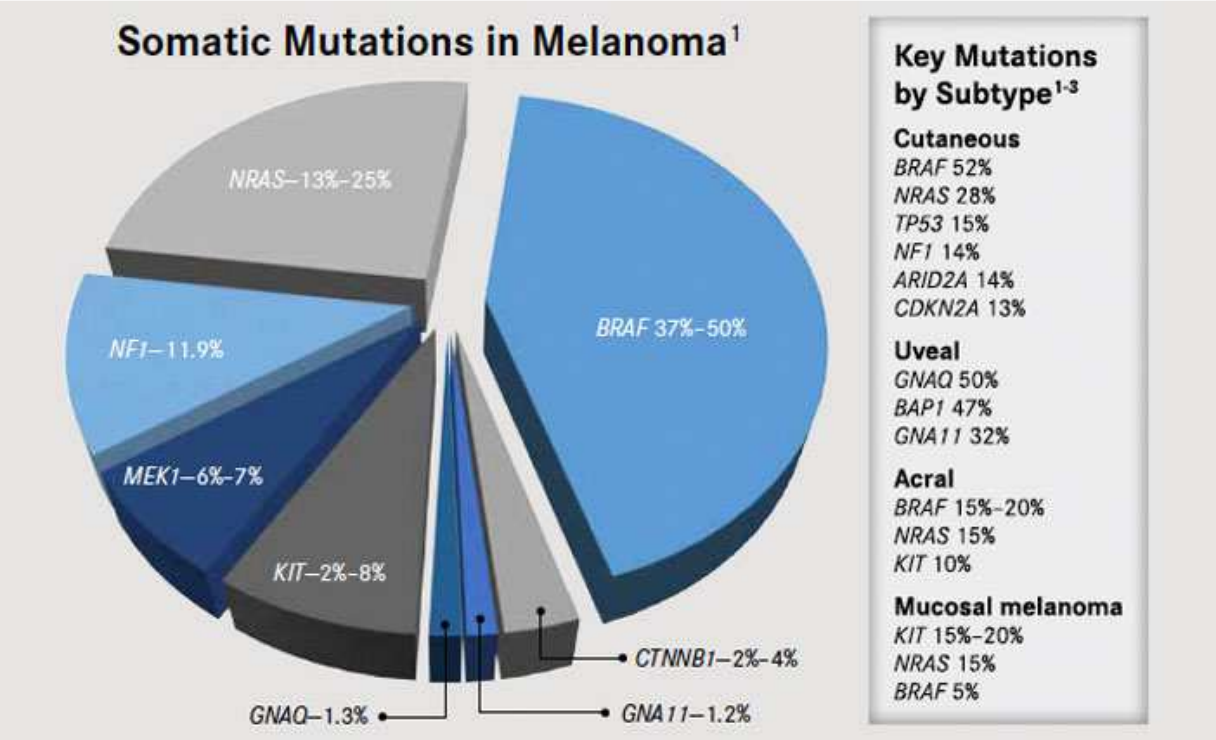


Figure 2 Classification of melanoma by genetics [1]

I will focus my work on ALM which is less common, but more aggressive. Interestingly it shares several characteristics with MM, since both arise on surfaces that are usually not exposed to sunlight. (Classification by sun exposure).

1.1.1 Melanoma Skin Cancer Stages

The different staging of a cancer describes how progressed the disease is. For melanoma, this includes its thickness in the skin, whether it has spread to nearby lymph nodes or any other organs, and certain other factors. The stages range from 0 to 4, often written as the Roman numerals. Generally, the higher the stage number, the more the cancer has spread. (AmericanCancerSociety, 2016)

1.1.1.1 Stage 0 (or melanoma in situ)

Stage 0 indicates that the cancer cells are only in the top or outer layer of the skin, so-called epidermis. This melanoma in situ is often described as a precancerous condition of the skin.

1.1.1.2 Stage I

The tumor thickness ranges from is 0.8 mm to 2 mm. There is no or only a slight ulceration of the tumor. There is no broken skin or open wound.

1.1.1.3 Stage II

Stage II can be subdivided in IIA, IIB and IIC, describing the advance of tumor thickness and ulceration.

1.1.1.4 Stage III

Stage III signals that the cancer has spread to one or more lymph nodes near the origin of the cancer (regional lymph nodes). After the lymph nodes are removed and examined by a pathologist, the cancer can be assigned stage IIIA, IIIB, IIIC or IIID, depending on the number of lymph nodes that contain cancer, the amount of cancer in the lymph nodes and the fact whether the cancer has spread to nearby areas of skin (satellite tumors) or lymph vessels (in transit tumors).

1.1.1.5 Stage IV

Last but not least, stage IV indicates that the tumor has spread to other parts of the body (called distant metastasis), such as to the liver or lungs. A different name for this progress is metastatic melanoma skin cancer.

1.1.2 Acral melanoma

Acral lentiginous melanoma (ALM or AM), short Acral melanoma is a rare subtype of melanoma that arises from pigment cells on non-sun-exposed (acral) skin surfaces. (Oakley, 2011) The term “acral” is derived from the Greek root "akren" meaning “affecting the extremities” or” Relating to the peripheral parts, e.g., limbs, fingers, ears, and other body parts ". (MedicalDictionary)

ALM appears mainly on the nail-beds, soles of the feet, palms of the hands and other hairless parts of the skin. It is the most common melanoma subtype in patients of African or Asian descent (Alina Goldenberg, 2015). There is a racial disparity in the incidence of melanoma histologic subtypes: black and Hispanic Americans are far more likely than white Americans (~40% vs. ~5%) to suffer from acral lentiginous melanoma (ALM). (Porcia T. Bradford, 2009)

This melanoma subtype that disproportionate affects US racial minority populations is biologically distinct from non-acral melanoma. Human cutaneous melanoma i.e. superficial spreading melanoma and nodular melanoma is typically associated with UV damage and primarily driven by activating mutations in BRAF (50%) or NRAS (17%). Many of the tumors that contain BRAF mutations also have inactivation of PTEN. In contrast, human acral melanoma harbors these mutations much less frequently. (James S. Goydos and Steven L. Shoen, 2016)

Unlike other more common types of melanoma, ALM is not caused by UV damage from the sun. (Lin Liu, 2016). The lack of mutational burden makes therapeutic targeting and research modeling of ALM potentially different from that of the cutaneous form in humans. Up to now, attempts to establish targeted therapies that improve therapy outcomes in metastatic ALM have unfortunately been mostly ineffective. This lack of therapy needs to be targeted, since this non sun-exposed melanoma is also more aggressive in comparison to other cutaneous melanomas (CMs). (R. Barra-Martínez, 2015)

As with most melanomas, early AM is usually diagnosed by visual inspection. Unfortunately it is often mistaken as subungual melanoma as either traumatic injuries or fungal infections, which leads to delay in diagnoses and a worse prognosis for patients with this subtype of melanoma. Surgical treatment after the appearance of acral melanoma is difficult, owing to the complexity and functional importance of the hands and feet, respectively. So far, endeavor to identify targeted therapies that can enhance treatment outcome in metastatic ALM have been unsuccessful. (Metzger S, 1998)

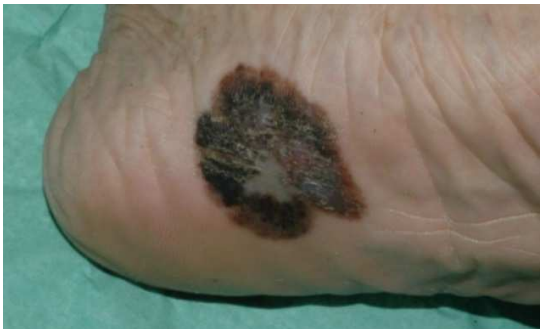


Figure 3 Acral lentiginous melanoma on the sole of a foot [2]

1.1.3 Mucosal melanoma

Mucosal melanoma (MM) is a rare form of melanoma that appears, as its name indicates, on mucosal surfaces. Mucous membranes are moist surfaces that line cavities within the body like the gastrointestinal tract, genitourinary tract and respiratory tract. Additionally, MMs occur most frequently in the head and neck, in the mouth, eyes, nasopharynx and larynx. They make up roughly 1% of all melanomas but appear to be mostly more complicated owing to late diagnosis due to their less visible locations and since they are often not pigmented.

This histologic subtype develops if uncontrolled growth of melanocytes (pigment cells) is present. It is similar to acral melanoma since both lack activating BRAF and NRAS mutations, in contrast to human cutaneous melanoma. The peak age of diagnosis of MM is between 70 and 80. However, younger people have a certain risk to develop mucosal melanoma, especially of the oral cavity.

The risk factors for mucosal melanoma include mutation and over-expression of the KIT gene, smoking, and ingested and inhaled environmental carcinogens.

Since the signs and symptoms of mucosal melanoma largely depend on its location, there are a wide variety of symptoms that patients may experience. If there is suspicion of mucosal melanoma, the most important test is a biopsy of the involved tissue. If the pathology report indicates melanoma, the patient will be fully examined with CTs, MRIs and PET scans. (Yeh, 2014)

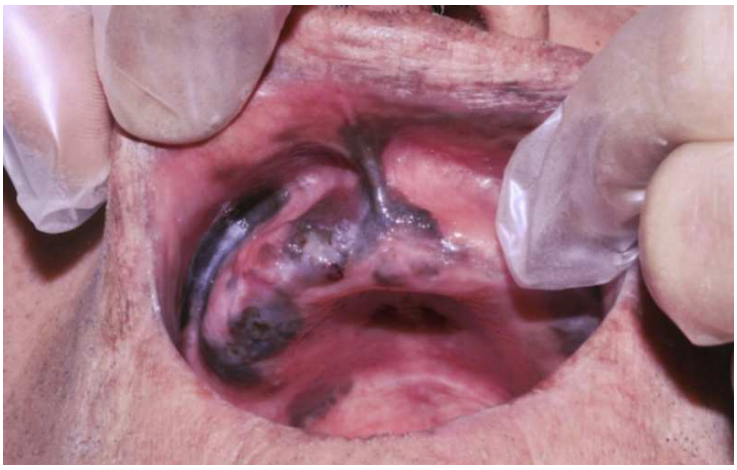


Figure 4 Mucosal melanoma in the mouth [3]

As described previously, mucosal and acral melanoma share numerous common features, including the presence of field cancerization cells, early chromosomal instability, a late age of onset, a broad radial growth phase with prominent lentiginous growth and a lack of a precursor nevus. Besides, many of the same genes are also involved in these two histologic subtypes. (Gerami, 2017)

1.1.4 Prognosis

In terms of prognosis, several studies have been performed examining melanoma-specific survival rates for patients with different subtypes of melanoma. After controlling for primary tumor thickness and stage, patients with ALM seem to have a worse overall outlook (five- and ten-year melanoma-specific survival rates of around 80 and 68%, respectively), than patients with superficial spreading and nodular melanomas (five- and ten-year melanoma-specific survival rates of 91 and 88 %, respectively ($p < 0.001$)). Interestingly, there does not appear to be a difference in prognosis between male and female tumor patients, in contrast to light-skinned Hispanic patients and patients from Asian or Pacific. Island inhabitants seem to have a worse forecast than other patients with ALM. (Judy Zhong, NYU Statistician, 2017)

The difference in prognosis between the non-sun-exposed melanomas like ALM and MM in comparison to other cutaneous melanomas emphasizes the demand for better patient screening in the course of physical examinations and increased effort in patient education particularly targeting vulnerable populations, with the result that these wounds can be diagnosed as early in their progress as possible.

There are several reasons for worse outcome, including the delay of diagnosis in MM and ALM, since it is mostly minorities who are affected and especially in the US they have less access to the health care system because of the huge socio-ecological status difference. Moreover, the delay in diagnosis might result in thicker tumors at presentation. Furthermore, the non-visible location of these tumors makes it difficult to determine its existence as well as the fact that these tumors tend to be biologically more aggressive. (Carrera C. , et al., 2017)

Besides, there is a lack of targeted therapy (BRAF targeted therapy) since they have a lower mutational burden. Not many patients can benefit from targeted therapy. There is a lack of therapy and less prospect with therapy.

There does not exist a lot of data showing the outcome with immunotherapy, whether it might be better or worse meaning the effectiveness of immunotherapy is unclear.

1.1.4.1 Melanoma survival

Figure 5 shows the ALM survival curve, created by Judy Zhong, the NYU statistician. It is unambiguous that acral melanoma is an independent negative predictor of Recurrence free survival (RFS) (HR, 2.24, p=0.001) & Overall survival (OS) (HR, 2.58, p=0.001) compared with non-acral melanoma. Also, there is a significantly higher recurrence rate for ALM vs. NAM for thinner tumors (<2.0 mm), p=0.048 (Priyanka V. Gumaste, 2014)

To evaluate whether ALM histologic subtype was an independent predictor of melanoma-specific survival, a multivariate Cox regression model was used. After adjusting for unmatched prognostic indicators such as sentinel lymph node status, Breslow thickness, ulceration and mitotic index, the results proofed the fact that ALM was an independent negative predictor of melanoma-specific survival (HR = 2.64, p = 0.001) compared to NAM. (Priyanka V. Gumaste, 2014)

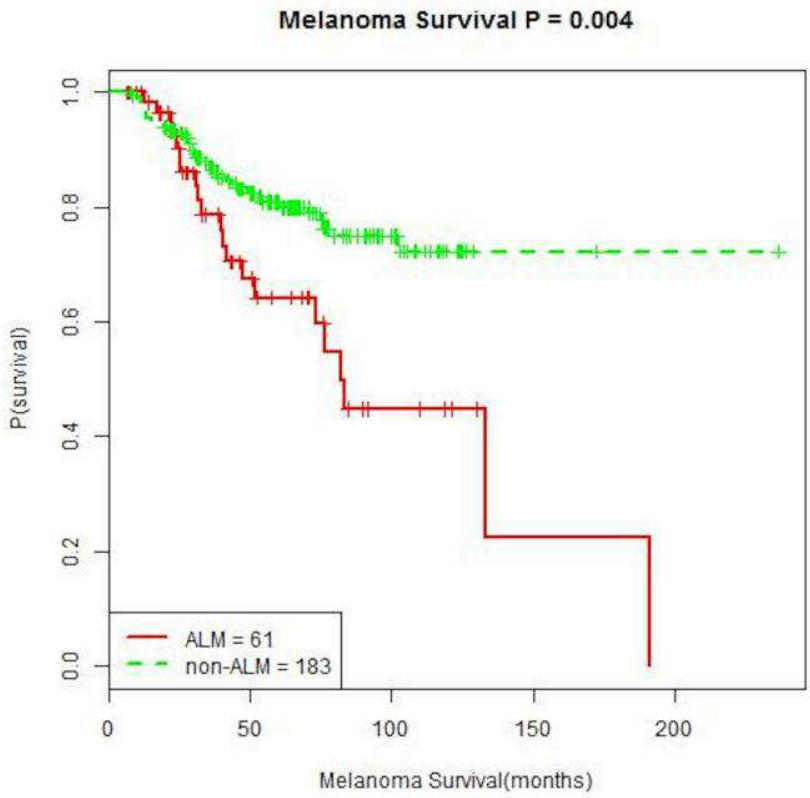


Figure 5 Graph showing significant worse survival with acral lentiginous melanoma

The Kaplan-Meier curve of Melanoma-Specific Survival in ALM and NAM in Figure 5Figure 21 makes clear that there is a significant overall survival effect with EZH2 in acral melanoma. Interestingly, the ALM cohort demonstrated a lower median melanoma-specific survival of 82.1 months, in contrast to the NAM group with more than 200 months (p=0.004). The average follow-up time for the NAM and ALM cohorts were 58 months and 33 months, respectively. Furthermore, the median melanoma-specific survival time has not yet been reached in the NAM group. (Priyanka V. Gumaste, 2014)

1.1.4.2 Recurrence free survival

Figure 6 shows the Kaplan-Meier curve of Recurrence-Free Survival in ALM and NAM. This analysis suggests that the median recurrence-free survival time in ALM is 47.1 months, compared to 213.5 months in NAM (p<0.001). After performing a multivariate analysis, the data revealed that the acral histologic subtype is an independent negative predictor of recurrence-free survival compared to non-acral melanoma (p < 0.001). (Priyanka V. Gumaste, 2014)

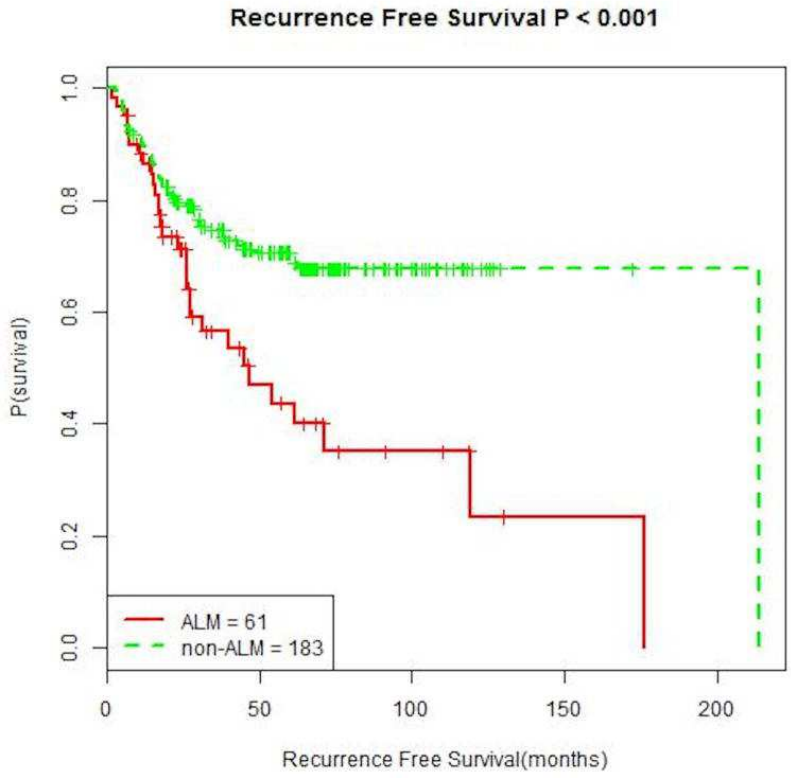


Figure 6 Kaplan-Meier curve of Recurrence-Free Survival in ALM and NAM showing that patients with ALM experienced a decreased recurrence- free survival compared to NAM and that ALM is an independent negative predictor of recurrence free survival.

In terms of demographic and primary lesion clinical parameters this analysis did not detect any significant differences in ALM and NAM patients who experienced a recurrence. Interestingly, the mean age of recurrence in acral melanoma was 61.0 years in contrast to 66.3 years in non-acral melanoma (p=0.06). The study could not identify a significant difference in mitoses (p=0.2), tumor thickness (p= 0.201), ulceration (p=0.82) or lymph node status (p=0.82) between ALM and MM. The specific analysis of a subset of recurred patients revealed that there is no significant difference in melanoma-specific survival time between acral and non-acral melanoma. (p = 0.67). (Priyanka V. Gumaste, 2014)

1.1.5 Epigenetic regulation and cancer

Figure 7 shows the genomics and epigenetics in the cell cycles as well as the epigenetic and genetic changes resulting in cancer. Furthermore, recent studies state that carcinogenesis cannot be accounted for by genetic alterations alone, but also involve epigenetic changes such as DNA methylation, histone modifications and microRNAs. During cancer formation, a large number of epigenetic modifiers are mutated or abnormally activated. At the same time, epigenetic changes such as DNA methylation, histone modifications and microRNAs lead to abnormal gene expression which evokes genome instability. (Chen, 2013)

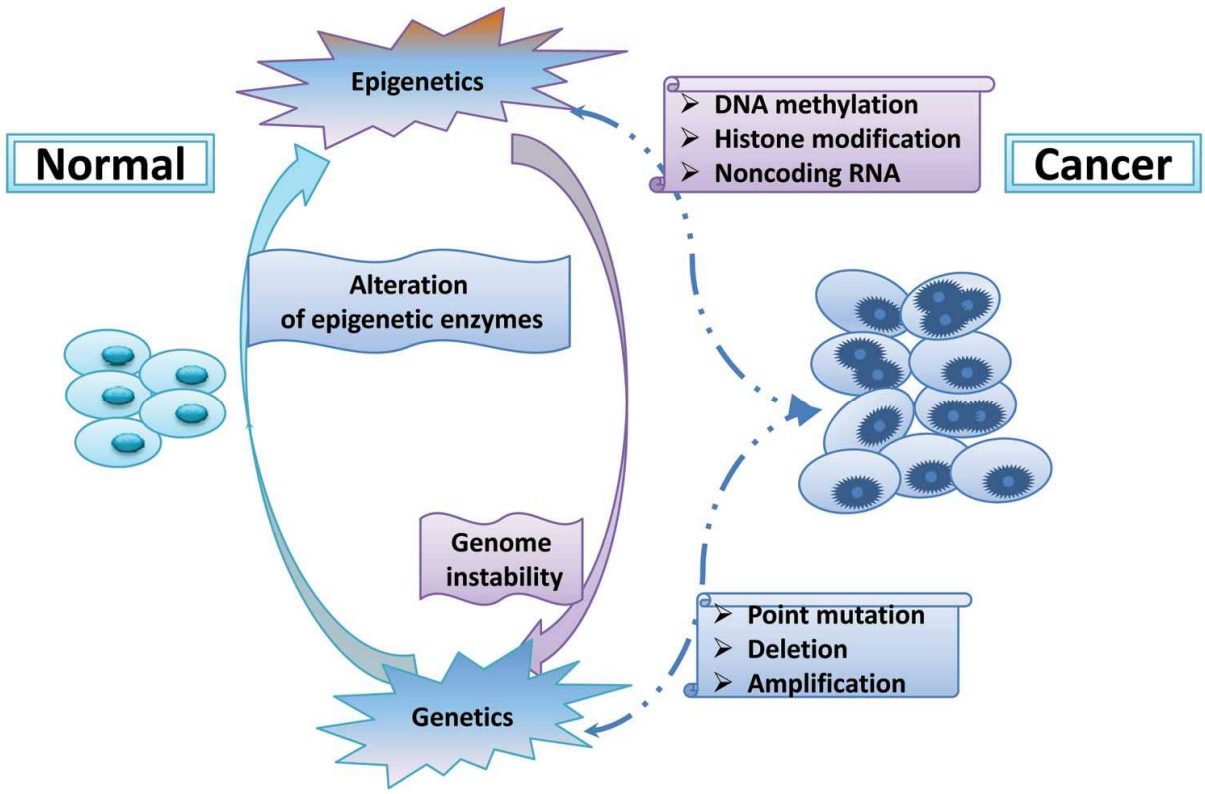


Figure 7 Epigenetic regulations in cancer [4]

Since ALM and MM have a lower mutational burden but usually a worse outcome, we came to our central hypothesis.

1.1.5.1 Central Hypothesis:

There is an epigenetic basis for ALM and MM development and progression.

Alterations of epigenetic enzymes feature in many cancers. EZH2, being an epigenetic regulator that caught a lot of interest in recent years, was the main subject in our investigations and might be one of the most prominent epigenetic enzymes. Enhancer of zeste homolog 2 has an important role in many cancers like melanoma. A lot of research has been done on it, but it has not been looked at in non-sun-exposed melanoma.

1.2 EZH2

Enhancer of zeste homolog 2, short EZH2, is the functional enzymatic component of the Polycomb Repressive Complex 2 (PRC2) see Figure 8. This highly conserved methyltransferase is often overexpressed in human and murine melanoma and its function is essential for melanoma initiation and growth. Furthermore it is crucial throughout several steps of melanoma metastasis, such as Epithelial-Mesenchymal Transition (EMT). EMT is a mechanism where epithelial cells lose their cell-cell adhesion and cell polarity, and gain invasive and migratory properties to become mesenchymal stem cells. MSCs

are multipotent stromal cells that can differentiate into a large number of cell types. (Barsotti, Ryskin, Kung, Verhelle, & Rollins, 2015)

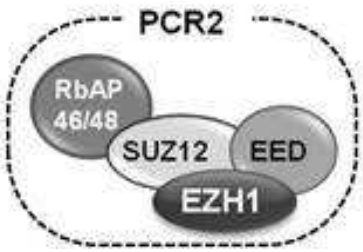


Figure 8 Schematic of the Polycomb Repressive Complex 2 [5]

Due to its frequent upregulation in cancer, EZH2 is a potential target for inhibition in many cancers. Its loss activates p21 and induces both: senescence and apoptosis in melanoma. (Mahmoud, 2016)

The enzyme´s primary role is to methylate histones, more precisely the trimethylation of Lysine 27 (H3K27me) on histone H3, Figure 10. This mechanism is PRC-2 Methyltransferase independent. The addition of the tri-methyl groups to histone 3 at lysine 27 functions by using the cofactor S-adenosyl-L-methionine. (Yoo KH, 2012)

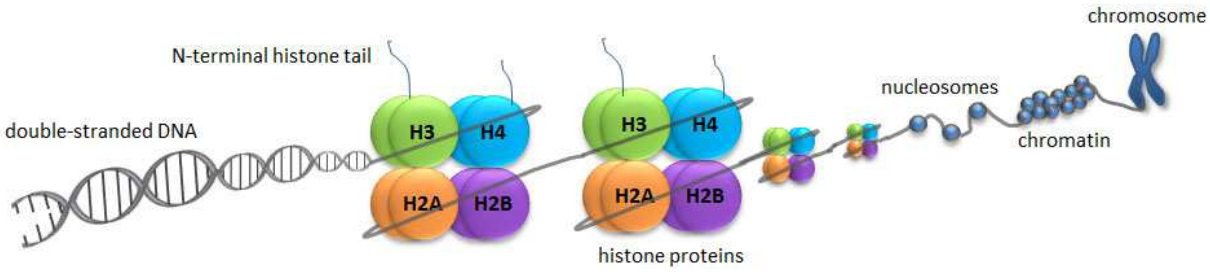


Figure 9 Schematic representation showing the organization and packaging of genetic material [6]

In Figure 10 the nucleosomes are represented in grey. The figure above shows how the DNA is wrapped around eight histone proteins, called H2A, H2B, H3, and H4 (green, blue, orange and purple circles). The N-terminal histone tails are shown in blue, they protrude from H3 and H4.

Figure 10 shows the highly conserved histone methyltransferase that targets lysine-27 of histone 3. [7]

Figure 10 is another representation showing how the nuclear DNA does not appear in free linear strands but is highly condensed and wrapped around histone proteins in order to fit inside of the nucleus, be packaged. They take part in the formation of chromosomes and give them its structure. EZH2´s canonical function, the trimethylation, leads to chromatin condensation. This compacting of the chromatin blocks the recruitments of transcriptional initiation machinery thereby silencing (transcriptional repression) of the affected target gene. These target genes tend to be genes responsible for suppressing tumor development, so-called tumor suppressor genes, which are involved in apoptosis. It also suppresses MHC II expression which helps melanoma cells to escape from immune surveillance and thereby avoiding senescence. It abnormally represses tumor suppressor genes.

During cell mitosis, remodeling of chromosomal heterochromatin by EZH2 is required. A mutation in EZH2 may cause a gain of function (= an activating mutation). This key player in promoting malignant melanoma progression has a particular role in melanoma growth. The EZH2 expression is strongly upregulated during malignant melanoma progression. The activity of this histone-lysine N-methyltransferase is regulated by methylation and acetylation.

As various EZH2 inhibitors have already entered clinical trials for other cancer types, EZH2 represents an actionable target in AM that can be rapidly translated to the clinic. In addition, the test of a novel compound that degrades EZH2 in cell lines in vitro (cell proliferation, apoptosis) may help the cure of ALM and MM. Mutation or over-expression of EZH2 has been linked to many forms of cancer. Therefore we hypothesize that blockage or depletion of EZH2 may slow tumor growth. (Irene Marchesi, 2013)

Figure 11 Biology of EZH2 overexpression in melanoma [8]

Figure 11 demonstrates the effects of EZH2 on the biology of melanoma. The positive effects are highlighted in green, the negative ones in red.

The enhanced B and T cell functions are favorable, as well as the regulation of the epithelial-mesenchymal transition and the enhanced interactions between melanoma cells and the microenvironment.

In contrast, the negative effects by far outweigh the positive effects. It has adverse effects on the immune system by suppressing MHCII expression and helping melanoma cells escape immune surveillance. As mentioned above, EZH2 silences tumor suppressor and apoptotic genes and helps melanoma cells escape senescence. EZH2 enhances the process of de-differentiation to neural-like stem cells and leads to EZH2- mediated silencing of cyclin-dependent kinase inhibitors (CdkI). (Mahmoud, 2016)

Preliminary data, obtained by the lab team of Prof. Iman Osman, MD, suggests that the frequency of EZH2 overexpression in acral and mucosal melanoma is higher than that previously reported for other melanoma subtypes, implying a particular role for EZH2 alterations in ALM and MM biology and supporting the rationale for targeting EZH2 in these tumors.

1.2.1 Specific Hypotheses:

a. **I hypothesize that EZH2 levels are increased in acral and mucosal melanomas.**

b. **I hypothesize that increased EZH2 levels are associated with tumor progression and a poor clinical outcome for patients.**

c. **I hypothesize that multiple mechanisms are responsible for increased EZH2 levels in acral and mucosal melanoma, including EZH2 gene copy number gain and protein stabilization.**

d. **Owing to the fact that the EZH2 enzyme is a histone methyltransferase, I hypothesize that increased expression of EZH2 provokes H3K27me3 upregulation.**

1.2.2 Aims:

The specific aims of the project can be divided into two parts to address the clinical and the biological significance of EZH2 in non-sun-exposed melanomas:

- ✓ Firstly, the validation of the role of altered EZH2 function, which can be subdivided into the testing of an expanded non-sun-exposed melanoma cohort for the presence of EZH2 mutations by DNA sequencing and the investigation of EZH2 gain of function by an alternative mechanism: EZH2 protein overexpression, using immunohistochemistry and western blotting.

- ✓ The second part consists of the investigation of EZH2 inhibition or degradation as a therapeutic approach for these distinct melanoma subtypes, by pharmacologically testing three small molecule drugs, two of them inhibiting the canonical enzymatic function of EZH2 and one of them, a novel compound that degrades EZH2. These experiments are performed using ALM and CM cell lines in vitro by testing cell proliferation and apoptosis.

2 Materials

2.1 Cell line culture

The cell lines used for the experiments are shown in the table below:

Table 2 Cell lines

Cell line	Type	Classification	Mutant	Reference	Source
BM09-085	ALM	brain metastasis	NRAS Q61K	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3329580/	NYU
WM4234	ALM	metastatic	BRAF V600E mutant	Unpublished cell line	Wistar Institute
WM4235	ALM	metastatic	NRAS Q61R mutant	Unpublished cell line	Wistar Institute
SKMEL173	CM	metastatic	NRAS Q61R	http://web.expasy.org/cellosaurus/CVCL_6090	MSKCC
SKMEL239	CM	metastatic	BRAF V600E	http://web.expasy.org/cellosaurus/CVCL_6122	MSKCC
SKMEL103	CM	metastatic	NRAS Q61R	http://web.expasy.org/cellosaurus/CVCL_6069	MSKCC
501MEL	CM	metastatic	BRAF V600E	http://web.expasy.org/cellosaurus/CVCL_4633	-
SKMEL28	CM	metastatic	BRAF V600E	http://web.expasy.org/cellosaurus/CVCL_0526	MSKCC
SK147	CM	metastatic	NRAS Q61L	https://web.expasy.org/cellosaurus/CVCL_3876	MSKCC
SK192	CM	metastatic	BRAF V600E	http://web.expasy.org/cellosaurus/CVCL_6101	MSKCC

The human cancer cell lines mentioned in Table 2 were maintained in DMEM (CORNING supplemented with 10% FBS and Pyruvate (1%) respective Penicillin-Streptomycin (1%).

BM09-085 was maintained in RPMI (CORNING supplemented with 10% FBS, Pyruvate (1%), Penicillin-Streptomycin (1%), L-Glutamine (1%) and Essential Amino acids (1%)). This cell line of patient-derived short term acral melanoma cultures was obtained by Eleazar Vega-Saenz de Miera PhD, a member of Dr. Osman's lab team. Since these cells are comparatively new and have not been passaged that often, they present more closely the real tumor cells in the patient.

For our experiments we use the 3 acral melanoma cell lines and the 7 cutaneous melanoma cell lines mentioned in Table 2. All of them are metastatic, indicating that they are from different areas of the body.

2.2 Drug treatments

2.2.1 EZH2 inhibitor

- **GSK126** was purchased from SelleckChem (#S7061) and re-suspended / diluted in media. GSK126 is a potent, highly selective inhibitor of EZH2 methyltransferase activity with an IC50 of 9.9 nM. The mechanism of action of this histone methyltransferase inhibitor, which is already in clinical development, is well understood. It blocks the EZH2 activity, resulting in a reactivation of the tumor-suppressor genes. This inhibitor is already on the market. [9]

- **JQEZ5 is a** tool compound, meaning it has sufficient potency, selectivity, cell permeability and bioavailability to be used for testing the principle and validation of a biological hypothesis when used in cellular models of disease. In contrast it cannot be used as a drug in humans because of its instability and the function of entering cells still needs optimization. Several EZH2 inhibitors have entered clinical trials for various cancer types. [10]. The drug was provided by Dr Kwok-Kin Wong, NYU School of Medicine. (Haikuo Zhang, 2016)

References for inhibitors hitting their targets (Inhibition of H3K27me3):

- ➔ Reference for **GSK126**: McCabe MT et al. – 2012 - EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature.
- ➔ Reference for **JQEZ5**: Haikuo Zhang et al. – 2016 – Oncogenic deregulation of EZH2 as an opportunity for targeted therapy in lung cancer

2.2.2 EZH2 degrader

- This EZH2 protein degrader that decomposes EZH2 in cell lines in vitro is still unpublished. We received this novel compound through a collaborator from Mount Sinai. This potent degrader was established to target EZH2 by destabilizing it, without interfering with its enzyme activity. The EZH2 protein initially emerges but is degraded in a following disassembling step. It has selectivity and specificity for EZH2 and kills the tumor after 5 – 10 days and is still in preclinical development. The

degrader is a tool compound, that knocks-down EZH2, meaning it still needs to be optimized in terms of stability, efficiency and effectiveness in entering the cells.

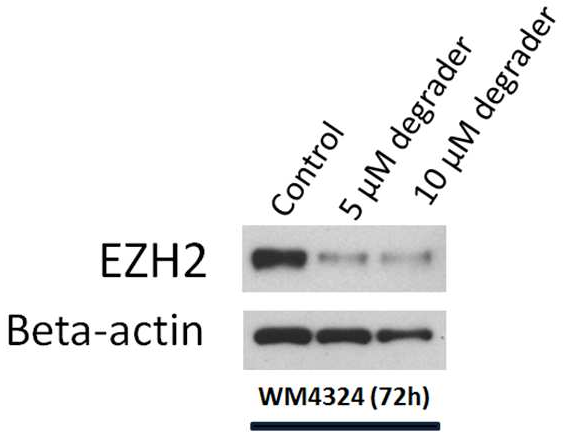


Figure 12 Immunoblot showing the potency of the EZH2 degrader by suppressing the protein expression

This Westernblot is a proof that the degrader is hitting its target and degrading the EZH2 protein. The treatment was performed in the WM4324 acral melanoma cell line.

2.2.3 HDAC Inhibitor

- **LBH589 (Panobinostat)** is a novel histone deacetylase (HDAC) inhibitor with a multi-targeted profile that silences EZH2. HDAC inhibitors have been developed as anticancer drugs. Panobinostat is a drug by Novartis for the treatment of various cancers by epigenetically modifying the cells. This so-called histone deacetylase inhibitor (HDACi) is a hydroxamic acid and acts non-selectively via blocking the removal of the acetyl groups from the lysine. This can result in hyper acetylation of histones, causing either an up-regulation or repression of genes. [11]

2.2.4 PI3K Inhibitor

- **LY294,002,** being a morpholine-containing chemical compound, is a very potent inhibitor of numerous proteins. In detail, it is the first synthetic molecule known to inhibit PI3Kα/δ/β (broad-spectrum inhibitor). This drug is generally considered a non-selective research tool, and should not be utilized for experiments aiming to target PI3K uniquely. It also blocks auto phagosome formation and potently inhibits CK2. [12]

2.2.5 Protein synthesis inhibitor

- **Cycloheximide** (CHX) is a eukaryote protein synthesis inhibitor, generated by the bacterium Streptomyces griseus. By interfering with the translocation step in protein synthesis (tRNA and mRNA movement in relation to the ribosome) Cycloheximide blocks the translational elongation, thereby inhibiting protein synthesis. Its big advantage is the price and the fast effect. [13]

- **Puromycin** is an antibiotic that inhibits translation by causing premature chain termination during translation taking place in the ribosome. This amino nucleoside antibiotic originates from a bacterium called Streptomyces alboniger. [14]

2.2.6 Tetracycline antibiotic

➢ **Doxycycline** is a tetracycline antibiotic that combats bacteria in the body and thus is used to treat many different bacterial infections, such as urinary tract infections, intestinal infections, eye infections, acne, gonorrhea and periodontitis. [15]

2.3 Laboratory equipment

Table 3 Laboratory equipment

instrument	supplier
CO_2 Incubator (37 °C)	Thermo Scientific
Microcentrifuge	Mandel
Microscope	Spencer
Red Rocker	Hoefer
TMS Microscope	Nikon
Vortex Mixer	Fisher Scientific

2.4 Cell Culture Media

Table 4 Cell Culture Media

Media components	supplier
Albumin Standard (Bovine serum albumin)	Thermo Fisher Scientific
DPBS 1X	Corning cellgro ®
Dulbecco's Modification of Eagles Medium (DMEM)	CORNING
RPMI 1640	CORNING
TrypLE Express Enzyme (1X), no phenol red	Life Technologies

2.5 Chemicals, solutions

Table 5 Chemicals, solutions

Reagents	supplier
CL-XPosure™ Film, 5 x 7 in. (13 x 18 cm) 34090	Thermo Fisher Scientific
Countess Cell Counting Chamber Slides	Thermo Fisher Scientific
Countess Cell Counting Chamber Slides	Thermo Fisher Scientific
DAB Peroxidase (HRP) Substrate Kit (with Nickel), 3,3'-diaminobenzidine	VECTOR LABORATORIES
EpiQuik In Situ Histone H3K27 Tri-Methylation Assay Kit	EpiGentek
Histone Extraction Kit	Active Motif
Histone Extraction Kit (ab113476)	Abcam
Immun-Blot PVDF Membrane	BIO-RAD
LDS Sample Buffer (4X)	NuPAGE
Micro BCA Protein Assay Kit	Thermo Fisher Scientific
NuPAGE 4-12% Bis-Tris Gel 1mmX12Well	Thermo Fisher Scientific
NuPAGE™ LDS Sample Buffer (4X)	Thermo Fisher Scientific
Pierce ECL Western Blotting Substrate	Thermo Fisher Scientific
Pierce TM BCA Protein Assay Kit	Thermo Fisher Scientific
Prot/Elec Tips	BIO-RAD

RNase-Free Water	Qiagen
Thick Blot Filter Paper, Precut, 7.5 x 10 cm	BIO-RAD
Tris Base (White Crystals or Crystalline Powder/Molecular Biology), Fisher BioReagents	Cell Signaling TECHNOLOGY
VECTASTAIN ABC HRP Kit	VECTOR LABORATORIES
CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS)	Promega

2.6 Antibodies

Table 6 Antibodies

material	supplier
Akt Rabbit mAb #4685	Cell Signaling Technology
Anti-rabbit IgG, HRP-linked Antibody #7074	Cell Signaling Technology
EZH2 Rabbit mAb #5246	Cell Signaling Technology
H3K27me3 Rabbit mAb #9733	Cell Signaling Technology
Histone H3 Rabbit mAb #4499	Cell Signaling Technology
HSP90 mAb #4874	Cell Signaling Technology
p27 mAb #3686	Cell Signaling Technology
Phospho-Akt (Ser473) Rabbit mAb #4060	Cell Signaling Technology

2.7 Drugs

Table 7 Drugs

material	supplier
Cycloheximide solution	SIGMA-ALDRICH
Doxycycline	SIGMA-ALDRICH
EZH2 degrader	Collaborator at Mount Sinai
GSK126	SelleckChem
JQEZ5	Dr Kwok-Kin Wong (NYU School of Medicine)
LBH589 (Panobinostat)	SelleckChem
LY294002	SelleckChem
Puromycin	Thermo Fisher Scientific

3 Methods

The project can be subdivided into two parts, a clinical and a biological one.

3.1 Clinical

3.1.1 Study Cohort

The study cohort comprises patients presenting to NYU with a pathological diagnosis of acral lentiginous melanoma and mucosal melanoma, who prospectively enrolled in the IMCG's biorepository protocol from 2002-2017 (n=115). These tissue samples are formalin-fixed paraffin-embedded (FFPE) tissue samples, ready to be used for immunohistochemical staining. We have quite a large number of samples, taking into account that these are rare tumor types. In addition, we have a certain amount of tissue samples of the same patient from Primary tumor (stage 1) and metastatic tumor (stage 2) in order to compare the EZH2 concentration of each patient in the course of the disease.

Table 8 is showing the clinical and pathological features for 101 patients who presented to NYU and consented to the study. The mucosal melanoma cases are de-identified; therefore no specific clinic pathological information is available.

Table 8 IMCG ALM patient demographics and clinic pathological characteristics (n=101)

IMCG ALM patient demographics	
and clinicopathological characteristics (n=101)	
Age at primary diagnosis (y), median (range)	
	65.3 (21.0-94.7)
Sex, n (%)	
Male	43 (42.3)
Female	58 (57.4)
Ethnicity, n (%)	
Caucasian	66 (65.3)
African American	13 (12.9)
Hispanic	13 (12.9)
Asian	7 (6.9)
Other/Unknown	2 (2.0)
Primary tumor thickness (mm), n (%)	
< 2	51 (50.5)
≥ 2	49 (48.5)
Cannot assess	1 (1.0)
Primary tumor ulceration, n (%)	
Present	44 (43.6)
Absent	48 (47.5)
Not classified	9 (8.9)
Clinical stage at primary diagnosis, n (%)	
Stage 0	7 (6.9)
Stage I	39 (38.6)
Stage II	23 (22.8)
Stage III	31 (30.7)

Stage IV	1 (1.0)
Disease Progression, n (%)	
Recurred	48 (47.5)
Did not recur	31 (30.7)
Unknown (<2 years of follow-up)	22 (21.8)

3.1.2 Immunohistochemical staining (IHC)

Immunohistochemistry or IHC is widely used in basic research and in the diagnosis of cancerous tumors. The name is derived from "immuno", in reference to antibodies used in the method, and "histo," meaning tissue.

Immunohistochemistry relates to the mechanism of detecting antigens, like proteins in cells of a biological tissue section by exploiting the principle of antibodies binding specifically to their target antigens. Specific molecular markers are indicative of particular cellular processes such as cell death or proliferation. Immunohistochemical staining helps identifying the localization and distribution of biomarkers and differentially expressed proteins in different parts of biological tissue. Visualizing an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as a peroxidase, that can catalyze a color-producing reaction. (Mass Histology Service)

Briefly, immunohistochemical staining of nucleic acids and proteins was performed using primary antibodies (Table 6 Antibodies), the VECTASTAIN ABC HRP Kit (Peroxidase, Rabbit IgG) in combination with the DAB Peroxidase (HRP) Substrate Kit, according to manufacturers' protocols. The DAB incubation was performed for a duration of 3 minutes. Anti-rabbit IgG HRP-linked antibody served as a secondary antibody and Haematoxylin for counterstaining. Lung tissue was used as a positive control, using the same protocol.

- The IMCG acral and mucosal FFPE fixed tissue samples were stained for EZH2 (antibody 1:200). My colleague, Allison Izsak, who had already been working on this project before me, stained a large amount of the acral cases. I performed IHC for EZH2 on all remaining acral and mucosal cases in the IMCG cohort, which gave a total number of 115 cases (90 acral and 25 mucosal). Out of these 115, 66 were primary 40 metastatic and 9 unknown.

- In addition, I stained a total number of 44 FFPE fixed slides with H3K27me3 (antibody 1:2000), to look at the levels in a sub-set of acral and mucosal tumors with high or low EZH2 levels. We wanted to determine whether there is a tight correlation between EZH2 and H3K27me3 in these tumors or not. All in all, 34 acral cases (25 Primary and 9 Metastatic) and 10 mucosal cases (6 Primary and 4 Metastatic) were stained with H3K27me3.

- As a control, in order to show that H3 (antibody 1:150) could be detected in the H3K27me3-negative tumors, a total number of 8 cases (2 metastatic, 6 primary) were stained using the H3 monoclonal antibody from Cell Signaling Technology, listed in Table 6. The high levels of H3 are the proof that a lack of H3K27me3 cannot be attributed to a lack of H3 total.

- The stainings were optimized using lung tissue, since it is known that lung contains high H3K27me3 and H3 levels.

The stained slides were subjected to immunohistochemical analyses, by the NYU Langone Health Pathologist Dr. Farbod Darvishian, MD. In a following step he took high resolution images of different tumors and levels of EZH2/ H3K27me3 expression.

There exist several ways how we could stratify EZH2 levels e.g. by the range of expression scores (0, 2, 3, 4), by the presence (2, 3, 4) vs absence (0), or high (3, 4) vs low (0, 2) expression. We chose the system described in Table 9 and Table 10.

The following scoring system was used:

Table 9 IHC Scoring system

Tumor	Staining intensity	Staining distribution	Score (Sum of Intensity and distribution)
Tumor present	0	0	**0**
Tumor present	1	1	**2**
Tumor present	1	2	**3**
Tumor present	2	1	**3**
Tumor present	2	2	**4**

.

Staining intensity:

- **Intensity - 1** shows weak staining

- **Intensity - 2** implies strong staining

Staining distribution:

- **Focal staining - 1** denotes that all the staining is concentrated on one spot, it is focal.
- **Diffuse staining - 2** indicates that the staining is spread among the whole tissue, it is diffuse.

Table 10 IHC Scores

Score=0	no staining intensity or distribution, the tumor is present
Score=2	weak protein levels, focally expressed
Score=3	weak protein expression, diffusely expressed OR strong EZH2, focally expressed
Score=4	strong protein expression, diffusely expressed

For the 115 ALM + MM cases stained for EZH2 we decided that a score of 2 – 4 is assessed as an increased EZH2 protein level.

3.2 Biological

3.2.1 Cell culture

Different acral and non-acral cell lines, see Table 2 Cell lines, were cultured in vitro in 10cm cell culture plates with the appropriate media in a CO_2 Incubator from Thermo Scientific, at 37 °C.

3.2.2 Histone and Total Protein extraction

Total Proteins:

The in vitro cultured cells were harvested and isolated with the Total Protein Lysis Buffer cleared and quantified. More specifically, the lysis consisted of a previous washing step with PBS buffer. Then the cells were lysed with the Total Protein Lysis Buffer and from the cell debris by centrifugation.

Total Protein Lysis Buffer Preparation:

In order to prepare 500 mL of lysis solution, we mix 25mL of 1M Tris pH7.5, 2mL of 0.5M EDTA pH 8.75 mL of 1M NaCl, 5mL of Triton x100, 398 mL of distilled H_2O and 5 tablets each of Complete Ultra (Roche #05 892 791001) and PhoSTOP (Roche #04 906 837001).

Histones:

For harvesting histones I tried two different histone kits, the Immunoblots are shown in Figure 13. The Abcam kit showed more background noise than the active motif kit. Therefore we decided on extracting the histones with the active motif acid precipitate histone extraction kit, which extracts histone proteins while preserving their modifications.

Figure 13 Histone Kit optimization – The new active motif kit shows less background

3.2.3 Protein quantification

The histones and total proteins, extracted in 3.2.2, were quantified with the help of the Pierce™ BCA Protein Assay Kit from Thermo Fisher Scientific, using the following working procedure:

Total Proteins were diluted 1:5, histones 1:3 with RNAse/DNAse free water. The substrate of the Micro BCA™ Protein Assay Kit was prepared by mixing reagents A + B in a 50: 1 ratio. 200 µL of this mixture was pipetted into the wells in the 96 well plate. The first two rows are reserved for the 9 different concentrations of the BSA Standards (A through I). Final BSA concentration ranging from 2000 – 0 µg/mL. We always perform repeat determinations.

Next measurements are done with a spectrophotometer with the programme: Soft Max Pro 6.1. at a wavelength of 562 nM.

3.2.4 Histone modifications

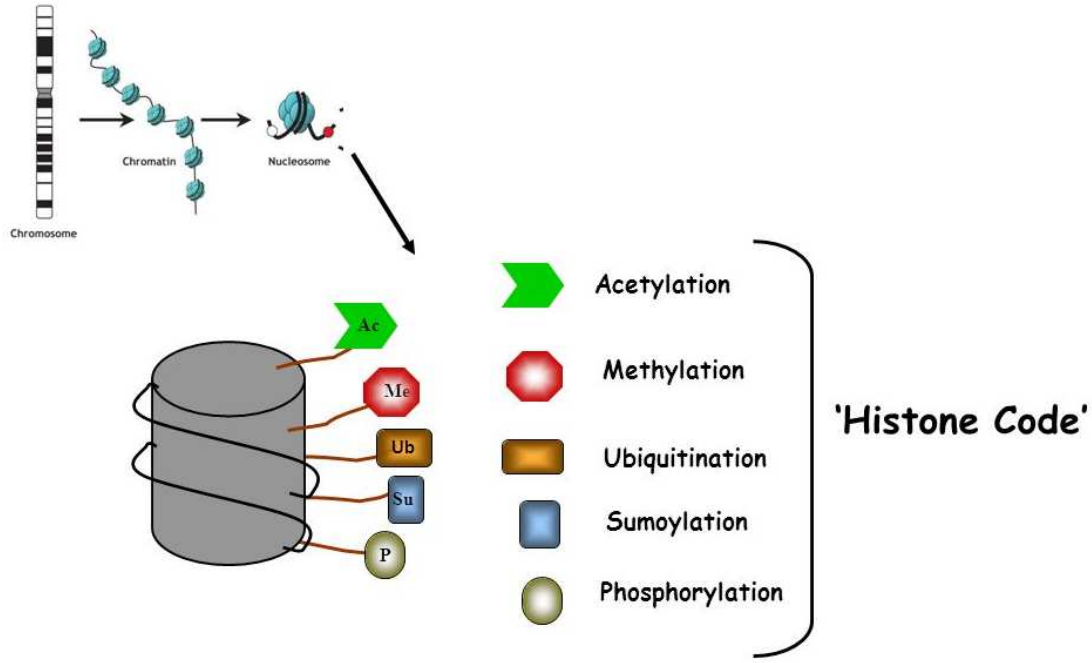


Figure 14 Possible histone modifications [16]

Figure 14 shows different histone modifications, these include covalent post-translational modifications (PTMs) to histone proteins which include acetylation, ubiquitylation, methylation, phosphorylation and sumoylation. These modifications may impact gene expression by recruitment of histone modifiers and altering chromatin structure. Histone modifications act in diverse biological processes such as DNA damage and repair, transcriptional activation and inactivation as well as chromosome packaging. [17]

Among all posttranslational protein modifications, the role of protein methylation in signal transduction remains rather unexplored. Methylation of lysine residues within the conserved N-terminal histone tail controls transcriptional activity. The Histone methylation is mediated by methyltransferases (HMTs) that catalyze mono-, di-, or tri-methylation of particular lysine residues. (I-hsin Su, 2005)

3.2.5 Westernblotting/ Immunoblotting

Gel and Membrane:

The quantified lysates were mixed in calculated amounts with a dye (4X) and RO-water and loaded on a 4-12% Bis-Tris Gel from Thermo Fisher Scientific. These polyacrylamide gels contained 12 wells and I made sure to load the outermost wells with dye only. Additionally, I loaded a ladder. In a following step the gel was plotted on an Immune-Blot PVDF Membrane by building a sandwich and exposing it to 80 volts for 1 hour.

Antibody incubation:

After the 1 hour blocking step in milk TBST, the membrane was subjected to Primary antibodies Table 6, mixed with milk-TBST in the appropriate concentration. This incubation usually constituted 1 hour. If the signal was too weak it was extended up to overnight at 4°C. In the subsequent step the antibodies were

visualized using secondary antibodies (Table 6) in milk TBST for 60 minutes at room temperature. The blots were exposed to Pierce ECL Western Blotting Substrate and scanned.

I also looked at the levels of different Read-outs of EZH2, e.g. Akt and p-Akt and studied how these levels change when blocking EZH2 expression.

3.2.6 Possible mechanisms of regulation

In the course of answering the question how EZH2 is being regulated, we tested several possible mechanisms:

- Copy number
- Protein stabilization
- Epigenetic regulation

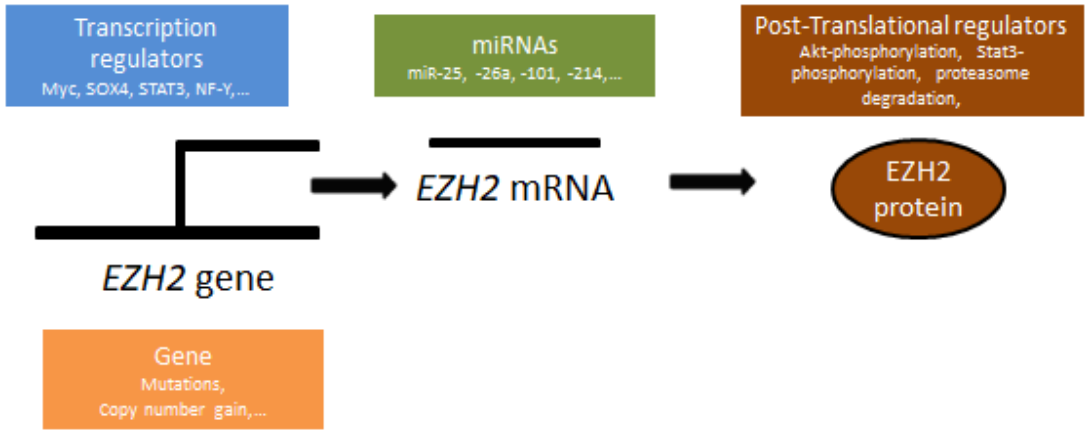


Figure 15 Molecular mechanisms by which EZH2 expression and activity is regulated

Figure 15 describes the regulation of EZH2 expression by various oncogenic transcription factors and tumor suppressor miRNAs. The adjustment can be on several levels, including transcriptional, post-transcriptional and translational regulations.

Additionally, the control can be via post-translational regulators and genetic regulators. The miRNAs can regulate and silence EZH2 expression. The regulators of EZH2 expression are also essential for cell proliferation, tumorigenesis, and stem cell maintenance. For example, myc binds to an EZH2 promoter and thereby directly activates its transcription. Furthermore, myc upregulates EZH2 expression by downregulating different miRNAs. SOX4, one of the key regulators of stem cells, directly controls the expression of EZH2 mRNA. Moreover, NF-Y, STAT3, and ETS transcription factors directly manage EZH2 transcription in different types of cancers. (Hirohito Yamaguchi, 2014)

The literature denotes that phosphorylation of EZH2 activates STAT3 signaling via STAT3 methylation, and promotes tumorigenicity of glioblastoma stem-like cells. In contrast, inhibition of EZH2 reverses the silencing of Polycomb target genes and decreases the activity of STAT3, proposing new therapeutic strategies. Summarizing the data demonstrates that targeting EZH2 effectively hinders H3K27 methylation, STAT3 methylation and STAT3 activation in GBM xenografts. (Eunhee Kim, 2013)

In addition, a paper from a group in France (Cavalli, 2012) mentions several possible mechanisms for EZH2 action like EZH2-mediated gene silencing via recruitment of the PRC2 complex (including EZH2, Suz12, and EED) by DNA-binding proteins and EZH2-mediated gene activation via EZH2 recruitment by the androgen receptor (AR) and methylation of it or by formation of a ternary complex with the RelA/RelB NF- κB components.

3.2.6.1 Testing the Half-life of EZH2

To assess protein stability, half-life experiments were performed in 6cm cell culture plates. By the addition of CHX we want to knock-down EZH2, meaning to stop its translation. After the treatment I want to chase the levels of EZH2 protein over time, taking samples at 0,2,4,6 and 8 hour time points. In addition, I will assess the decay of other proteins with a short half-life, like p27 and p53.
We want to test the half-life of EZH2 in WM4235 or BM09-085, the two acral cell lines containing high levels of EZH2. My hypothesis is that EZH2 is overexpressed in these acral melanomas because the protein half-life is increased (i.e. there is decreased turnover of the protein). The literature indicates a half-life of EZH2 of 1.5 – 4 hours. (Wu & Zhang, 2011) (Jia Wang, 2017) (Yung-Luen Yu, 2013)

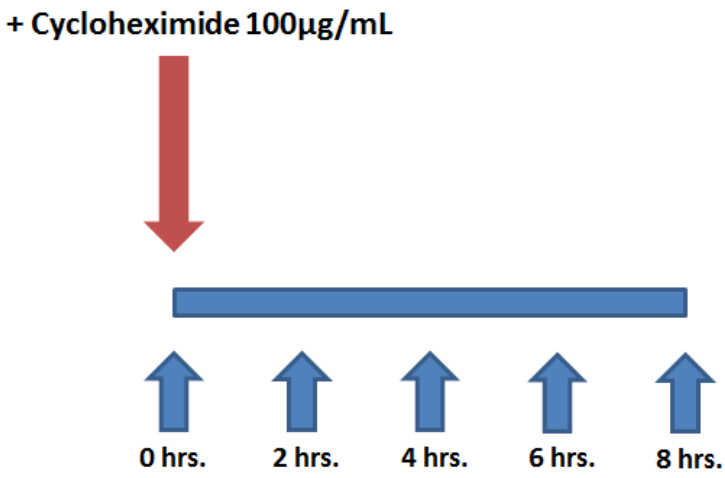


Figure 16 Schematic of the EZH2 half-life experiment

We used a concentration of Cycloheximide of 100µg/mL and harvested protein at 0,2,4,6 and 8 hrs. CHX blocks translation, indicating it is a potent protein synthesis inhibitor. I harvested proteins and analyzed the decay of the EZH2 protein.

3.2.6.2 Copy number PCRs

Copy number variation (CNV) describes the condition where parts of the genome are repeated. In Figure 17, this gene duplication has generated a copy-number variation, meaning the chromosome now possesses two copies of this section of DNA.

CNV is a type of deletion or duplication incident that impacts a notable number of base pairs. This structural variation means that the number of repeats in the genome alters between people in the populace. In mammalians, copy number variations play an important role in generating fundamental variation in the population.
Interestingly enough, recent research reveals that around two thirds of the whole human genome is built up of repeats and 5-10% of the genome can be categorized as copy number variations. [18]

Figure 17 Gene duplication, indicating a copy number increase [19]

Copy number gains or losses, meaning focal aberrations, drive the development of cancer. (Oscar Krijgsman, 2014) (Keiichi Ohshima, 2017) . Another paper, published in Bioinformatics states that copy number abnormalities (CNAs) such as somatically-acquired chromosomal deletions and duplications are common events in cancers. (Newman, 2015)

3.2.6.3 Hotspot mutation sequencing

The frequency of mutations tends to vary along nucleotide sequences such that there often exist certain hotspots where the rates of recombination are highly elevated. The recombination hotspots follow from

higher DNA break formation in these regions and the recombination rate at these hotspots can be many times that of the close regions.

These hotspots might reflect sequence specificity as well as functional and structural characteristics of the sequence of DNA. These specific areas could mirror the properties of the gene product and the mutant selection scheme. In addition, analysis of the nucleotide sequence context of hotspots can provide information on the molecular mechanisms of mutagenesis. (Igor B.Rogozinab, 2003)

By extending the notion of recurrence of mutations from acral and mucosal melanoma to all melanomas, one immediately identifies several papers, stating activating respective inactivating hotspot mutations as a common event in cancer. (Matthew T. Chang, 2016) (Tenghui Chen, 2016) (Martin L. Miller, 2015)

3.3 Assays

3.3.1 Colony Assay

The Colony assays, also called clonogenicity assays, is a cell biology technique commonly used in cancer research, since it helps investigating the efficiency of specific drugs on proliferation and survival of cells. The term "clonogenic" refers to the fact that these cells are clones of one another.

These assays were performed in cell culture plates. 2 – 3000 cells were evenly distributed and incubated for 3 weeks at 37°C. The colonies formed were counted. Hereby we can show the clonogenicity of the cancerous cells. Normal cells would die because they need cell-cell contact.

3.3.2 Proliferation Assay

Different terms for Proliferation assay are cell growth-, apoptosis-, titration-, cytotoxicity-, growth- and cell viability- assay. This test is designed to efficiently stain live cells for excellent resolution of each cell division generation. We are interested in the cell viability, since aberrations in cell proliferation can give rise to malignant transformation and cancer pathology.

Acral melanoma cells were seeded in equivalent, low-density cultures in 96-well plates in normal media. Twenty-four hours after seeding, the medium was removed and replaced with media containing serial dilutions of the EZH2 degrader and the two enzyme inhibitors, yielding final drug concentrations ranging from 0 to 5 µM. Cultures were incubated with this mixture for 7 - 11 days. Cells were treated only initially and even in terms of the long-term incubation the media was not changed. Later on we measure at 490nm with a photometer to determine the OD.

3.3.3 Genetic approach – Hairpins

Short hairpin RNAs (shRNAs) elucidate the functions and interactions of genes by specifically silencing target genes, indicated in Figure 18. These transfected stable cell lines help studying gene function analysis, target discovery, target validation, assay development, and compound screening. In principle it

functions similar to the EZH2 degrader, in the sense that the EZH2 protein initially emerges but is degraded in a following disassembling step.

They work as precursors for short interfering RNAs (siRNAs) which are powerful mediators of RNA interference (RNAi).

Lentiviruses, such as the human immunodeficiency virus (HIV) are capable of infecting cells. Short hairpin RNAs can be expressed from lentiviruses, allowing for high efficiency transfection of a variety of cell types. The virus works via integration into the gene.

After cloning the short hairpin sequence into the lentivirus plasmid, this Lentivirus is used to infect the target cell lines, in our case different acral cell line strains. (UCSF Viracore)

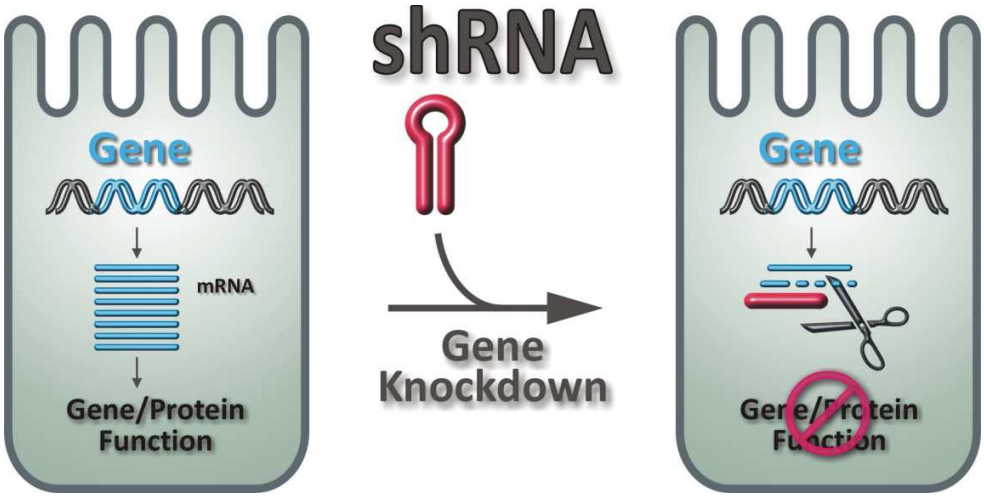


Figure 18 Schematic showing the short hairpin RNA gene knock-down [20]

Our vector contains a puromycin antibiotic selection marker, used for selection of the cells that have been successfully infected with the hairpin.

The shRNAs we used in our experiments have a aoxycycline inducible promoter.

Two of the 3 acral cell lines we have, contain high levels of EZH2. The transduction of BM09-085 did not successfully work out and caused several off-target-effects and changes in the cells, like growth inhibition. In contrast, the transduction of WM4235 with the shRNA containing Lentivirus was successful.

4 Results

4.1 1st Hypothesis - EZH2 levels are increased in acral and mucosal melanomas

4.1.1 Clinical part

The clinical experiments to test the 1st hypothesis include immunohistochemical staining with EZH2 specific antibodies in different patient tissue samples from the IMCG cohort.

A total number of 115 ALM + MM cases were stained for EZH2, specifically 90 ALM + 25 MM slides, containing the tissue samples. We decided that a score of 2 – 4 is assessed as an increased EZH2 protein level. For a detailed description of the scoring system, please see Table 10 IHC Scores. The EZH2 staining is nuclear, since EZH2 is mostly in the nucleus, indicated in Figure 19.

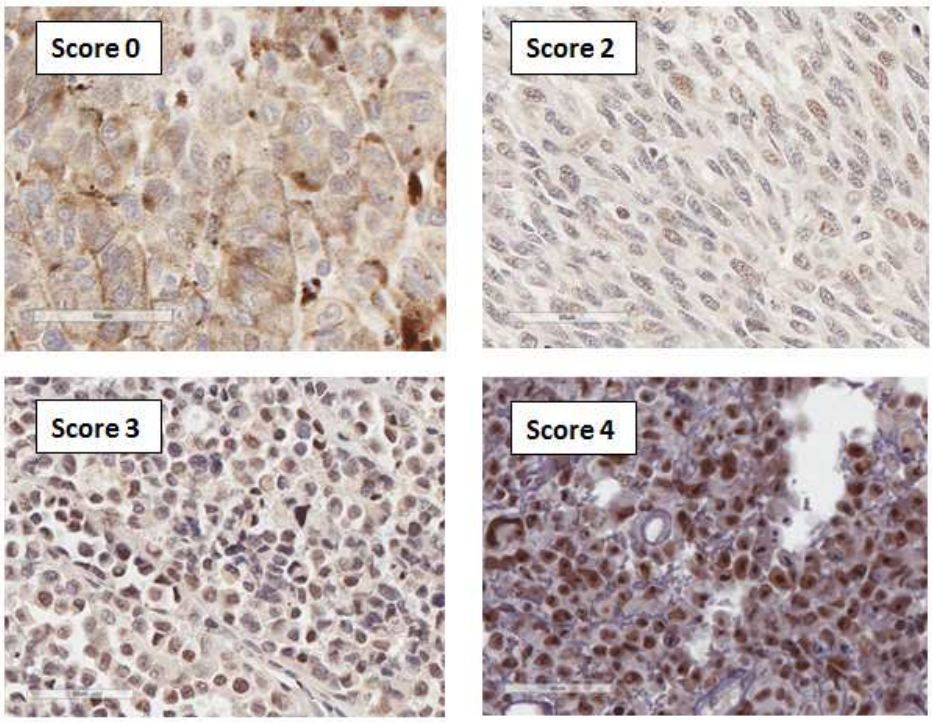


Figure 19 Different Scores of EZH2 stained slides, antibody dilution 1:200; Score=0 meaning no EZH2 expression; Score= 2 representing weak EZH2, focally expressed; Score=3 signifying weak EZH2, diffusely expressed OR strong EZH2, focally expressed and a Score=4 meaning strong EZH2, diffusely expressed

The Scale bar on the bottom left in Figure 19 accounts for 60 µM.

Table 11 Scores of 115 cases stained for EZH2, 90 acral melanoma cases and 25 mucosal melanoma cases

EZH2					
ALM	#	%	MM	#	%
Score 0	23	26	Score 0	5	20
Score 2	19	21	Score 2	6	24
Score 3	34	38	Score 3	6	24
Score 4	14	16	Score 4	8	32
Total	90	100%	**Total**	25	100%

74% (67/90) **80% (20/25)**

Table 11 indicates that in 74% (67/90) of the ALM and 80% (20/25) of the MM cases, the level of EZH2 is enhanced, meaning that the EZH2 levels are quite high in these tumors.

4.1.2 Biological part

EZH2 protein expression in melanoma cell lines was determined by immunoblotting. Immunoblots were performed with the antibodies mentioned in 2.6.

We used a panel of melanoma cell lines, see details in Table 2, to look at the level of EZH2. Three of these cell lines are acral melanoma ones, the rest are cutaneous melanoma cell lines. Unfortunately, we do not have a mucosal cell line for these experiments since our lab only possessed 1 mucosal melanoma cell line which sadly did not express EZH2. For future investigations we might additionally want to try to acquire a panel of different MM cell lines.

EZH2 is already well studied in cutaneous melanoma, which has been well described in the literature; therefore we used them alongside with the ALM cell lines. The literature claims high levels of EZH2 in CM. Still, there is a lack of information of EZH2 levels in acral cell lines.

We also performed tests of determining the EZH2 levels in normal melanocytes, but they were undetectable (not shown in the thesis).

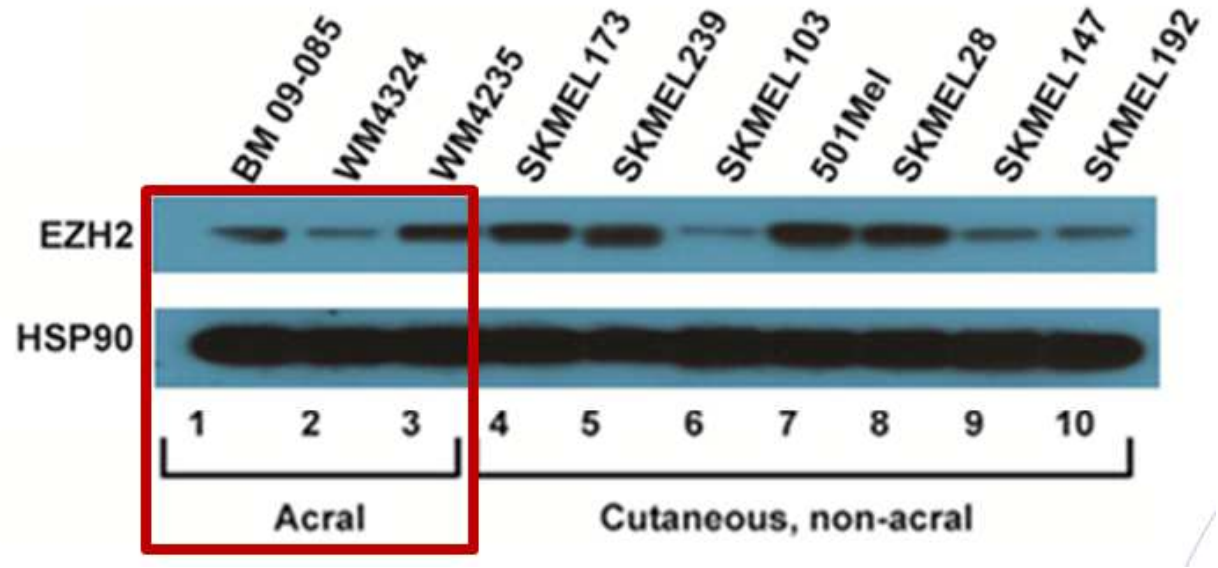


Figure 20 Epigenetic landscape of EZH2 in different melanoma cell lines showing rather high levels of the protein

What we can appreciate in the Immunoblot in Figure 20 is consistent with the IHC staining in the clinical part. We can identify enriched levels of EZH2 in all 2 out of the 3 ALM cell lines (boxed in red) and 4 out of the 7 cutaneous melanoma cell lines.

This technique is only semi-quantitative, since we can see differences in the colors and intensities, respectively, but we do not have exact numbers. After scanning, these bands can be quantified with a program called ImageJ.

The literature supports our findings, stating that high expression of EZH2 is associated with features of aggressive tumors. (Yaser R. Hussein, 2012)

4.2 2nd Hypothesis - increased EZH2 levels are associated with tumor progression and a poor clinical outcome for patients

In our second hypothesis we want to study the association between the EZH2 score (0, 2, 3, 4 – with 4 being the highest expression, either primary or metastasis) and the clinical and pathological features listed below to determine whether they are prognostic factors for acral melanoma.

- ✓ Ethnicity
- ✓ Lymph node positivity
- ✓ Initial clinical stage
- ✓ Primary tumor thickness
- ✓ Primary tumor ulceration

- ✓ Location of primary tumor
- ✓ BRAF, NRAS, C-kit mutation status
- ✓ TILs (presence or absence)
- ✓ TILS – brisk vs non-brisk vs none
- ✓ Number of mitoses in tumor
- ✓ Recurrence (yes/no)
- ✓ Recurrence-free survival
- ✓ Overall survival

Unfortunately, the mucosal cases we received from the IMCG data base were mostly de-identified, so we could not use them for any statistical analysis taking into account the clinicopathological variables.

Dr. Zhong ran the statistical analysis of the clinical data and could identify a statistically significant overall survival (OS) effect with EZH2 in acral melanoma. High EZH2 protein expression is associated with significant worse survival. This can be seen in the Kaplan-Meier curve in Figure 21.

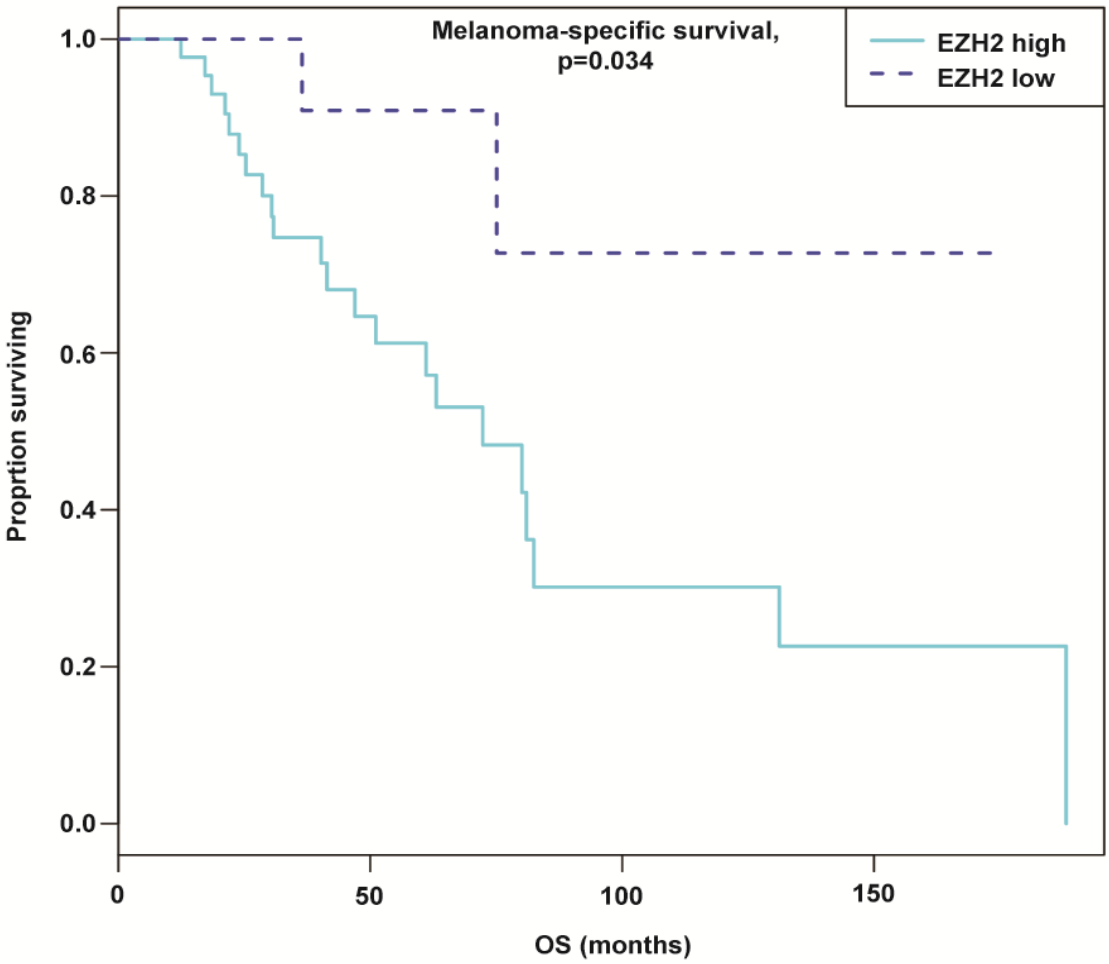


Figure 21 Kaplan Meier graph showing that high EZH2 expression predicts shorter overall survival in acral melanoma

Figure 21 demonstrates that high EZH2 expression predicts shorter overall survival in acral melanoma, meaning high expression is associated with significant worse survival. Even when corrected for stage (since EZH2 levels increase with stage), the worse survival effect is still significant, indicating that the levels of EZH2 are an independent prognostic marker. EZH2 levels do increase with stage, which we saw before. These are only preliminary results that need validation. For future experiments we will increase the sample size.

Additionally, an interesting paper published in the Journal of Clinical Oncology (Thai Huu Ho, 2017) suggests that patients in The Cancer Genome Atlas cohort with high EZH2 gene expression were 1.5 times more likely to experience overall death than patients with low EZH2 expression (95% CI, 1.1 to 2.3; P = .028). Patients in the University of Texas Southwestern Medical Center cohort with high EZH2 protein expression levels were even two times more likely to experience overall death than patients with low EZH2 protein levels (95% CI, 1.1 to 4.4; P = .034).

A paper published in the Blood Cancer Journal suggests that in multiple myeloma upregulation of EZH2 is also associated with poor prognosis and dysregulation of cell cycle control (C Pawlyn, 2017).
cyc

There are associations of EZH2 with lymph node positivity, recurrence, thickness and mitoses that approach significance. This lack in significance with these parameters is possibly due to the sample size.

Re-defining the cutoffs might be helpful, i.e. 55% of EZH2 2, 3, 4 tumors had moderate/many mitoses vs. only 31% of EZH2 0 tumors. Also, Dr. Zhong set the cutoff for primary tumor thickness as <4.0 mm or >4.0 mm, but in reality most of the tumors were less than 4.0 mm. It might be helpful to set the cutoff at more than or less than 2.0 mm, and also at <2.0 mm, 2-4 mm, and >4.0 mm to determine whether there is an association at all.

Figure 22 indicates the primary and recurrent EZH2 IHC expression scores of 106 ALM + MM cases. For 9 cases no survival information was given.

Figure 22 EZH2 expression increases with progression of non-sun-exposed melamoma (ALM + MM, n=106)

There is a significant difference in the mean EZH2 IHC score between the primaries and metastases (p= 0.05).

We could identify 7 matched pairs (primary and recurrent) and compared their levels of EZH2.

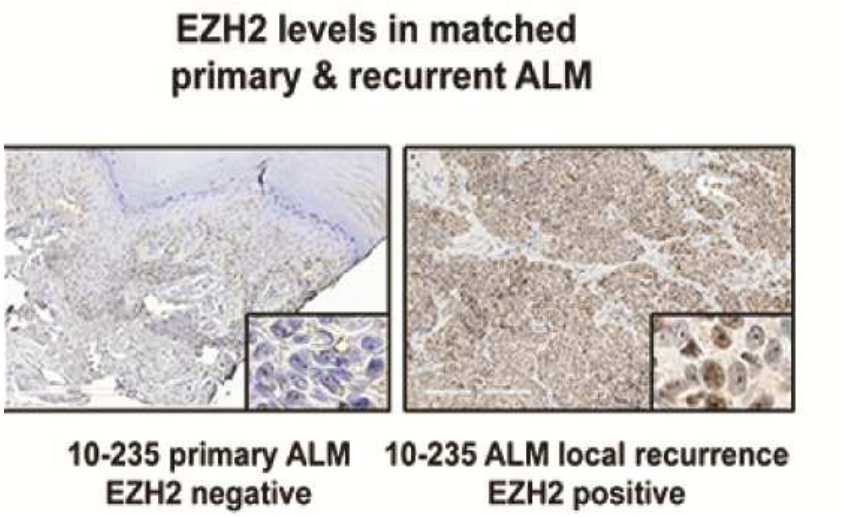


Figure 23 EZH2 levels in matched primary & recurrent ALM pairs

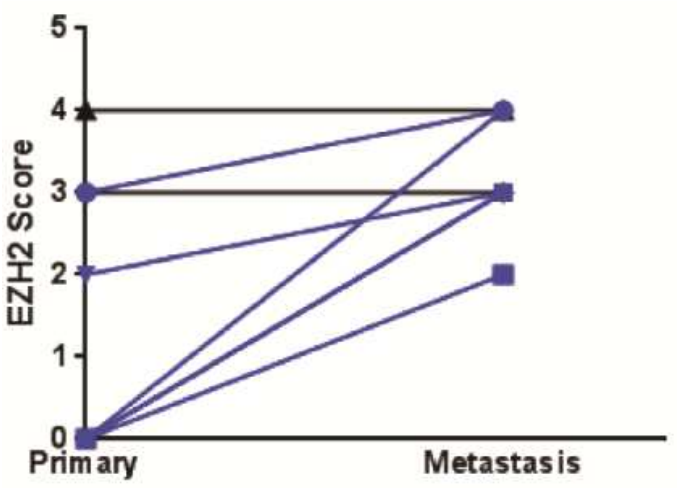


Figure 24 ALM matched pairs: EZH2 expression increases from primary to metastasis

Figure 24 suggests that the levels of EZH2 are increased from primary to metastasis in 5 out of 7 cases. However, the protein expression did not change in 2 out of 7 metastatic tumors as compared to their primaries.

4.3 3rd Hypothesis - multiple mechanisms are responsible for increased EZH2 levels in acral and mucosal melanoma, including EZH2 gene copy number gain and protein stabilization

4.3.1 Possible mechanisms of (up-) regulation

In the course of answering the question how EZH2 is being regulated, we tested several possible mechanisms:

- Copy number
- Protein stabilization
- Epigenetic regulation

It might also be a regulation at a number of levels, suggesting a combination of several mechanisms.

4.3.1.1 Testing the Half-life of EZH2

In this part of the project I wanted to determine whether an increased stabilization of the protein is the reason for EZH2 overexpression in ALM and MM. The experiments were carried out in 6cm cell culture plates and 6-well plates, cells were cultured in submerged media conditions. By the addition of CHX we wanted to knock-down EZH2, meaning to stop its translation.

Cycloheximide, the protein synthesis inhibitor, was used at a concentration of 100µg/mL and protein was harvested at 0,2,4,6 and 8 hrs. I analyzed the decay of the EZH2 protein. p27 serves as a control since it is known to be very unstable. HSP90 is the loading control.

For these assays I used 2 different acral cell lines (BM 09-085, WM4235) and show a MCF-7 breast cancer cell line as a control.

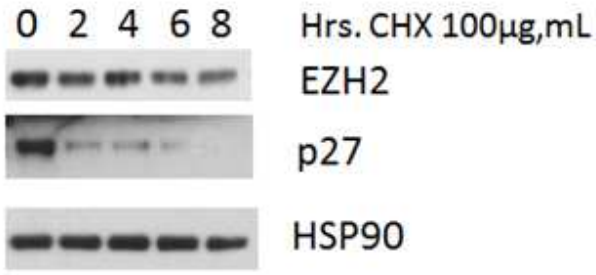


Figure 25 Immunoblots in BM09-085 suggesting a prolonged EZH2 protein half-life

The rapid decay of the p27 protein levels in Figure 25 indicates that the experiment worked. There is only a minimal level of decay of EZH2, suggesting a half-life of 4-6 hours. The HSP90 housekeeping gene levels are constant.

Figure 26 Western blots in WM4235 suggesting an increased EZH2 protein stability

Figure 26 suggests that the decay of EZH2 in WM4235 when treated with Cycloheximide is not apparent. The rapid loss of p27 and HSP90 as house-keeping genes for human is shown for comparison. According to this figure, it seems like the half-life of EZH2 accounts for more than 8 hours.

In parallel, I searched in the literature to determine the half-lifes previous research has detected. Published studies showed that the half-life in MCF-7 breast cancer cells amounts to 4-6 hours Figure 27 (Sahasrabuddhe, 2014) and in another breast cancer cell line it is reported to be only 60 minutes (Manjari Dimri, 2015).

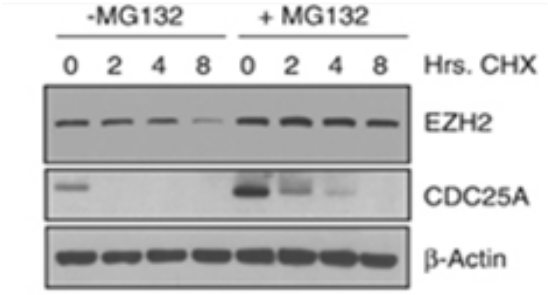


Figure 27 Immunoblots in the breast cancer cell line named MCF-7 suggesting an increased half-life of EZH2 (Sahasrabuddhe, 2014)

It is interesting to note that the prolonged half-life in the two acral cell lines I tested suggests that protein stabilization might be a mechanism of EZH2 upregulation.

This is only preliminary data with many limitations that requires further investigation and validation. In addition, it needs to be tested in normal melanocytes for comparison.

4.3.1.2 Copy number PCRs

Another approach to test the 3rd hypothesis claiming the high levels of EZH2 lie in several mechanisms, Keith Giles, PhD performed a PCR based copy number assay with the 28 DNA samples I isolated from FFPE dissected sections. He used the applied Biosystems™ TaqMan™ assays designed for the detection and quantitation of copy number variation targets. Out of the 28 cases, 11 are mucosal melanoma cases and 17 acral melanoma cases.

Fehler! Ungültiger Eigenverweis auf Textmarke. indicates all 28 DNA samples that were used to run the PCR copy number assays.

Table 12 Copy number assay with 28 DNA samples, 36% show a copy number gain, 14% a significant gain in copynumber

ID	Histology	EZH2 IHC score	EZH2 copy number gain
03-050	ALM	2	no
09-241	ALM	2	4.46
11-148	ALM	2	no
13-133	ALM	2	no
07-021	ALM	3	2.44
08-235	ALM	3	no
09-093	ALM	3	2.46
09-085	ALM	3	no
09-263	ALM	3	no
10-106	ALM	3	no
11-285	ALM	3	no
13-276	ALM	3	2.38
16-183	ALM	3	no
06-057	ALM	4	no
08-109	ALM	4	no
10-235	ALM	4	no
13-051	ALM	4	3.01
ts-12-15250	MM	0	2.85
ts11-11371	MM	2	no
ts04-7724	MM	2	no
ts-13-08586	MM	2	no
ts-17-05829	MM	2	6.79
ts11-04660	MM	3	3.98
b14-13215	MM	3	2.69
s06-1779	MM	4	no
ts16-515-421	MM	4	2.66
ts16-11193	MM	4	no
ts-13-13766	MM	4	no

Cases that have a copy number higher than 2 are listed with their exact number and highlighted in orange or grey. We decided on the threshold of 3 for a significant EZH2 copy number gain. These are the samples highlighted in orange. In this genomic screening, we identified a significant copy number amplification of EZH2 in 14% of ALM + MM cases (4/28). 10 out of 28 (36%) show a copy number gain. According to our data genomic rearrangements are more common than mutations.

In this genomic screening, we identified copy number amplification of EZH2 in 14% of ALM and MM cases, a higher frequency than previously reported in cutaneous melanomas (5%). (Jessamy Tiffen, 2014)

It is important to emphasize that the data shown is only preliminary that warrants further investigations! An independent validation is very important! The poor quality of the DNA might be a limitation. Some of the cases have even a copy number loss.
Our data suggests that EZH2 copy number gain is not a major mechanism of EZH2 activation.

4.3.1.3 Hotspot mutation sequencing

To systematically analyze somatic mutations, we retrieved whole-exome sequencing data from 28 DNA samples. Eleazar Vega-Saenz de Miera, PhD hereby tested the assumption that the EZH2 protein upregulation could have its origin in a genetic component, more specifically in mutations. The hotspot mutation sequencing was performed with the DNA I had previously extracted from FFPE tissues. He used primers that covered exons 12, 16, 17 and 18, which is where most of the well-described "hotspot" mutations associated with cancer have been described in EZH2.

Table 13 Hotspot mutation sequencing with 28 DNA samples, only 1 activating mutation found at Y646N (3.5%)

ID	Histology	EZH2 IHC score	EZH2 hotspot mutation
03-050	ALM	2	WT
09-241	ALM	2	Y646N
11-148	ALM	2	WT
13-133	ALM	2	WT
07-021	ALM	3	WT
08-235	ALM	3	WT
09-093	ALM	3	WT
09-085	ALM	3	WT
09-263	ALM	3	WT
10-106	ALM	3	WT
11-285	ALM	3	WT
13-276	ALM	3	WT
16-183	ALM	3	WT
06-057	ALM	4	WT
08-109	ALM	4	WT
10-235	ALM	4	WT
13-051	ALM	4	WT
ts-12-15250	MM	0	WT
ts11-11371	MM	2	WT
ts04-7724	MM	2	WT
ts-13-08586	MM	2	WT
ts-17-05829	MM	2	WT
ts11-04660	MM	3	WT
b14-13215	MM	3	WT
s06-1779	MM	4	WT
ts16-515-421	MM	4	WT
ts16-11193	MM	4	WT
ts-13-13766	MM	4	WT

Table 13 shows the hotspot mutation sequencing results, indicating only 1 out of the 28 cases contains a hotspot mutation (3.5%). This activating mutation was found at Y646N, denoting a substitution of a Tyrosine at position 646 with an asparagine. This was present at an allele frequency of 21%, meaning that only 21% of the DNA present in the reaction contained the mutation. This could be because the

tumor was heterogeneous (a mixture of wild-type and mutant EZH2), that the sample was contaminated with DNA from normal (non-cancerous) cells that are present in the FFPE tissue, and/or that the sample is heterozygous for the mutant allele. The Y646N mutation is relatively common in lymphomas (blood cancers) and makes us predict high levels of H3K27me3.

This activating mutation enhances the EZH2 enzyme activity, meaning it causes an increased Histone methyltransferase activity. Tumors that have this mutation are highly sensitive to enzyme inhibitors like GSK126. In any case, since only 1/28 (3.5%) of all acral and mucosal melanomas analyzed, carried a known activating mutation in EZH2, this tumor type is a poor candidate for inhibition of EZH2 methyltransferase activity.

Our preliminary data suggests that the frequency of EZH2 mutation in ALM and MM, respectively, is lower than that previously reported for other melanoma subtypes, claiming a unique role for EZH2 alterations in this melanoma subtype biology and supporting the rationale for targeting EZH2 in these tumors.

This result is consistent with the low mutational burden I discussed in the introduction. So most probably the mutation is not a main mechanism of upregulation of EZH2.

4.3.2 TCGA data

We also looked at the TCGA data set in cBioPortal to search for mutations and putative copy-number alterations from tConut in melanoma. - Paired-exome sequencing of acral melanoma (TGEn, Genome Res 2017). [21]

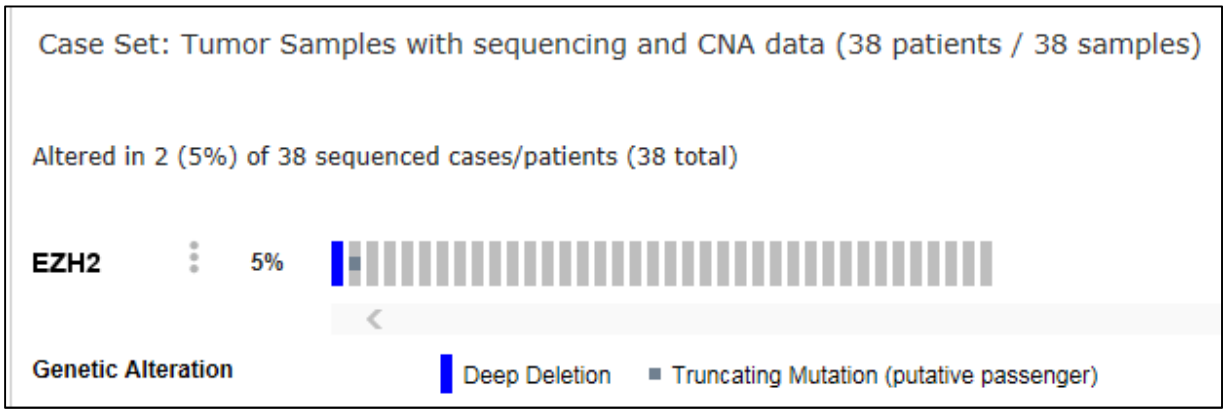


Figure 28 TCGA data set on Paired-exome sequencing for Mutations and Putative copy-number alterations in Acral Melanoma (n=38) (TGen, Genome Res 2017) [21]

This TCGA data set in Figure 28 has recently been added and only comprises a small number of 38 patients. It is only a little collection and does not support the idea of mutations or copy number gain being the mechanisms of EZH2 upregulation. The data only identified one single mutation which is not even an activating one, and one deep deletion.

4.4 4th Hypothesis- increased expression of EZH2 provokes H3K27me3 upregulation

4.4.1 Clinical

To test the 4th hypothesis, stating that high levels of EZH2 imply high levels of H3K27me3 since EZH2 is a histone methyltransferase, we used a mixed population of MM (10 cases) and ALM tissue samples (34 cases). The slides were stained with an H3K27me3 antibody and scored by Dr. Farbod Darvishian. Since the histones are found in the nucleus, H3K27me3 entails nuclear staining.

Figure 29 shows examples for different H3K27me3 scores.

Figure 29 Different Scores of H3K27me3 (antibody 1:2000) stained slides, nuclear staining, Score=0 meaning no H3K27me3 expression; Score=3 signifying weak H3K27me3, diffusely expressed OR strong H3K27me3, focally expressed and a Score=4 meaning strong H3K27me3, diffusely expressed Scale bar = 60 µM

We made sure our detections were right by optimizing the antibody in lung, which we know contains high H3K27me3 levels.

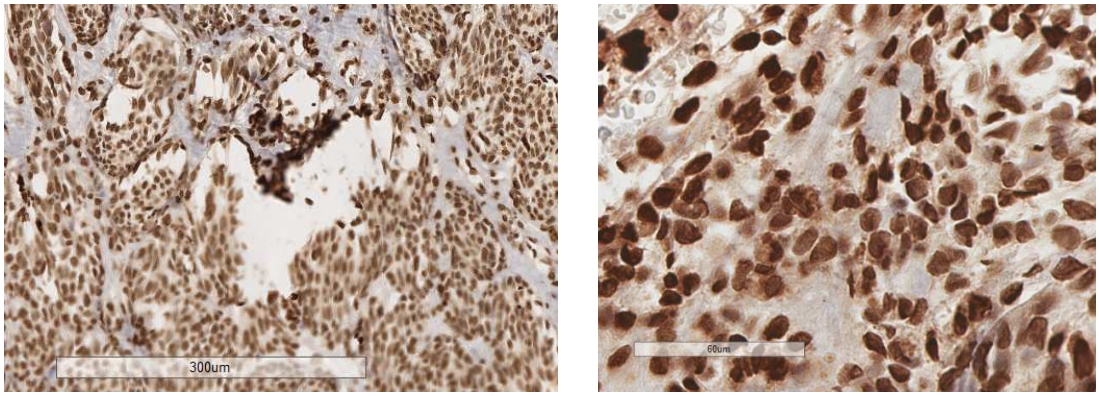


Figure 30 Positive lung tissue stained for H3 (antibody 1:150) as a positive control

Much to our surprise, in the 44 cases stained for H3K27me3 the levels were low in the majority of the tumors, even though they had high EZH2.

Table 14 A total of 44 ALM and MM cases stained for H3K27me3

H3K27me3					
ALM	**#**	**%**	**MM**	**#**	**%**
Score 0	20	59	Score 0	9	90
Score 2	5	15	Score 2	1	10
Score 3	7	21	Score 3	-	-
Score 4	2	6	Score 4	-	-
Total	34	100%	**Total**	10	100%

Figure 31 34 ALM and 10 MM cases stained for H3K27me3

Surprisingly we found an inverse correlation in the tissues with EZH2 and H3K27me3, see Table 14 and Figure 31. We would think H3K27me3 should be high since EZH2 is a Histone Trimethyl Transferase on Lysine 27.

As a positive control we performed the detection of total H3 in a small set of tissues, including some with no H3K27me3. All the 6 randomized picked patient tissues of different H3K27me3 scores showed a strong staining with H3 of a score of 4. These results illustrate that the lack of H3K27me3 cannot be attributed to a lack of H3 total, since H3 could be detected in the H3K27me3-negative tumors as well.

Among the 115 cases that had been stained for EZH2 and the 44 for H3K27me3, we could determine an overlap of 36 slides that had been stained with both antibodies. Figure 32

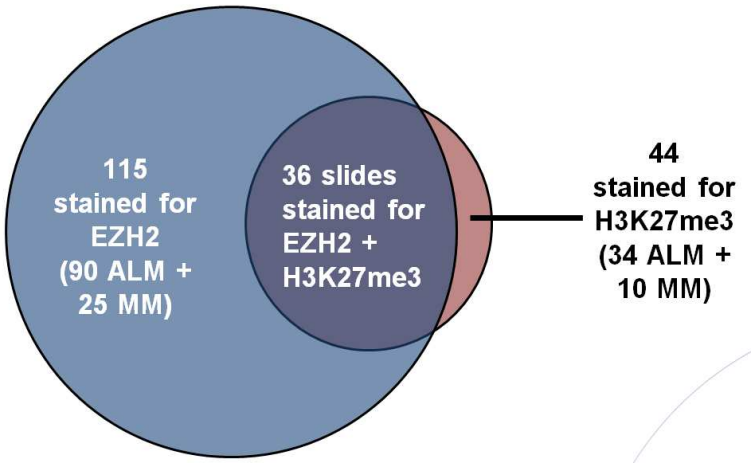


Figure 32 Schematic of the EZH2 and H3K27me3 stained slides

Next we tried to correlate the EZH2 and H3K27me3 scores of the 36 samples we had previously identified.

Table 15 EZH2 and H3K27me3 scores

	EZH2		H3K27me3	
Score	#	%	#	%
0	7	19	25	69
2	9	25	4	11
3	13	36	5	14
4	7	19	2	6

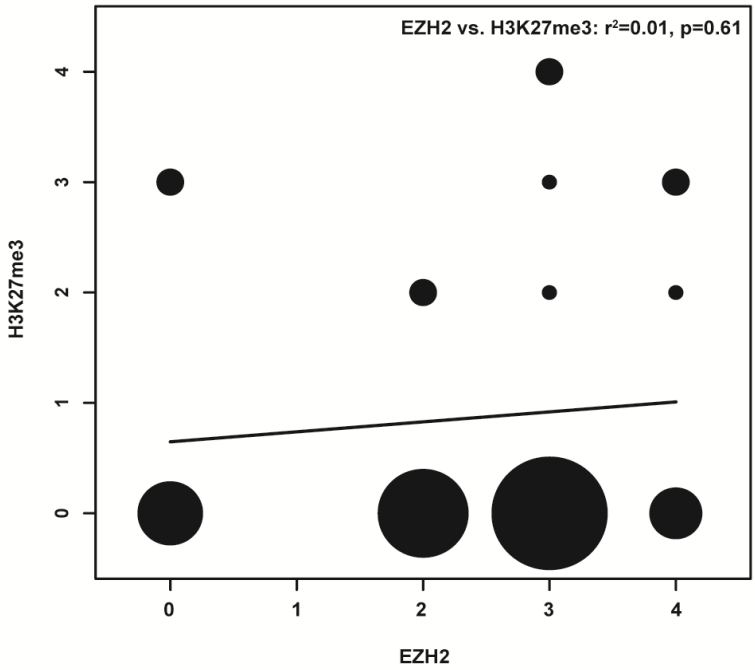


Figure 33 Correlationplot of the 36 EZH2 and H3K27me3 IHC scores showing an inverse correlation

Figure 33 and Table 15 indicate the inverse correlation we identified between the levels of EZH2 and H3K27me3. Much to our surprise, the majority of the tumors show low levels of H3K27me3, suggesting a non-canonical function of this enzyme. The size of the circle is proportional to the amount of cases matching these two scores. The r^2 value indicates that there is no positive correlation between the two variables. Additionally the p value is not significant (p=0.61). In cutaneous melanoma high levels of EZH2 and H3K27me3 have been reported. Counter intuitively, it is the opposite in these tissues. EZH2 high and H3K27me3 low indicate the worst outcome in this histologic melanoma subtype.

4.4.2 Biological

Next, I try to confirm our findings by western blotting. The basal levels of H3K27me3 were assessed by immunoblotting using purified histone preps (Abcam / acid extraction) from both ALM and non-ALM cell lines, see Figure 34. HSP90 and H3 are the loading controls.

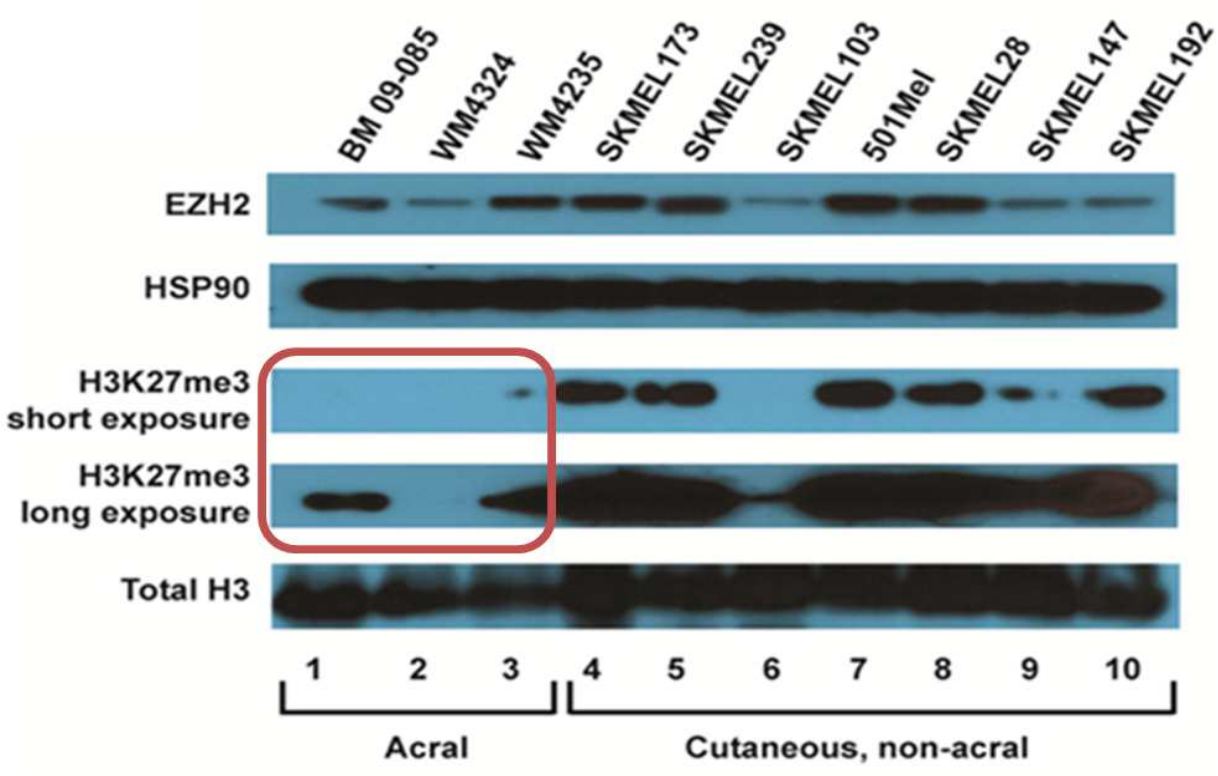


Figure 34 Immunoblot showing low levels of H3K27me3 in all 3 acral cell lines, in contrast to the cutaneous melanoma cell lines

As expected, the Western blot analysis in the biological part of the project, Figure 34, is consistent with what we previously determined with the IHC stained slides. The acral lentiginous melanoma cell lines have significantly lower basal H3K27me3 levels than other cutaneous melanoma cell lines, despite high levels of EZH2. In the CM cell lines the levels are generally higher and the abundance of H3K27me3 across all cell lines is following the pattern of EZH2 expression. There is no correlation between high levels of EZH2 and high levels of H3K27me3, if anything, it is the other way around and the cells with high EZH2 have low levels of H3K27me3. This is consistent with the IHC data.

There are other papers claiming high levels of EZH2 matching with low levels of H3K27me3. (Holm K, 2012) (De Donatis GM, 2016)

We also identified high expression EZH2 was associated with poor distant disease-free survival whereas high expression of H3K27me3 was linked with better survival. Interestingly enough, there is worse prognosis for low levels of histone 3-lysine 27 Trimethylation.

As the levels of H3K27me3 are pretty low in all of the acral cell lines we hypothesize that EZH2 might have in part a histone methyltransferase-independent (non-canonical) function in ALM and MM.

4.5 Non-canonical function of EZH2

Like I described in the previous chapter, increased expression of EZH2 does not necessarily correlate with enhanced abundance of H3K27me3. Therefore, EZH2 probably has effects beyond epigenetic silencing of target genes in these histologic subtypes.

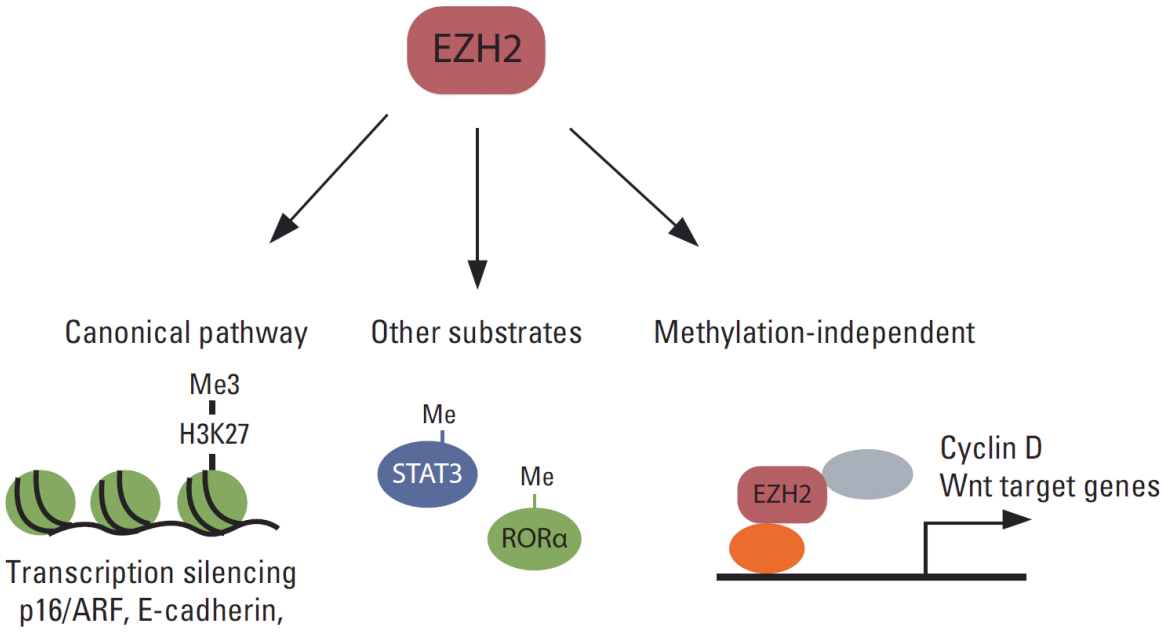


Figure 35 Mechanisms of action of EZH2 in cancer

Figure 35 of a publication in Cancer Research and Treatment (Hirohito Yamaguchi, 2014) shows the various functions of EZH2 in human cancer. One of them is the silencing of EZH2 of several tumor suppressors such as E-cadherin and INK4A/ARF via canonical histone trimethylation at lysine 27. Next, the EZH2 protein also methylates substrates other than H3K27, such as STAT3 and RORα. Supplementary Figure 50. Furthermore, EZH2 has a methylase-independent function like its interaction with the estrogen receptor (ER) α and β-catenin. Thereby the complex regulates c-Myc and cyclin D1 expression in breast cancer cells. A paper published in Cancer Research showed that acral melanoma has frequent amplification of the CD1 locus. (Edward R. Sauter, 2002)

There might be several other non-canonical functions of EZH2 that have not been revealed yet.

Recently there is emerging more supporting literature, talking about a non-canonical function of EZH2. (Holm K, 2012) (De Donatis GM, 2016). (Xu Wang, 2017)

In the introduction, I mentioned the urgent need for therapies against ALM and MM, since they lack targeted therapies and there is less prospect with the existing therapy. Considering the epigenetic basis, we thought about ways to target these tumors.

Figure 36 shows a schematic of epigenetic histone modifications and different drugs available to interact with these processes on several different levels.

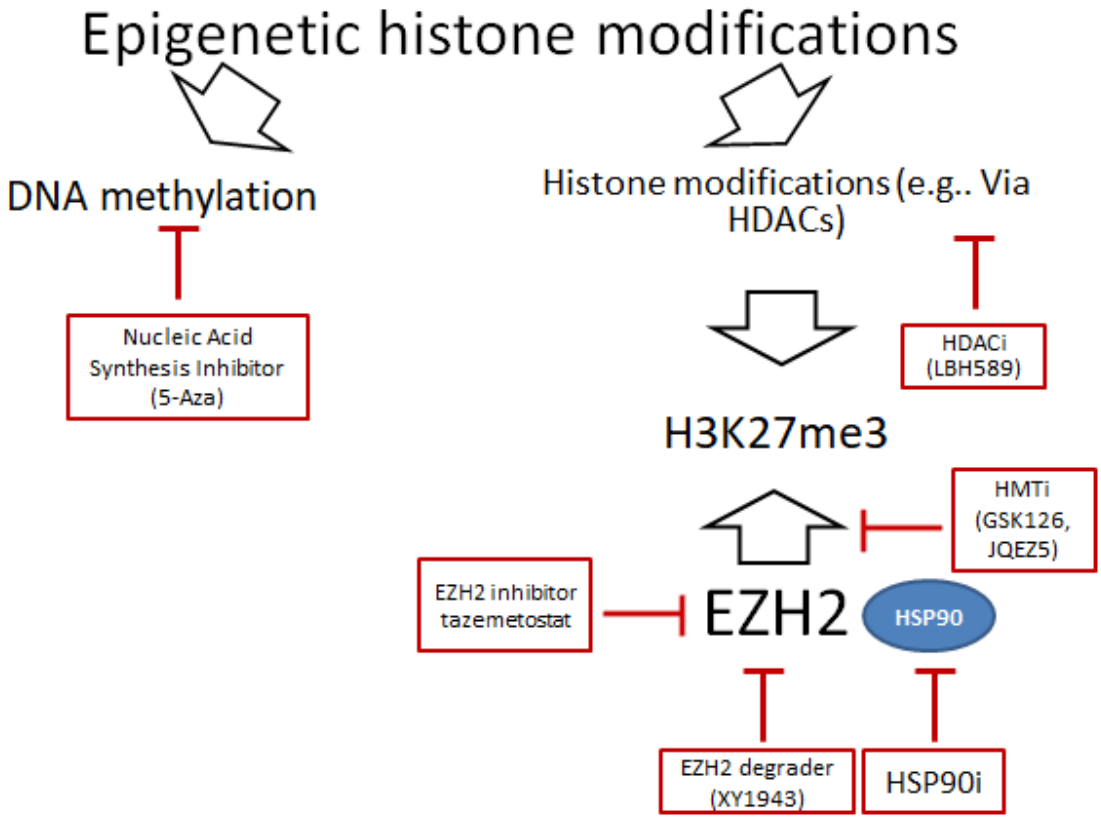


Figure 36 Schematic of epigenetic histone modifications and different drugs available to interact with these processes

Epigenetic modifiers, like the Nucleic Acid Synthesis Inhibitors and the HDAC inhibitors have been in use for a long time, but unfortunately lack effectiveness. (Berkley E Gryder, 2012)

There are new drugs arising that target the enzyme activity, like GSK126 and JQEZ5. Fortunately, they are already in clinical development. Since we suggest that EZH2 might work in part non-canonically, we are interested in degrading the protein. We received a tool compound from a collaborator from Mount Sinai with the ability to degrade EZH2.

Taking into account our previous findings, we come up with another hypothesis: Acral melanoma cells are sensitive to EZH2 degradation but not to H3K27me3 inhibition. This implies a histone methyltransferase – independent mechanism of action.

4.6 5th Hypothesis- acral melanoma cells are sensitive to EZH2 degradation but not to H3K27me3 inhibition, implying a histone methyltransferase – independent mechanism of action.

- ✓ The aim is to test the canonical vs non-canonical function of EZH2 via a pharmacological approach. We want to examine 2 different EZH2 inhibitors (GSK126 + JQEZ5) that reduce H3K27me3, which means they target the enzymatic histone methyltransferase activity of EZH2. In addition we will do experiments with a novel EZH2 degrader from a collaborator at Mount Sinai (unpublished) that degrades the EZH2 oncoprotein. Therefore we performed several different assays.

Table 16 showing the 3 different drugs used in the experiments as well as their mechanism of action

GSK126	JQEZ5	EZH2 degrader
inhibitor	inhibitor	degrader

The two inhibitors, GSK126 and JQEZ5, block the H3K27me3 function whereas the decreases the EZH2 protein level.

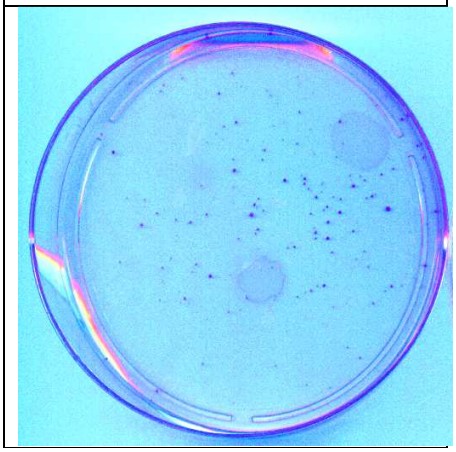
4.7 Assays

4.7.1 Colony Formation Assay

In order to test the hypothesis of the non-canonical function and the influence of the drugs on colony formation, I performed colony assays with the two acral cell lines that form colonies: BM09-085 and WM4235. A total number of 2000 cells was plated into 10cm cell culture plates and incubated for 2 weeks at 37°C. Next, the colonies that developed were counted.

The plates containing the colonies of BM09-085 can be seen in Figure 37.

Control 0 µM
>300 colonies



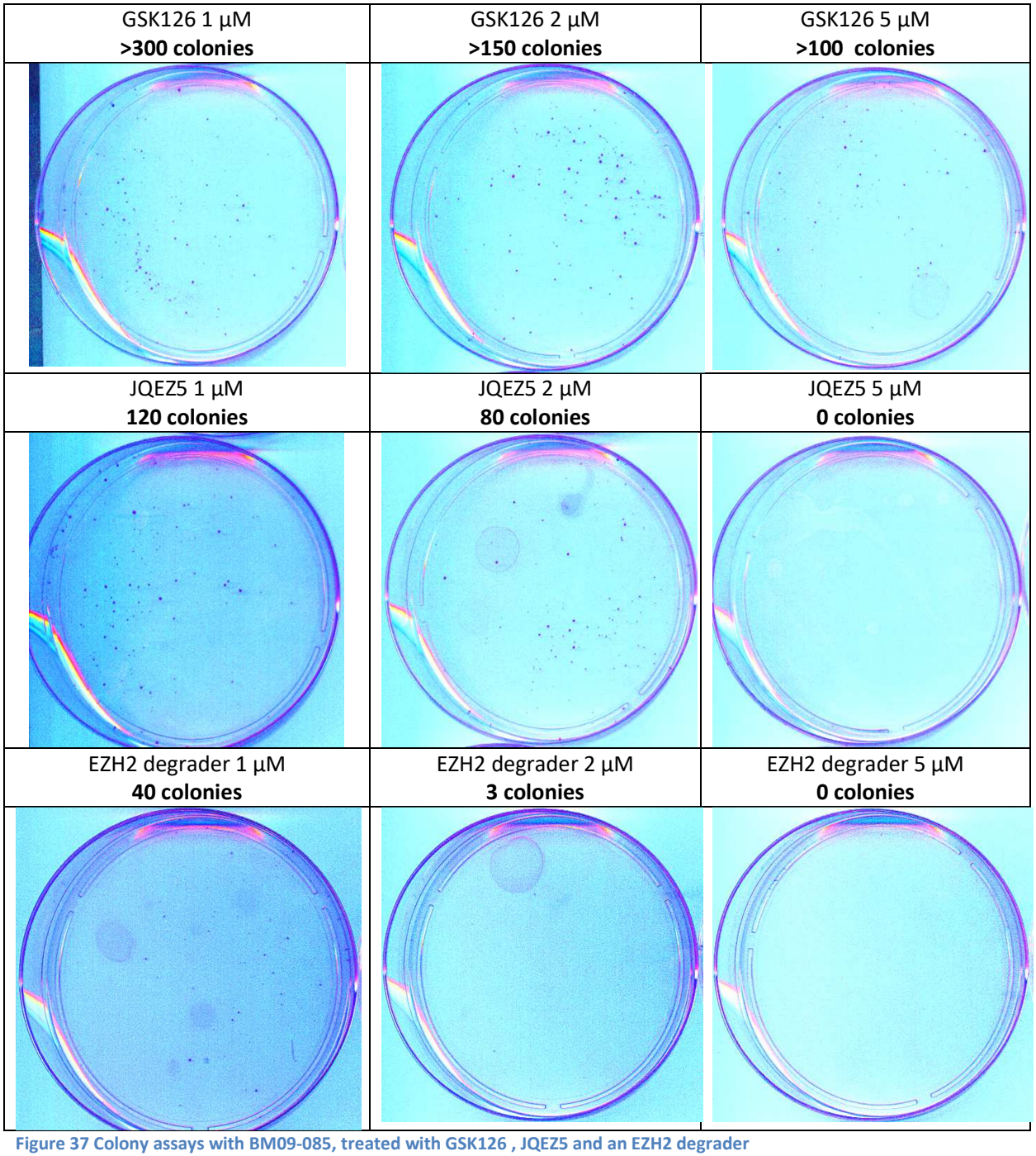


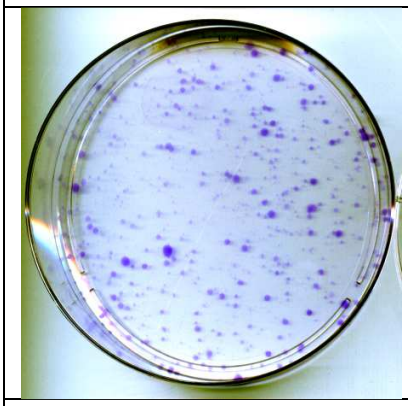
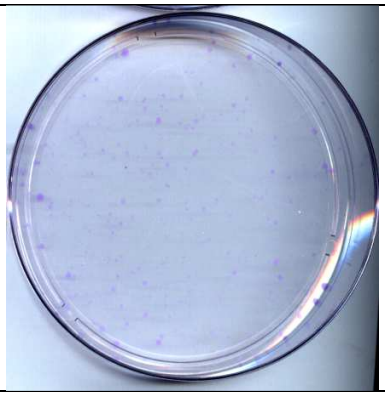
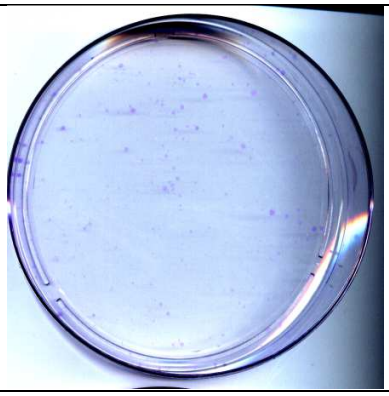
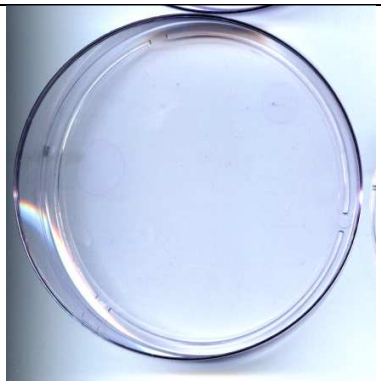
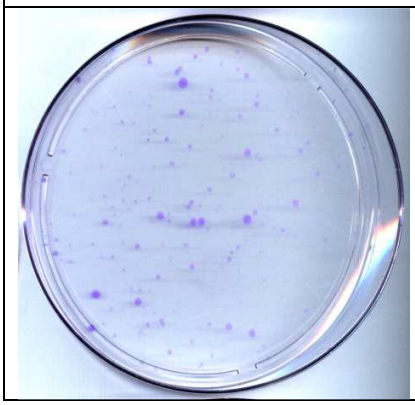
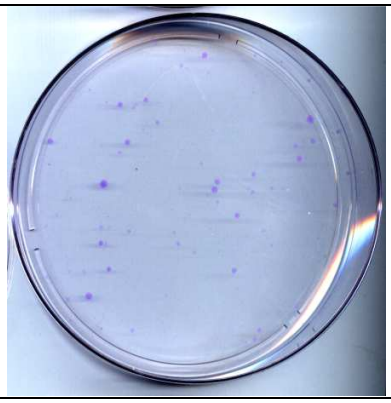
Figure 37 Colony assays with BM09-085, treated with GSK126 , JQEZ5 and an EZH2 degrader

These drug sensitivity assays were performed to test the therapeutic rationale for EZH2 inhibition in ALM in vitro and in vivo. It appears that the enzyme inhibitors have some amount of efficacy, the degrader even more. The assays reveal that GSK126 has a very weak effect. Even at a concentration of 5 µM there are still comparatively many colonies (<100).

JQEZ5 has some amount of efficacy; especially at 5 µM it impedes colony formation.

The EZH2 degrader has the biggest effect, even it lower concentrations it significantly decreases colony formation.

Figure 38 shows the colony assays I performed with WM4235, again treated with the two inhibitors and the EZH2 degrader.

Control 0 µM **>400 colonies**		
		
GSK126 1 µM **>300 colonies**	GSK126 2 µM **>150 colonies**	GSK126 5 µM **7 colonies**
		
JQEZ5 1 µM **100 colonies**	JQEZ5 2 µM **60 colonies**	JQEZ5 5 µM **0 colonies**
		

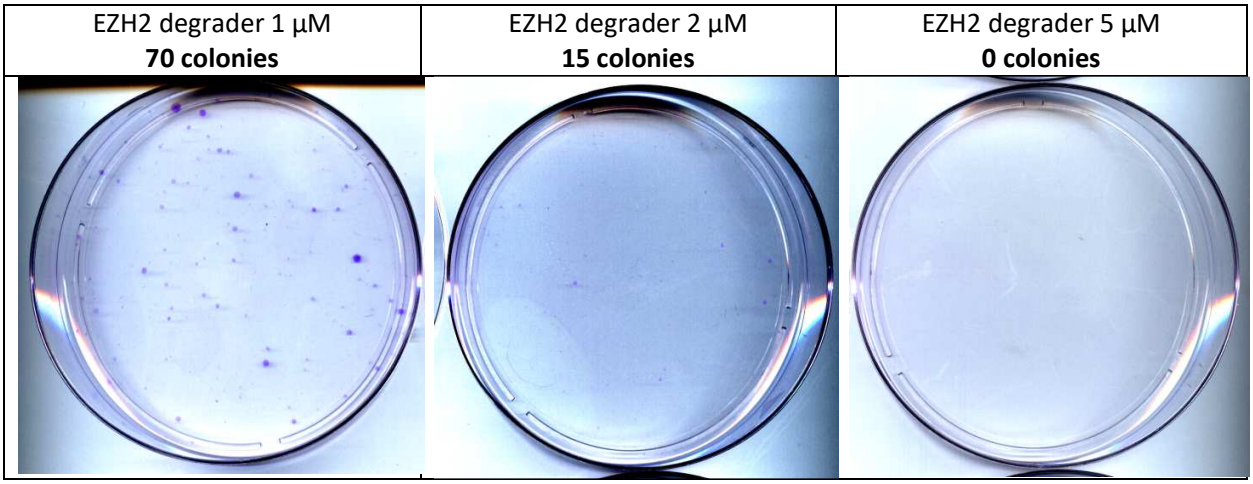


Figure 38 Colony assays with WM4235, treated with GSK126 , JQEZ5 and an EZH2 degrader for 2 weeks

Figure 38 reveals that at 5 µM all the 3 drugs have a significant effect on colony formation. In contrast, lower doses of GSK126 do only slightly decrease colony formation.

The size of the colonies in the control plate is bigger, letting us assume that the drugs decrease the speed of cell growth.

JQEZ5 at lower doses has still some amount of effectiveness.

In addition, the selective sensitivity of the ALM cell line to the EZH2 degrader supports the therapeutic potential of EZH2-targeted therapy in ALM.

Summarizing, the significant difference in inhibition of cell viability between the two histone methyltransferase inhibitors GSK126 and JQEZ5 and the EZH2 degrader suggests that there is, at least in part, an EZH2 histone methyltransferase independent function in this melanoma histotype.

4.7.2 Proliferation Assay

Finally, we performed proliferation assays to determine whether pharmacological treatment with different drugs would result in decreased proliferation in the acral lines. This appeared to be a viable treatment strategy based on the observation that there might be a non-canonical function of EZH2. Specifically, the cells were treated using different concentrations (1, 2 and 5 µM) of GSK126 and JQEZ5, two EZH2 histone methyltransferase inhibitors, as well as a tool compound that degrades EZH2.

The Promega MTS assay we used is a colorimetric method which measures cell metabolism as a surrogate measure of the number of viable cells that are present in the well. Cells were incubated for 7 and 11 or 14 days before subjection to 200 µL of the CellTiter 96® AQueous One Solution Reagent and incubation for 1–4 hours. In the following step the absorbance at 490nm is recorded with a 96-well plate reader. The amount of formazan product as determined by OD measurement is directly proportional to

the number of living cells in culture. This assay serves the investigation of EZH2 knock-down over a long period of time.

Figure 39 shows the pipetting scheme table of the cell viability assay. Figure 40 displays the color change when performing this MTS assay. The reagent contains a tetrazolium compound (MTS) and an electron coupling reagent (PES). PES, being very stable, allows forming a stable solution when combined with MTS. The original color of the reagent is red, when the nutrients are metabolized by healthy cells the color changes to yellow. Hence, the brighter the color, the more cells are alive.

MOAT											
MOAT	Control	1 µM degrader	2 µM deg.	5 µM deg.	1 µM GSK126	2 µM GSK126	5 µM GSK126	1 µM JQEZ5	2 µM JQEZ5	5 µM JQEZ5	MOAT
	Control	1 µM degrader	2 µM deg.	5 µM deg.	1 µM GSK126	2 µM GSK126	5 µM GSK126	1 µM JQEZ5	2 µM JQEZ5	5 µM JQEZ5	
	Control	1 µM degrader	2 µM deg.	5 µM deg.	1 µM GSK126	2 µM GSK126	5 µM GSK126	1 µM JQEZ5	2 µM JQEZ5	5 µM JQEZ5	
	Control	1 µM degrader	2 µM deg.	5 µM deg.	1 µM GSK126	2 µM GSK126	5 µM GSK126	1 µM JQEZ5	2 µM JQEZ5	5 µM JQEZ5	
	Control	1 µM degrader	2 µM deg.	5 µM deg.	1 µM GSK126	2 µM GSK126	5 µM GSK126	1 µM JQEZ5	2 µM JQEZ5	5 µM JQEZ5	
	Control	1 µM degrader	2 µM deg.	5 µM deg.	1 µM GSK126	2 µM GSK126	5 µM GSK126	1 µM JQEZ5	2 µM JQEZ5	5 µM JQEZ5	
MOAT											

Figure 39 Schematic indicating the pipetting scheme table of the 96-well plate proliferation assay

Figure 40 Picture of the 96-well plate proliferation assay after 7 day incubation

The plots of the titration assay were created with GraphPad Prism 7.02. They show cell viability in different acral cell lines for 7 days and 11 or 14 days incubation.

4.7.2.1 WM4235 (acral cell line)

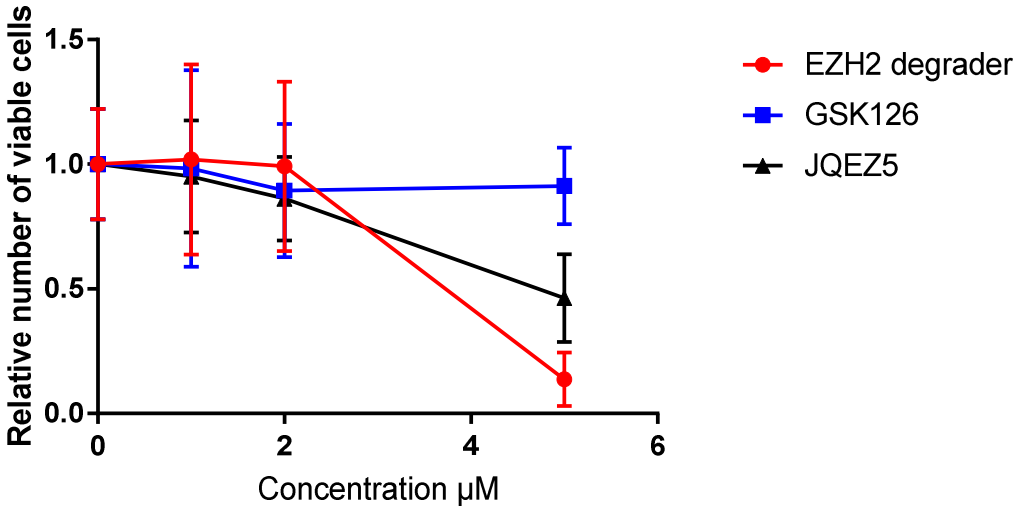


Figure 41 WM4235 acral melanoma cells are highly sensitive to targeted degradation of EZH2 after 7 days of drug treatment

In the graph in Figure 41 we noticed a consistent and statistically significant reduction in proliferation with the EZH2 degrader. In addition, JQEZ5 has an effect that is comparable to the degrader. It seems like it is suppressing H3K27me3 stronger than GSK126.

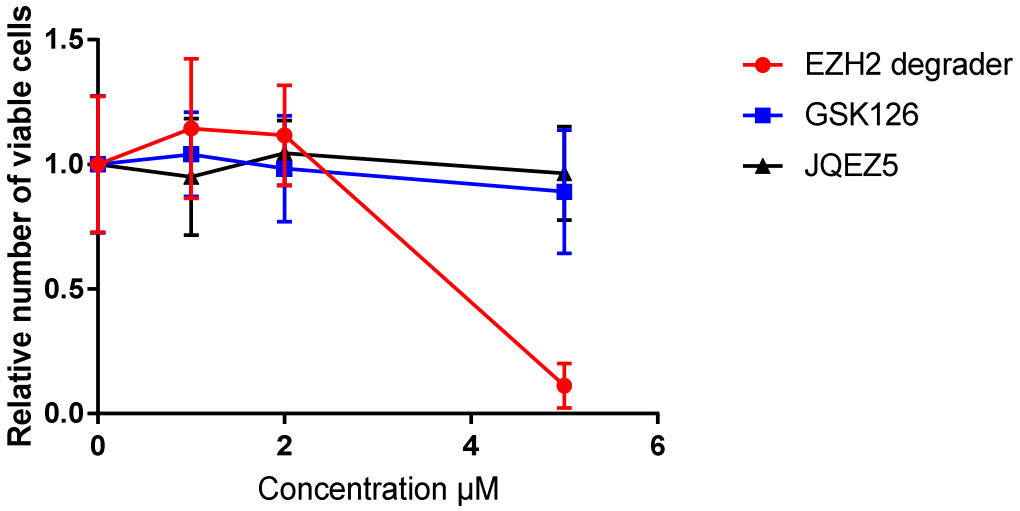


Figure 42 11-day cell growth assays in WM4235, indicating that the acral cells reover with the H3K27me3 inhibitor in contrast to the EZH2 degrader

It is important to note that at longer time points at 5µM, Figure 42, the different effect between the degrader and inhibitor becomes clearer. The cells treated with JQEZ5 recover, in contrast to the cells

treated with the drug from the collaborator at Mount Sinai. This supports other work on the H3K27me3 independent role. Since there are fewer viable cells than the control and the majority of the cells are dead, we conclude that the degrader is stopping proliferation. This graph proofs that monotherapy of the H3K27me3 inhibitor GSK126 on its own is ineffective. However the degrader, an epigenetic modulator, can reduce growth of the cells and/or cause their death.. JQEZ5, being an EZH2 inhibitor, also has some effect on the growth of the cells.

Confirming the non-canonical function of EZH2, we found that the comparison of Figure 41 with Figure 42 justifies degrading EZH2 vs. suppressing the histone methyltransferase function. Maybe by day 11 there is a rebound in H3K27me3 levels, which does not happen when EZH2 is being degraded. Or possibly the acral cells are more sensitive to EZH2 degradation than they are to enzymatic inhibition.

The MTS assays we performed do not reveal whether the degrader is affecting cell growth or death. Hence, future experiments to investigate the anti-tumor effects would be needed to delineate the exact effects of the degrader targeting the questions whether it promotes apoptosis. Therefore we could measure caspase or PARP cleavage and look at BrdU incorporation assays to look at the specific effects of the degrader on cell proliferation.

Next we performed cytotoxicity assays in BM09-085.

4.7.2.2 BM09-085 (acral cell line)

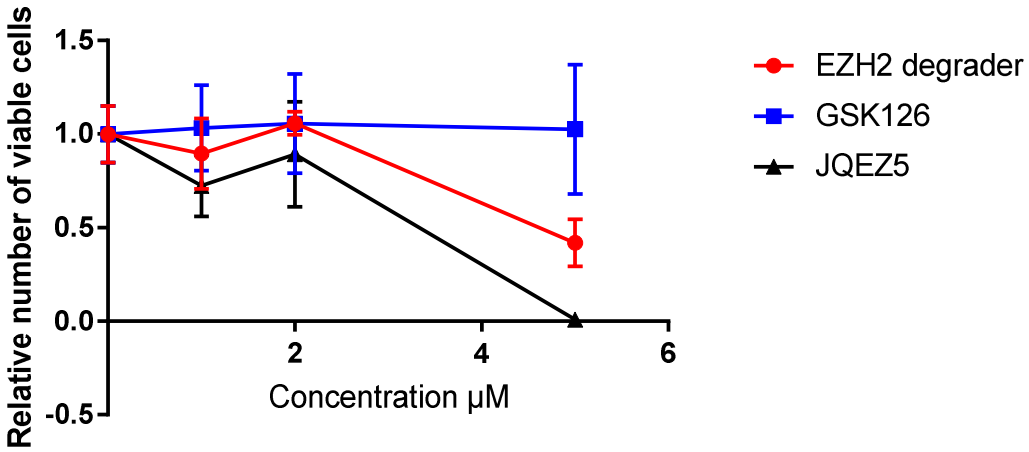


Figure 43- 7-day Proliferation assays in BM09-085 showing strong effects with JQEZ5

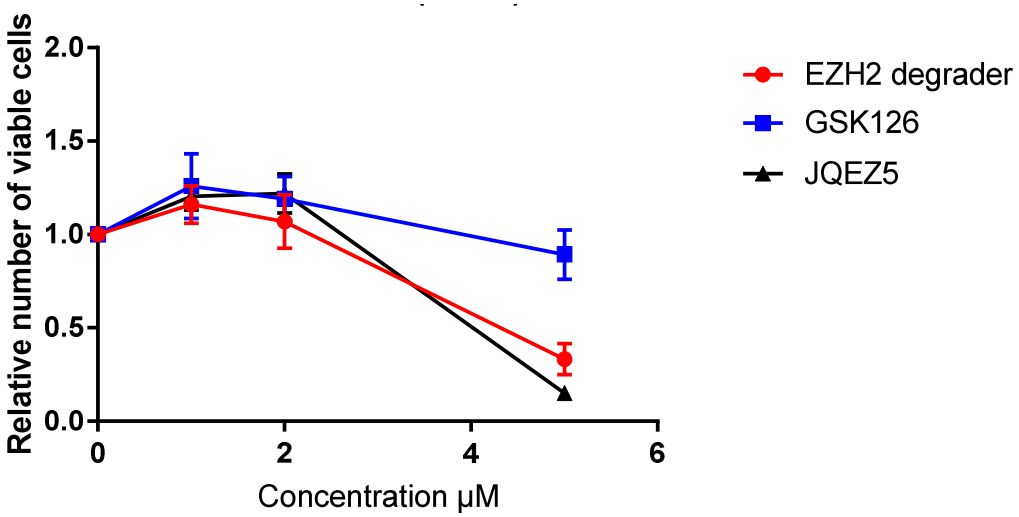


Figure 44- 14-day Proliferation assays in BM09-085 indicating sensitivity for EZH2 degradation and inhibition via JQEZ5

Figure 43 and Figure 44 is a proof for the little impact the histone methyltransferase inhibitor GSK126 had on the growth of ALM cell lines. In this cell line JQEZ5 had even a stronger effect than the EZH2 degrader. Both, JQEZ5 and the degrader are significantly inhibiting growth in BM09-085 at 5µM, especially in the long treatment.

We also tested these 3 drugs in WM4324 for 7 days. The 2-week drug treatment in order to study cell growth in this acral cell line needs to be repeated, since it did not work out.

4.7.2.3 WM4324 (acral cell line)

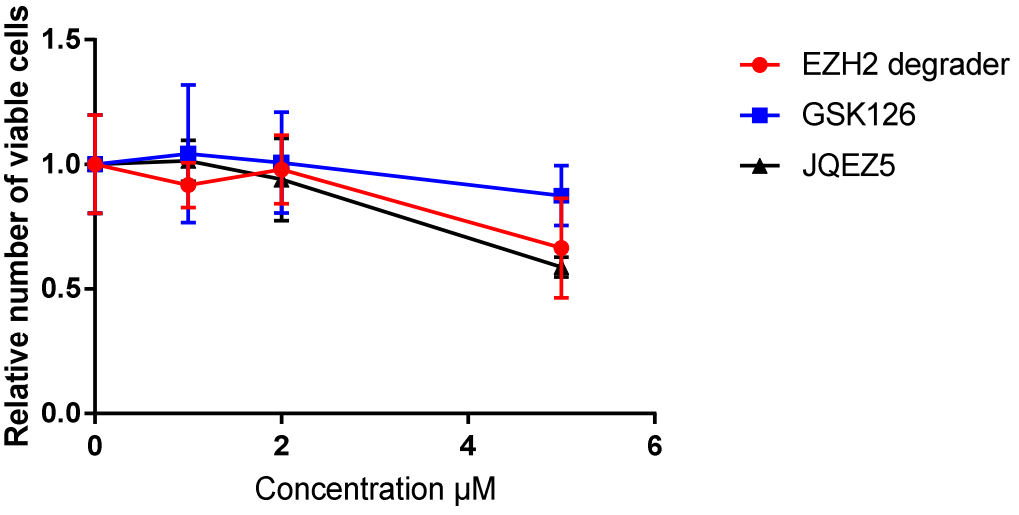


Figure 45- 7-day Proliferation assays in WM4324 supporting the rational of degrading EZH2 or using JQEZ5

Basically, the plot of the proliferation assay in Figure 45 shows the same results as in the other cell lines. There is a huge effect of the degrader and JQEZ5 and almost no effect of GSK126.

In conclusion, we can say that the growth assays in the ALM cell lines suggest that there is certain sensitivity to targeted degradation of EZH2 vs Inhibition, but also a difference between the two enzyme inhibitors.

Since this is only preliminary data, it needs to be validated in more ALM cell lines. In addition, it seems that EZH2 inhibition is having a stronger effect with fewer cells. Future work might include plating fewer than 2000 cells per 10 cm cell culture plate.

4.7.3 Genetic approach – Hairpins

We thought about different ways how to target EZH2 and came up with a genetic approach. For this purpose we used EZH2 protein targeting short hairpin RNA (shRNA).

Table 17 Overview of the genetic vs the pharmacological approach for EZH2 knock-down

Sh RNA	EZH2 degrader	GSK126 / JQEZ5
genetic	Pharmacological / chemical	Pharmacological / chemical
degrader	degrader	inhibitor

We used different hairpins targeting EZH2 (Hairpin 2, 3 and 4) as well as a control hairpin (hairpin 1) which does not target the protein.

The shRNAs are Doxycycline-dependent inducible vectors, meaning it is a reversible RNA interference mediated by a single lentivirus vector. DOX induces the promoter and hence silence genes of interest. Reversible refers to the fact that withdrawal of Dox after transient treatment results in complete recovery of target gene expressions to usual levels. (Matsushita N, 2013)

To show the effect of depletion of EZH2 we induce with DOX.

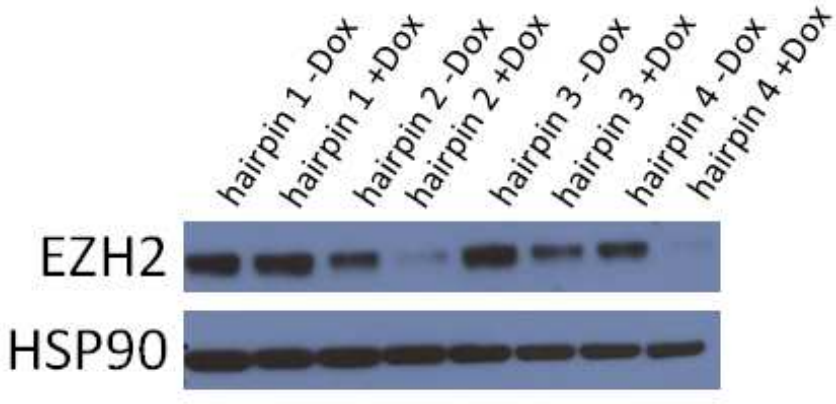


Figure 46 We confirmed knockdown with the shRNA using Westernblot analysis

Figure 46 shows that we were able to successfully deplete the expression of EZH2 in WM4235 using small hairpin RNAs. The control hairpin 1 does not silence EZH2 when being induced with Doxycycline, in contrast to hairpin 2, 3 and 4. Hairpin 2 and 4 induce a stronger knock-down than hairpin 3.

Future work to study the impact of EZH2 shRNA in acral melanoma would include infecting all available ALM cell lines and then treating the cells infected with these hairpins with Dox. This would induce the hairpin and then we could measure the viability of this histologic subtype.

By inducing only over a short period, like less than 5 days, we try to mimic the effect of a single dose of EZH2 degrader. Next we will assess cell growth and colony formation.

4.7.4 HDACi

Epigenetic alterations by histone deacetylases (HDACs) are one of the many mechanisms that cancer cells use to alter gene expression and promote growth. In general, genetic mutations and loss of PTEN, the Phosphatase and tensin homolog, are mostly irreversible and lead to many cancers. On the contrary, epigenetic changes are reversible and hence an attractive target for cancer therapy. (Hagelkruys A, 2011)

The rational for testing a HDAC inhibitor, like LBH589, in the cells is, that they could function as a negative regulator by changing the acetylation.

This drug down-regulates members of the PRC2 in a time- and dose-dependent manner in cultured acral melanoma cells. The HDAC inhibitor changes the acetylation status of many proteins, like HSP90, which is a chaperone to protect proteins from degradation, including EZH2. By this change the HDACi prevents the association of the complex with EZH2, thereby decreasing the proteins stability and rapidly degrading it.

P21 serves as a positive control, since it is being induced when treated with LBH589. In contrast, cell lines that already have p21 do not induce it again. This protein restricts the cell cycle in the acral cell lines. Since WM4235 grows way quicker, a stronger effect can be seen.

Figure 47 shows a 1 day treatment with Panobinostat in WM4235.

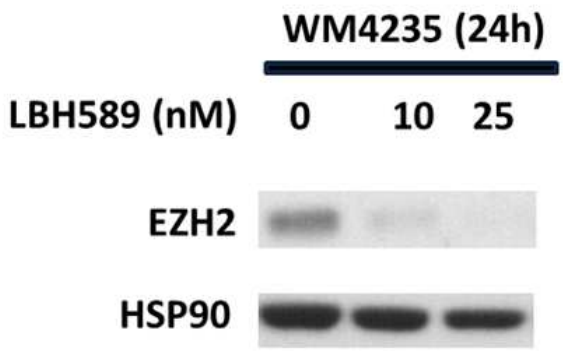


Figure 47 24h LBH treatment shows a change in the immune relating genes, meaning EZH2 goes down

The experiment in Figure 47 indicates that the short term (24h) treatment in an acral cell line (WM4235) to LBH589 at 10 and 25nM has a dose-dependent effect. The EZH2 expression is significantly depleted in the lower concentration and completely silenced at 25nM, suggesting that Histone-Deacetylase inhibitors (HDACi) repress negative regulators of EZH2 and thereby lower its levels.

Literature suggests that the loss of HDAC1,2 activity increases global H3K27ac and hence impairs proliferation of the EZH2. HDAC targets both histone and non-histone proteins within tumor cells. (Danielle P. Johnson, 2015).

Since we do not have normal melanocytes we searched in the literature and found a reference supporting the fact that low nanomolar concentrations of LBH589 do not inhibit the growth of normal melanocytes. (Woods DM, 2013)

To sum up, LBH589 exerts a dual effect upon melanoma cells by affecting not only growth and survival but also by increasing melanoma immunogenicity, meaning it provokes an immune response in the body.

These effects provide the basis for further evaluation of this HDAC inhibitor in melanoma treatment.

There exists some literature, supporting the use of LBH589 in order to reduce the protein expression of EZH2. (Warren Fiskus, 2009)

4.7.5 Combination of HDAC- and EZH2- inhibitor

Our previous results suggest that panobinostat is a potential drug in the treatment of melanoma.

Furthermore, GSK126 has shown a lack in effectiveness so far. In order to tackle this problem, we did some research and found that the HDAC inhibitor LBH589 has proven to be effective in the treatment of specific malignancies, particularly in combination with other anticancer agents. (Ling, 2017).

Hence we tried a combination of different concentrations of GSK126 (0, 0.5,1,2 and 5 µM) and LBH589 (0, 2.5, 5 and 10 nM).

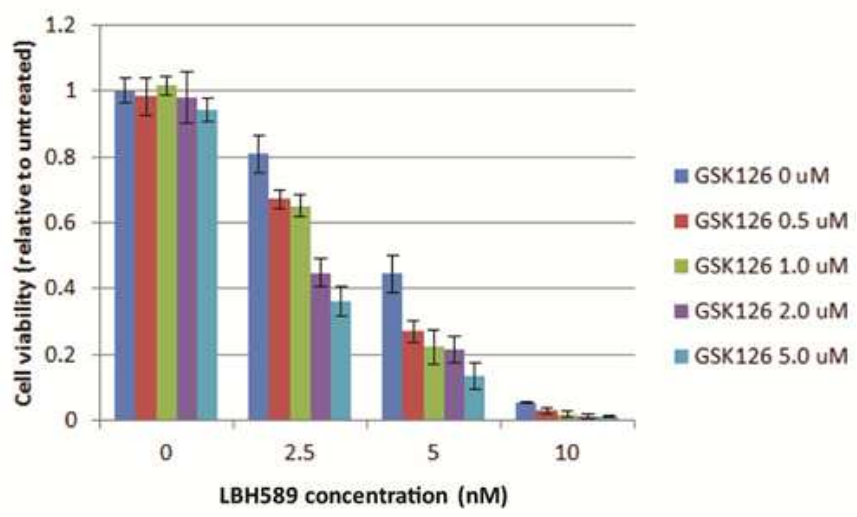


Figure 48 Chronic low-dose LBH589 treatment can sensitize acral melanoma tumor cells to GSK126

Figure 48 is a proof that chronic low-dose LBH589 treatment, reducing EZH2 protein, can sensitize acral melanoma tumor cells to otherwise-ineffective doses of GSK126, an EZH2 HMT inhibitor. At higher concentrations of the enzyme inhibitor, cell viability is dramatically decreased.

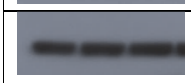
On its own LBH589 is degrading EZH2. In contrast, GSK126 is not very effective by itself. Both drugs being in clinical development, this combinational therapy could rapidly be translated into treatments.

4.7.6 LY294,002

Treatment with this PI3K inhibitor, described in 2.2.4, blocks Akt at Serine 21. The blockade works through histone methylation via the Erk Pathway. (Barancík M, 2006)

We want to look at the Akt and p-Akt (mAb: Phospho-Akt (Ser473)) levels in an acral cell line when EZH2 is upregulated.

Table 18 LY294,002 treatment in WM4235 showing decreased levels of p-Akt

	**p-Akt (short exposure)**
	**p-Akt (long exposure)**
	**Akt**
	**HSP90**

WM4235 was treated for 1 hour with 0, 10 and 20 µM (left to right) of LY294,002, see Table 18. The PI3K inhibitor immediately shows an effect on the p-Akt levels. This proofs the blockade of the drug via the Erk Pathway. In contrast, the Akt and HSP90 levels, as controls, are constant.

Future experiments include harvesting histones after a longer drug treatment, approximately 24hrs. Since we are blocking p-Akt, we are expecting to see an increase in H3K27me3 levels in the Immunoblots.

A paper released in Oncotarget talks about the functional interaction between EZH2- and PIK3CA (PIK3 Catalytic Subunit Alpha) dependent signaling pathways. In detail, PIK3CA alterations mediate firstly the increased expression of different miRNAs, which in a following step post transcriptionally downregulate EZH2 expression. Furthermore, alterations in this complex facilitate the activation of Akt which phosphorylates EZH2 on Ser21, precluding the trimethylation of histone H3 in K27. (Cristina Segovia, 2017)

It is important to mention that these are only preliminary results that warrant further validation and an increased sample size.

5 Discussion

To recapitulate, our data identified an EZH2 protein overexpression in 75 – 80% of a cohort of primary and recurrent ALM and MM tissues. Our data demonstrate that EZH2 up regulation is common in the clinical patient tumor tissues and cell lines of these histological subtypes (ALM + MM). Interestingly, the frequency of EZH2 overexpression observed in our ALM and MM cohort is far higher than previously reported for non-acral melanoma (i.e. ~80% vs. ~20%, (Jessamy Tiffen, 2014)), which suggests that EZH2 overexpression may be important to ALM development and progression. In this project, we utilized melanoma patient tissues with extensive clinic pathological annotation, and protein expression between melanoma subtypes (non-sun exposed vs sun exposed melanoma) to determine the clinical impact of EZH2 overexpression in these histological subtypes. The translational impact of my work is to address the disparity in ALM prevalence among black and Hispanic Americans, by identifying a novel role for EZH2 as a biological driver and a therapeutic target in ALM.

Additionally, this study explores the association between high levels of EZH2 and worse survival and progression. Interestingly, increased EZH2 expression correlates with poor outcome, meaning that high transcript levels are indicative for poor patient survival. Our preliminary data identified an increased EZH2 expression with oncogenesis of ALM from primary to recurrent ALM and MM tumors. Hence, our working hypothesis is that there is a subtype-specific dependence of ALM upon EZH2 to sustain tumor development and progression, and that EZH2 represents a novel, actionable target to develop a targeted therapy approach for treatment of ALM and MM.

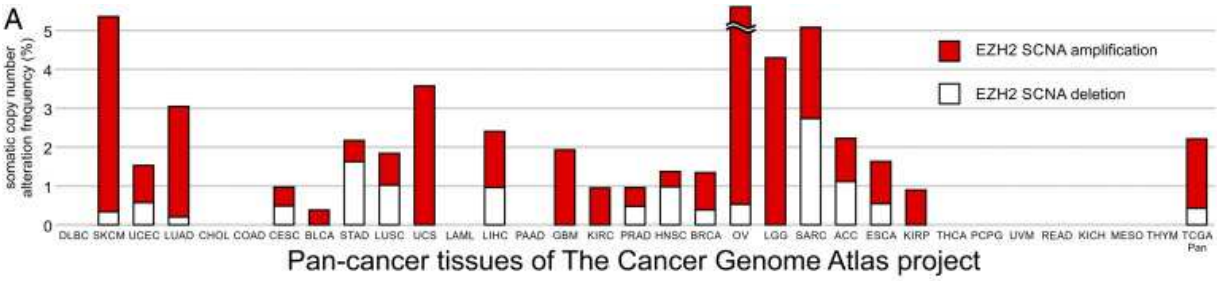
Our data suggests there is, at least in part, a non-canonical function, other than the canonical H3K27me3 enzymatic function of EZH2. Looking at the mutational burden, we assume that most likely somatic mutations are not a mechanism of activation. This result is consistent with the low mutational burden discussed in the introduction. Our findings exhibit that the EZH2 locus is over-expressed at the level of somatic copy number alterations of 14 %. A paper published in Neoplasia, in February 2016 (Jessamy Tiffen, 2014) suggests 5.0% and the TCGA data claims an average of 1.8%. (Supplementary Figure 49). We connect somatic copy number alterations (SCNAs) of EZH2 to epigenetic and transcriptional control of its target genes. Our preliminary data identified an EZH2 copy number gain in ~15% of all ALM and MM cell lines and tumor samples. The prolonged half-life in the two acral cell lines I tested denotes that protein stabilization might be a mechanism of EZH2 protein upregulation. EZH2 might be high due to a combination of several mechanisms including increased copy number and protein stabilization.

Beyond this assertion, IHC and western blots show a significantly distinct expression of both markers, EZH2 and H3K27me3 across all subtypes with high abundance of EZH2 and a lack H3K27me3 in most of the cases. We would think H3K27me3 should be high since EZH2 is a histone trimethyl transferase on lysine 27. However, intriguingly there is no positive correlation between the levels of EZH2 and H3K27me3, if anything, it is the other way around and the cells with high EZH2 have low levels of H3K27me3. In contrast, in cutaneous melanoma high levels of EZH2 and H3K27me3 have been reported. Counter intuitively, it is the opposite in these tissues. High expression EZH2 was associated with poor distant disease-free survival whereas high expression of H3K27me3 was linked with better survival.

As a consequence, we hypothesize that EZH2 might have, at least in part, a histone methyltransferase-independent (non-canonical) function in ALM and MM. Hence, there is a rational for using EZH2 degraders and combinational therapy. Our preliminary data identified reduced colony formation of ALM cell lines upon treatment with an EZH2 degrader and two EZH2 inhibitors in vitro. Blocking of EZH2 in human melanoma cells affects their growth as well as their invasive capacity. Deletion of EZH2 does not interfere with normal melanocyte function. (Daniel Zingg, 2015). This data together provides a preliminary justification for investigating the use of an EZH2 degrader, EZH2-, PI3K- and HDAC- inhibitors and for genetically silencing the gene of interest.

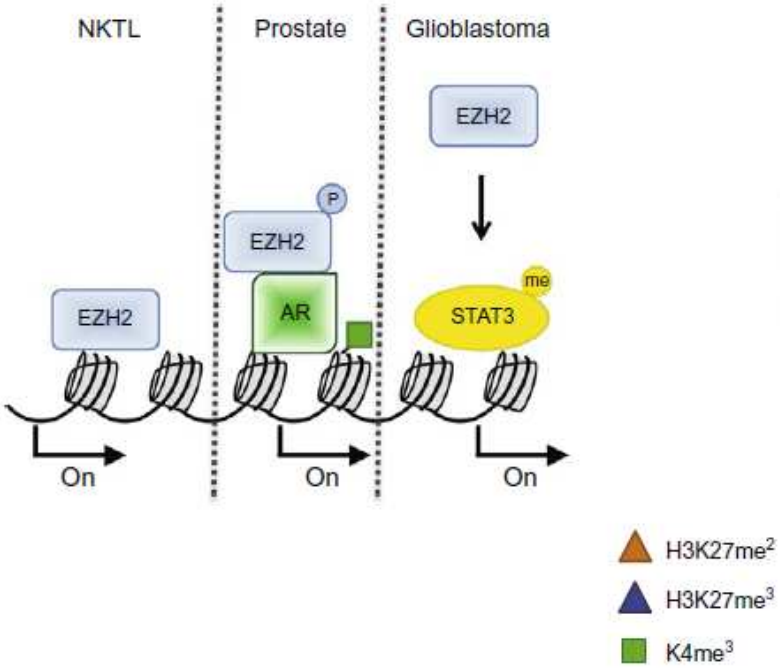
6 Supporting information / Appendix

6.1 Supplementary figures



Supplementary Figure 49 showing that EZH2 is predominantly amplified at the somatic copy number level across a comprehensive panel of TCGA Pan-cancer patients. (Jessamy Tiffen, 2014)

The frequency of EZH2 somatic copy number alterations (SCNAs) is shown in white for deletions and red for amplifications. The SCNAs frequency of Ovarian serous cystadenocarcinoma (OV) has a SCNAs frequency exceeding the chosen y-axis range (11.4%). This has been indicated by the tilde. At the right the TCGA Pan-cancer average across 9833 specimens is being displayed. (Jessamy Tiffen, 2014)



Supplementary Figure 50 suggesting EZH2 has non-canonical activities when overexpressed

Supplementary Figure 50 shows a model of the activities EZH2 overexpression causes beyond H3K27 methylation, since EZH2 is overexpressed in several cancers. (J.N. Nichol, 2016)

7 Conclusion

This Master thesis provides insight into the clinical and biological relevance of EZH2 in two distinct melanoma subtypes, acral and mucosal melanoma.

Our current model demonstrates that EZH2 up regulation is common in ALM and MM. Furthermore, the findings in this manuscript support that high EZH2 expression is a poor prognostic feature underlining the particular role of protein levels in the tumor´s metastatic progression. We have demonstrated that there is a significant overall survival effect with EZH2 in acral melanoma.

This analysis may also shed light on significantly lower basal H3K27me3 levels in ALM and MM cell lines compared to other cutaneous melanoma cell lines despite high levels of EZH2, which supports the hypothesis that EZH2 has in part a histone methyltransferase-independent function in ALM.

In conclusion, we can confirm that EZH2 protein degradation may be a good strategy to pursue. A rational for inhibiting EZH2 in this tumor might be targeting the canonical and non-canonical function by using a degrader. Selective sensitivity of ALM cell lines to an EZH2 degrader supports the therapeutic potential of EZH2-targeted therapy in this histological subtype. Based on the observations that

Recapitulating, most probably a combination of several mechanisms, including somatic copy number alterations and a prolonged half-life might be the reason for EZH2 protein upregulation. In addition, our findings denote that there is a rational for using EZH2 combinational therapy and for genetically silencing the EZH2 via shRNAs.

8 Outlook

Our current work adds further layers of information to the epigenetic characteristics of EZH2 in non-sun-exposed melanomas. We demonstrate EZH2 to be a key player in promoting melanoma growth and progression to metastatic disease. In line with these findings, we identified a correlation between high EZH2 transcript levels with poor survival. Intriguingly, we elucidate the lack of H3K27me3, especially in the acral and mucosal samples. Hence, we focus on the non-canonical function of EZH2 by testing an EZH2 degrader and two EZH2 enzyme inhibitors. Accordingly, our study reveals that temporary pharmacological inhibition or degradation of EZH2 counteracted growth of the cells. In support of this, EZH2 inhibition and especially degradation might be promising strategies for future therapies of human acral and mucosal melanoma patients.

Future work could include an EZH2 targeting shRNA in a mouse xenograft. We would aim to create a mouse model with an inducible genetic knockdown of EZH2 to test if EZH2 degradation inhibits tumor growth in vivo. At first we want to inject this type of tumor into mice and let the tumor grow until it reaches a certain size. Then we would induce in vivo via feeding the mice with Doxycycline. The EZH2 degrader would be delivered by intraperitoneal injection. If we want to image in the mice we might need to add GFP to being able to show the protein expression. We would monitor the tumor size.

Importantly, CRISPR/Cas9 would be another approach to genetically knocking out the EZH2 protein.

Relatively few studies on EZH2 overexpression have been published. Therefore, an important next step would be to artificially express this gene in increased quantity and then treat the cells with the EZH2 degrader to identify its effect in contrast to normal cells.

Additionally, we could overexpress EZH2 Future work will aim to quantify the H3K27me3 and total H3 levels, respectively, for acral and non-acral cell lines with an ELISA assay. Also we could perform mass spec analysis of total H3 and H3K27me3 from selected acral and non-acral cell lines.

Another interesting approach could be targeting HSP90 by using an HSP90i. Hsp90 inhibition destabilizes the EZH2 protein.

Future Directions include experiments with Ki67, a proliferation mark. This nuclear protein that is necessary for cellular proliferation and associated with ribosomal RNA transcription. Its inactivation gives rise to inhibition of ribosomal RNA synthesis.

Moreover, we want to assert whether there exist other repressive marks than H3K27me3. If they are high, our results would be even more believable, since there must be repressive marks in a tumor. We could test this in the cell lines. The low levels of H3K27me3 are counter dogma and very impressive. Therefore we would need to proof it is true and believable by showing that there exist other suppressive marks.

Furthermore it will be of interest to do invasion experiments with 3D skin.

Novel therapeutic strategies effectively testing whether the MEK-ERK1/2-Elk-1 pathway leads to EZH2 overexpression could be very useful. This is what the literature suggests. (Fujii S, 2011)

In future studies we could look at tumors which are deficient in the SWI/SNF core component INI1 (SMARCB1), to test whether they are selectively killed by inhibitors of the H3K27 histone methyltransferase EZH2. (Chan-Penebre E, 2017)

In addition to the protein knock-down with DOX, we also could show the mRNA knock-down to show the independency of EZH2 from H3K27me3 in acral and mucosal melanoma. This can be shown in a few different ways, e.g. via EZH2 mRNA knockdown.

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