A ROLE FOR GENOME-NUCLEAR LAMINA INTERACTIONS IN THE ESTABLISHMENT OF RESISTANCE TO VEMURAFENIB IN BRAF-MUTANT MELANOMA

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A Role For Genome-Nuclear Lamina Interactions in the Establishment of Resistance to Vemurafenib in BRAF-Mutant Melanoma

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ABSTRACT

In the recent years, there has been a rapid development of new agents in the management of patients with metastasized melanoma. Targeted therapy with vemurafenib shows an improved response in comparison to conventional chemotherapy; however, its efficacy is limited in time as resistance inevitably occurs.

Several studies have investigated mutational mechanisms driving tumour resistance, however, these occur late in the course of treatment under selective pressure imposed by the drug. When performing short-term response curves on various tumour cell lines, it was discovered that a significant portion of cells survive the treatment at a dose that otherwise kills the majority of the population, suggesting that a nonmutational mechanism of resistance develops on the early stages of treatment. This subpopulation of surviving cells was termed “drug tolerant persisters” (DTPs). Clinical observations suggest that a similar situation happens in vivo, as relapses often occur on the sites of residual tumours.

In this study, our goal was to characterize the early drug-tolerant state and investigate a possible role for interactions between the genome and the nuclear lamina in the establishment of this state. For this, we attempted to create an in vitro model for DTPs in two cell lines – M026x1 and SK-MEL-28. We have also carried out DamID with LaminB1 on the DTP model established from one of our cell lines.

We were able to successfully establish DTPs and resistant cells for SK-MEL-28. Our findings suggest that upon treatment with vemurafenib, cells enter a state of cell cycle arrest, which can be reversed upon withdrawal of the drug. We have carried out tests for DamID infections and are currently optimizing conditions for the biological replicate, from which we will start preparing the samples for deep sequencing.
ACKNOWLEDGEMENTS

After this challenging project, I would like to express my gratitude to the people that helped me make this work possible. Firstly, I thank Professor Daniel Peeper, who gave me the opportunity to carry out my thesis project in his research group and my supervisor Dr Christelle Lenain for her sharing of knowledge and experience, constructive criticism and guidance during these past months.

I am grateful to Sirith Douma for her enthusiasm, willingness to help and assistance with cell culture and Western Blots. I would like to thank all other members of Daniel Peeper’s laboratory for sharing their expertise during group meetings, as well as people from the collaborating Bas van Stenseel’s group for providing constructs for the DamID experiments.

Thank you to my friends and family for supporting me throughout this whole semester. Last but not least, I would like to express my deepest gratitude to Mr Rudolph Talens for his guidance, feedback and encouragement from the beginning until the end.
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................................I

ACKNOWLEDGEMENTS .........................................................................................................II

TABLE OF CONTENTS ...........................................................................................................III

ABBREVIATIONS ......................................................................................................................IV

1. INTRODUCTION ..................................................................................................................1

2. THEORETICAL FRAMEWORK ............................................................................................3
   2.1. BRAF in melanoma ........................................................................................................3
   2.2. Mechanisms of Resistance .........................................................................................4
   2.3. Nuclear lamina and LADS .........................................................................................5
   2.4 DAMID .............................................

3. METHOD AND MATERIAL ....................................................................................................7
   3.1. Cell culture and drug treatments ................................................................................7
   3.2. BrdU incorporation and FACS .................................................................................7
   3.3 SA-b-GAL staining .....................................................................................................8
   3.4. Protein isolation and Western blot analysis ...........................................................8
   3.5. RNA isolation and RT-qPCR ..................................................................................9
   3.6. Immunofluorescence and confocal microscopy ......................................................10
   3.7. Generation of DamID lentiviruses ..........................................................................10
   3.8. Lentiviral transduction and induction of Dam proteins expression in SK-MEL-28 cells ..........................................................11
   3.9. DpnII assay ............................................................................................................11

4. RESULTS .............................................................................................................................13
   4.1. Establishment of DTPs and DTPPs .........................................................................13
   4.2. Characterization of DTPs and DTPPs .....................................................................15
   4.3. The drug-tolerant state-associated cell cycle arrest is reversible .........................17
   4.4. Nuclear lamina characterization and test of DamID infection and Dam-fusion protein expression ...........................................18

5. DISCUSSION .......................................................................................................................21

6. CONCLUSIONS ....................................................................................................................23

REFERENCES ...........................................................................................................................24
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>BRAF</td>
<td>v-raf murine sarcoma viral oncogene homolog B</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Dam</td>
<td>DNA adenine methyltransferase</td>
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<tr>
<td>DamID</td>
<td>DNA adenine methyltransferase identification</td>
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<tr>
<td>DH</td>
<td>drug holiday</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DTPs</td>
<td>drug tolerant persisters</td>
</tr>
<tr>
<td>DTPPs</td>
<td>drug tolerant proliferative persisters</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IC</td>
<td>inhibitory concentration</td>
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<tr>
<td>iLADs</td>
<td>inter lamina-associated domains</td>
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<tr>
<td>LADs</td>
<td>lamina-associated domains</td>
</tr>
<tr>
<td>LamB1/LMNB1</td>
<td>Lamin B1</td>
</tr>
<tr>
<td>LBR</td>
<td>Lamin B1 receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
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<tr>
<td>NE</td>
<td>nuclear envelope</td>
</tr>
<tr>
<td>NL</td>
<td>nuclear lamina</td>
</tr>
<tr>
<td>NT</td>
<td>non-treated</td>
</tr>
<tr>
<td>OIS</td>
<td>oncogene-induced senescence</td>
</tr>
<tr>
<td>PARP</td>
<td>poly ADP ribose polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>propium iodide</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>reverse-transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SA-β-GAL</td>
<td>senescence associated β-galactosidase</td>
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1. INTRODUCTION

The incidence of skin cancers such as melanoma, a malignant tumour of the melanocytes, is increasing [2, 3]. Due to growing public awareness of the earliest signs of skin melanomas, most cases nowadays can be cured surgically [4]. Nevertheless, the ability of melanomas to metastasise relatively quickly has earned the disease a reputation of one of the most dangerous and aggressive types of cancer, being associated with ¾ of all deaths caused by skin cancers [5]. The prognosis of patients with metastasised melanoma had long remained poor, the only standard treatment being dacarbazine, a DNA alkylating agent that achieved complete but short-lived responses in less than 5% of cases. In the recent years, however, there has been a rapid development of new drugs for managing patients with metastasised melanoma [6].

In 2002, large-scale sequencing of melanoma cell lines and primary tumours lead to the discovery of BRAF (HGNC:1097), an oncogene that is mutated in more than half of the cases [7]. Therapy targeting the transcribed protein, such as vemurafenib, had been developed and showed a clinical response in 48% of the patients during clinical trials [8]. The drug received FDA approval and is routinely prescribed for BRAF-mutant melanoma patients as of 2011 [9]. Nevertheless, the long-term prognosis of these patients still remains poor due to the inevitable development of resistance.

The proposed models of resistance mechanisms leading to targeted therapy failure fall into two groups. Late/acquired resistance is usually associated with the development of secondary mutations in the tumour. The main focus of this project, however, is the second group – early/adaptive resistance, that cannot be explained by mutations. It manifests when performing short-term response curves with vemurafenib in cultured melanoma cell lines, during which a significant portion of cells survive the treatment at a dose that otherwise kills the majority of the population [10]. This subpopulation of cells termed “drug tolerant persisters” (DTPs) [11] does not divide, eventually giving rise to proliferating clones called “drug-tolerant proliferating persisters” (DTPPs). Several studies point to the involvement of epigenetic mechanisms in the establishment of DTPs [11-14], leading to the hypothesis that a certain chromatin configuration and the resulting expression profile underlies the drug-tolerant state. One important aspect of chromatin organization and its influence on gene expression is the interaction of particular chromosomal regions with the nuclear lamina (NL). These regions called lamina-associated domains
(LADs) were previously identified and characterized by the research group of Bas van Steensel [15].

The research question of this project is the following: Is there a role for genome-nuclear lamina interactions in the development of early resistance to vemurafenib in BRAF-mutant melanoma?

There are two main goals of this project:
1. Determine whether there are any differences in the genome-nuclear lamina interactions between the parental cell line, DTPs and DTPPs.
2. Explore the relationship between changes in genome-nuclear lamina interactions and changes in gene expression that may occur during these transitions.

For my graduation period, I have only concentrated on the first goal due to time constraints. My main objectives were the following: (a) to establish a model for DTPs and DTPPs in vitro; (b) to characterize the generated DTPs and DTPPs; and (c) to optimize the DamID technique with LaminB1 in the parental cell line, the DTPs and the DTPPs.
2. THEORETICAL FRAMEWORK

2.1. BRAF in Melanoma

It has long been established that the melanomas comprise a group of biologically distinct subtypes that differ in various aspects, including histology, anatomy and genetics [16]. The first classification system, proposed by Wallace Clark and colleagues [17], distinguished melanoma subtypes based on factors such as histopathology, anatomical site, and degree of sun damage. It is still regularly applied in clinical practice [18], however, the advent of next-generation sequencing technologies has resulted in the discovery of crucial somatic alterations underlying melanomagenesis, leading to a revolution in the management of melanoma patients.

One such recent development is targeted therapy with BRAF inhibitors. In 2002, Andrew Futreal and colleagues [7] have carried out a large-scale sequencing of DNA from various melanoma tumours and cell lines, finding that in around 70% of the cases, one gene carried gain-of-function mutations. This gene was BRAF (HGNC:1097), which encodes serine/threonine kinase that acts in the mitogen-activated protein kinase (MAPK) pathway to transduce pro-proliferative signals. Normally, the kinase forms homodimers, which then lead to phosphorylation and activation of components downstream the pathway. However, an activating mutation in the gene, most commonly a substitution of glutamic acid for valine at position 600 (V600E), results in the BRAF kinase being constitutively active as a monomer, stimulating the pathway regardless of signals from the environment (FIG.1).

![FIG.1: Schematic of the MAPK pathway showing differential signaling in oncogenic (left) and physiological (right) conditions. Adapted from Sullivan et Flaherty, 2013 [1].](image-url)
BRAF\textsuperscript{V600E} can transform cells in the context of additional genetic lesions [19]. However, it should be noted that the activating mutation of BRAF alone is not sufficient to induce malignant transformation. In 2005, the research group of Daniel Peeper has shown that many melanocytic nevi carry the activating mutation, all the while not leading to tumour development [19]. This phenomenon was attributed to a tumour suppressing mechanism known as oncogene-induced senescence (OIS) – a state of permanent cell cycle arrest during which the cell is still able to perform its basic functions, the full mechanics and causal pathways of which still remain poorly understood [20]. It was also later shown by the research group of Daniel Peeper that a secondary somatic alteration, such as loss of tumour suppressor PTEN, leads to melanocytes evading senescence and progressing to melanoma [21]. In addition, it was shown that RNAi-mediated inhibition of BRAF\textsuperscript{V600E} in melanoma cell lines carrying this mutation results in impaired proliferation and apoptosis [22], indicating that melanoma cells are addicted to the BRAF\textsuperscript{V600E} oncogene.

On the basis of this dependency of melanoma on mutant BRAF, several agents targeting the mutated BRAF kinase have been developed. Amongst them, BRAF inhibitors such as vemurafenib (specific for the V600E variant) and dabrafenib (which targets the V600K variant in addition) have shown a greater and faster response in a larger portion of patients during clinical trials when compared to conventional chemotherapy [8, 23]. As a result, vemurafenib received FDA approval in 2011 and since then is routinely administered to patients with BRAF melanoma [9]. Dabrafenib received FDA approval in 2013 as a single-agent and in 2014 as in combination with trametinib (which targets MEK, a kinase downstream the MAPK pathway) [24, 25]. Unfortunately, the efficacy of these targeted therapies is limited in time – after several months of response, the tumours become resistant.

2.2. Mechanisms of Resistance

There have been multiple studies investigating the possible mechanisms of resistance, most of them focused on the development of somatic mutations [26-28]. By analysing whole-exome sequencing data from multiple patient samples, Roger Lo and colleagues [26] have determined that the most common mechanism of acquired resistance is the reactivation of the MAPK pathway throughout mutations in components upstream and downstream of BRAF. The second most common mechanism was mutations resulting in the activation of the PI3K–PTEN–AKT pathway, which also transduces growth and proliferation signals. Different resistance mechanisms may arise within different tumours of the same patients and even multiple resistance mechanisms within one tumour are possible.
What the described resistance mechanisms have in common is that they arise late in the course of treatment. However, when performing short-term response curves in cultured melanoma cell lines, it was discovered that a significant portion of cells survive the treatment even at high doses that otherwise kills the majority of the population, suggesting that a non-mutational mechanism of resistance occurs on early stages of treatment [10]. These findings parallel those observed in patients, where the response to the drug is partial and relapses often occur on sites of residual tumours.

The phenomenon of a subpopulation of cells being irresponsive to high drug doses was first noted by Settleman and colleagues [11] for a lung cancer cell line in response to EGFR inhibitors. They were also the ones to coin the term "drug-tolerant persisters" (DTPs) after similar observations in various other cell lines, including melanoma. Most DTPs are in cell cycle arrest, however, a fraction of them eventually resumes normal proliferation in the presence of drug, yielding colonies of cells referred to as "drug-tolerant proliferative persisters" (DTTPs). After prolonged culturing in a drug-free media, DTPs reacquire sensitivity, meaning the resistance state is reversible. The study was also the first to suggest the involvement of epigenetic mechanisms in the formation of DTPs – in a series of experiments, inhibition of HDAC activity led to failure of development of DTPs and treatment of already established DTPs with HDAC inhibitors resulted in rapid cell death. The group also showed that expression of histone demethylase RBP2/KDM5A/Jarid1A is necessary for the establishment of the drug-tolerant state. In another study, Herlyn and colleagues described a subpopulation of cells in melanoma that express Jarid1B, a H3K4 demethylase [14], that displays lower sensitivity to several drugs, including vemurafenib [29].

2.3. Nuclear Lamina and LADs

The studies described above point to a role for changes in histone post-translational modifications during the establishment of early resistance. We formulated the hypothesis that, next to histone mark alterations, changes in the spatial organization of chromatin may also contribute to the establishment of the early drug-tolerant state. A particular aspect of chromatin organization is the interaction of large genomic regions with the nuclear lamina. The nuclear lamina, located on the inner membrane of the nuclear envelope, is a thin meshwork of filament protein named the nuclear lamins. Lamins consist of two classes – A-type (comprising Lamins A, C, C2 and AΔ10) and B-type (comprising Lamins B1, B2 and B3) [30]. By carrying out DamID with Lamin B1, Bas van Stenseel's group has identified elements of the genome anchored to the nuclear lamina – lamina-associated domains (LADs) [15]. These 0,1-
10 MB long domains are distinctly demarcated by CpG islands or CTCF and characterized by low levels of gene expression, representing a repressive chromatin environment [15]. In agreement with this, artificial integration of reporters to the NL result in their repression [31]. Moreover, genes located in LADs become derepressed once positioned away from the nuclear lamina [32, 33]. Regions of the genome not anchored to the nuclear lamina are termed inter-lamina associated domains (iLADs). In comparison to LADs, iLADs were found to be enriched for H3K4me2, a histone mark associated with active transcription [15]. As this mark is removed by the histone demethylases Jard1A and Jarid1B [34], we speculated that the previously described upregulation of these histone demethylases in DTPs could lead to depletion of H3K4me2 from iLADs, possibly affecting their genome-NL binding patterns. This may result in a distinct expression profile, possibly contributing to the establishment of the adaptive resistance phenotype.

### 2.4 DamID

In order to investigate whether our speculations hold true, we would first need to investigate whether there are any alterations in genome-NL interactions to begin with. We will do this by carrying out DamID – a technique originally developed by Bas van Stenseel in 2000 [35] that is used for genome-wide mapping of protein-DNA interactions in vivo [36]. A full description of the principle may be found in articles by Greil et al [37] and Vogel et al [36]. In short, DamID employs a fusion protein consisting of the protein of interest, deoxyadenosine (Dam) methylase and a spacer linking the together. This construct is introduced into cells, where the protein of interest binds to certain locations in the genome, while the fused Dam methylates adenosine in GATC sequences located nearby. Since adenosine methylation is a modification not present in most eukaryotes [38], the Dam-methylated fragments can be selectively amplified from isolated genomic DNA and identified by next-generation sequencing. In order to correct for the intrinsic binding capacity of Dam, infection with a construct containing Dam alone is performed in parallel. In order to achieve controllable levels of expression, an inducible system called ProteoTuner was used [39]. In this system, a destabilising domain targeting the fusion protein for degradation by proteasome is added to the Dam-fusion protein. Under normal conditions, the fusion protein is mostly degraded. However, treatment with DNA Shield™, a molecule that binds the destabilizing domain, prevents targeting to the proteasome, resulting in increased abundance of the fusion protein and hence higher methylation levels.
3. METHOD AND MATERIAL

3.1. Cell Culture and Drug Treatments

For the establishment of DTPs and DTPPs, we used cell lines M026x1 and SK-MEL-28. Cells were cultured in DMEM (Gibco) supplemented with 9% FBS (Sigma) and refreshed with medium containing vemurafenib (Selleckchem) at the indicated doses every 3 to 4 days. Non-treated parental cells were maintained in parallel. For harvests, non-treated cells and M026x1 DTPs were trypsinised and re-seeded at indicated densities the day before. SK-MEL-28 DTPs were harvested without prior set-up, excluding the final harvest of the established DTPP cell line.

For the drug holiday experiment, cells were washed once with PBS and put on medium without vemurafenib. For harvests, the drug holiday cells were trypsinised and re-seeded at indicated densities the day before.

For the proliferation curve of the vemurafenib-treated cells, cells were seeded at 0.5 \( \times 10^6 \) cells (M026x1) or 0.6 \( \times 10^6 \) cells (SK-MEL-28) per 6 cm and treated with vemurafenib at the indicated doses. On the indicated days, cells were harvested by trypsinisation and counted using the CASY Model TT Cell Counter and Analyser (Roche).

For the dose response curves, samples were seeded in 96-well plates at 2500 cells (SK-MEL-28 NT, DTPPs) or 3500 cells (M026x1) per well. The next day, a ten-point threefold dilution series of vemurafenib, ranging from concentrations of 0.003 to 50 \( \mu \)M, was added. PAO at a concentration of 20 \( \mu \)M and medium without vemurafenib were used as controls. Viability was assessed after 3 days of vemurafenib treatment using the CellTiter-Blue® Cell Viability Assay (Promega) and the TECAN Infinite M200 scanner. The fluorescent signal from PAO and medium without vemurafenib were used for normalization against 0% viable cells and 100% viable cells respectively.

Pictures of cells were taken on the Zeiss Axio Vert.A1 microscope using the 20X objective and AxioVision Rel. 4.8.2 software.

3.2. BrdU Incorporation and FACS

BrdU/PI staining is an assay used for quantification of the percentage of cells in different phases of the cell cycle. Bromodeoxyuridine (BrdU) is a synthetic nucleoside analogous to thymine that incorporates only in cells that replicate their DNA, while as propium iodine (PI) stains the DNA of the whole cell population and is used to quantify cells in G1 and G2.
One day prior to labelling, cells were set-up at $0.5 \times 10^6$ cells (M026x1 all conditions), $0.6 \times 10^6$ cells (SK-MEL-28 parental; drug holiday) or $0.67 \times 10^6$ cells (SK-MEL-28 DTPPs) per 6 cm dish. Cells were refreshed with medium supplemented with 10 µM BrdU (Roche) and incubated for three hours at 37°C. Then, the cells were harvested by trypsinisation, fixed with 70% ethanol and stored at 4°C. Staining was done simultaneously for samples from all time points of one experiment. Firstly, cells were spun down and the supernatant removed. The pellet was resuspended in 200 µl of 0.5 mg/ml RNaseA in PBS and incubated for 30 minutes at 37°C. After removal of RNaseA, the DNA was denatured by 20 minutes of incubation in 2M HCl, 0.2% Triton X-100 solution, followed by neutralization with 1M Tris pH 7.5. After one wash with PBS-Tween 0.2% and centrifugation, the pellet was resuspended in PBS-Tween 0.2% containing 1% BSA and anti-BrdU antibody (DAKO) at a 1:40 dilution and incubated for 30 minutes. The cells were washed twice with PBS-Tween 0.2% and incubated for 30 minutes with PBS-Tween 0.2% containing 1% BSA and FITC-conjugated anti-mouse IgG (DAKO) at a 1:20 dilution. After two final washes with PBS-Tween 0.2% and centrifugation, the cell pellets were resuspended in 150 µl of PBS containing 20 µg/ml PI. The cell suspension was transferred to tubes for fluorescence-activated cell sorting and kept at 4°C in the dark until further analysis.

Samples were analysed at the NKI FACS facility on the BD Biosciences FACSCalibur flow cytometer using BD CellQuest™ Pro software.

### 3.3 SA-β-GAL Staining

Cells were seeded on collagen-coated slides (Millicell® EZ SLIDES) at a density of $3 \times 10^4$ (M026x1 all conditions), $2 \times 10^4$ (SK-MEL-28 parental; DTPs; drug holiday) or $2.375 \times 10^4$ (SK-MEL-28 DTPPs) cells per well. The following afternoon, samples were stained using the Senescence β-Galactosidase Staining Kit (Cell Signalling) according to instructions provided by the manufacturer.

Samples were imaged in the digital microscopy facility on the Zeiss Axiovert S 100 microscope using a 20X phase contrast objective and AxioVision Rel. 4.8.2 software.

### 3.4. Protein Isolation and Western Blot Analysis

Cells were seeded at $1.5 \times 10^6$ (M026x1 all conditions), $1.8 \times 10^6$ (SK-MEL-28 parental; drug holiday) or $2 \times 10^5$ (SK-MEL-28 DTPPs) cells per 10 cm dish. The following day, cells were washed and harvested in 1 ml cold PBS by scraping with rubber policemen. After centrifugation, the pellets were snap frozen in dry ice with
ethanol and stored at -80°C. Protein isolation was done simultaneously for samples from all time points of one experiment. Pellets were suspended in RIPA buffer (50 mM Tris-HCl Buffer pH 7.5, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS; phosphatase and protease inhibitor cocktails added freshly). After 30 min of incubation on ice, the cells were lysed by 1 minute of sonication at 4°C, the lysates cleared by centrifugation. The concentration of protein was determined using the Bradford assay (Bio-Rad). Samples were then brought to the same concentration by adding the appropriate amount of RIPA and NuPAGE® LDS Sample Buffer (Novex) supplemented with 2.5% β-mercaptoethanol. Samples were boiled prior to loading.

For the Western Blot, a sample volume corresponding to 20 µg of total protein was separated on 4-12% NuPAGE® Bis-Tris Precast Gels (Novex) and transferred to nitrocellulose membranes (Whatman, Pall Corporation). Staining with Ponceau S (Sigma) was done in order to assess equal loading, the dye then washed off with PBS-Tween 0.2%. The membranes were blocked for 45 minutes at room temperature in 4% non-fat dry milk in PBS-Tween 0.2% and subsequently incubated overnight at 4°C with primary antibodies diluted 1000 times in blocking solution. The following day, membranes were washed several times with PBS-Tween 0.2% and incubated for 30 minutes at room temperature with HRP-conjugated secondary antibodies diluted 7500 times in blocking solution. After washing off the secondary antibody, the blot was revealed by using ECL.

The primary antibodies used were against: MEK (4694S, Cell Signalling), MEK Ser217/221 (41G9) phosphorylated (9154S, Cell Signalling), ERK (9102S, Cell Signalling), p-ERK Thr202/Tyr204 phosphorylated (9106S, Cell Signalling), PARP (95425, Cell Signalling), p15 (sc-612, Santa Cruz), p16 (sc-56330, Santa Cruz), p21 (sc-397, Santa Cruz), Lamin B1 (ab16048, Abcam), Lamin A/C (sc-6215, Santa Cruz), Emerin (10351-1-AP, Proteintech), Lamin B1 Receptor (ab122919, Abcam) and HSP 90α/β (sc-7947, Santa Cruz) or β-actin (A5316, Sigma) as a loading control.

3.5. RNA Isolation and RT-qPCR

M026x1 cells were seeded at 1,5 × 10⁶ cells per 10 cm dish. The following day, cells were washed with PBS, harvested in 3 ml TRIzol® Reagent (Invitrogen) and stored at -20°C. RNA isolation was carried out simultaneously for samples form all time points according to instructions provided by the manufacturer.

The concentration of RNA was determined by measuring on the NanoDrop 2000 (Thermo Scientific). For cDNA synthesis, 0.6 µg of sample was reverse transcribed using the SuperScript™ II Reverse Transcriptase and the First-Strand
Synthesis System (Invitrogen) according to instructions provided by the manufacturer.

For the qPCR, the reaction mixture consisted of 1X SensiFAST SYBR Hi-ROX Mix (Invitrogen), 1 mM primers (sequences can be found in table 1 of the supplementary materials), 60 ng of the reverse-transcript and water to 10 µl. The thermal cycler program consisted of pre-incubation at 96°C for 2 minutes, followed by 40 amplification cycles (melting at 96°C for 15 seconds, primer annealing and elongation at 60°C for 30 seconds) and a melting curve from 60°C to 96°C. Samples were run on StepOnePlus™ (Applied Biosystems), using provided software for analysis. Quantification was done using the delta-delta-Ct method, normalising values to RPL13.

3.6. Immunofluorescence and Confocal Microscopy

M026x1 was seeded on collagen-coated slides (Millipore EZ SLIDES) at a density of 3 × 10⁴ cells per well. The following day, cells were washed with PBS, fixed with 2% formaldehyde in PBS and stored at 4°C in PBS with 0.02% sodium azide until staining. For staining, the cells were first permeabilised with 0.5% Triton X-100 in PBS for 10 minutes. After once washing with PBS-Tween 0.2%, cells were blocked for 30 minutes in PBS-Tween 0.2% supplemented with 5% FBS. Afterwards, cells were incubated for one hour in Lamin B1 antibody (ab16048, Abcam) at a dilution of 1:300 in blocking solution, washed three times and incubated for 30 minutes with a Alexa Fluor® 488 goat anti-rabbit antibody (Invitrogen) at a 1:500 dilution in blocking solution. After two washes, the cells were stained with DAPI (1:5000 dilution in PBS-Tween 0.2%) (Invitrogen) for 5 minutes, the slides then mounted with VECTASHIELD® (Vector Laboratories).

Stained slides were imaged at the digital microscopy facility on a Leica TCS SP5 II Confocal microscope using the 63X oil immersion objective and LAS AF software. Images were acquired as Z-stacks using sequential scanning mode of excitation wavelengths 405 nm and 488 nm.

3.7. Generation of DamID Lentiviruses

Expression plasmids pCCL-hPGK-DD-Dam-V5/Wpre (DD-Dam) and pCCL-hPGK-DD-Dam-V5-hLMNB1/Wpre (DD-Dam-LMNB1) were used for the DamID experiments. The production of lentiviruses was performed in the HEK293T packaging cell line using Lipofectamine 2000 (Invitrogen). Cells were seeded at 3,3 × 10⁶ cells per 10 cm dish. Twenty four hours later, cells were transfected with a mix of 1,6 ml Opti-MEM (Gibco) containing 0,3 µg/ml of an EGFP-encoding plasmid (used
as a transfection efficiency marker), 8 µg of DamID expression plasmid, 3 µg of each of the packaging plasmids (pKRev, pMD2.VSVG and pMDG) and 30 µl of lipofectamine. These were prepared in two separates mixes: the first consisted of Opti-MEM with all the plasmids and the second consisted of Opti-MEM with lipofectamine. The two mixes were then combined. After incubation for 20 minutes at room temperature the transfection mix was added drop wise to the cells. The dishes were then incubated overnight at 37°C and refreshed the following morning with 10 ml of DMEM (Gibco) supplemented with 9% FBS (Sigma). Harvests were done at two time points - around 48 and 64 hours after transfection. The medium containing virus was collected with a syringe, filtered through 0.45 µm filters (Whatman) in order to remove cell debris and distributed in 1.5 ml cryotubes. The virus was then snap frozen in dry ice with ethanol and stored at -80°C.

3.8. Lentiviral transduction and induction of Dam proteins expression in SK-MEL-28 cells

At days 4, 18 and 53 after vemurafenib treatment, non-treated and treated cells were transduced with DamID lentiviruses. The day prior to transduction, cells were seeded at 0.2 ¥ 10^6 (non-treated) or 0.5 ¥ 10^6 (DTTPs) cells per 6 cm dish. For the first two time points, DTPs that had been seeded at 0.6 ¥ 10^6 cells per 6 cm dish the day before the start of the treatment were transduced without prior set-up. Cells were infected overnight with the DamID viruses at indicated dilutions in medium supplemented with polybrene at 8 µg/ml. For DTP and DTPP cells, infection was performed in the presence of 3 µM vemurafenib. The next morning, the cells were refreshed using fresh medium with or without Shield™ (Zymo Research) at 0.5 µM. After 24 hours, the cells were washed, harvested in 1 ml PBS by scraping, pelleted and resuspended in 600 µl of lysis buffer. Samples were stored at 4°C until DNA isolation.

3.9. DpnII Assay

The DpnII assay is a PCR-based method for quantifying the amount of A^6-methylation in cells infected with the DamID constructs [35]. The principle is the following: genomic DNA is digested with DpnII, a restriction enzyme that cleaves the sequence only when the A in the GATC restriction site is unmethylated. A real-time quantitative PCR is then carried out using primers that amplify LAD or iLAD loci containing a GATC site. DNA with unmethylated GATC sites gives no product due to cleavage, while a methylated GATC site is protected from DpnII digestion and will therefore be amplified. Thus, the amount of product positively correlates with the amount of methylation.
The procedure used was as follows. First, genomic DNA was isolated using the Gentra® Puregene® Kit (Qiagen) according to instructions provided by the manufacturer. The concentration was determined by measuring on the NanoDrop 2000 (Thermo Scientific). For the digest, 0.9 µg per sample was taken, diluted with water and NEBBuffer DpnII (New England Biolabs) to a total volume of 75 µl. The volume was then separated into two equal fractions and 1 µl of the DpnII enzyme (New England Biolabs) was added to one of the fractions (“+”), while the other fraction remained non-digested (mock samples, “−”) and the samples were incubated at 37°C for 4 hours.

For the qPCR, the reaction mixture consisted of 1X LightCycler® 480 SYBR Green I Master (Roche), 0.15 mM primers (sequences can be found in table 2 of the appendices), 25 ng of digest and water added to a total volume of 10 µl. The reaction was run on the LightCycler® 480 Instrument II (Roche). The thermal cycler program consisted of pre-incubation at 95°C for 5 minutes, followed by 45 amplification cycles (melting at 95°C for 10 seconds, primer annealing at 60°C for 10 seconds, elongation at 72°C for 10 seconds), a melting curve from 65°C to 97°C during 1 minute and finally cooling down to 40°C. Quantification was done using the following. First, the Ct values were normalised to the ones of an amplicon containing no GATC sites (LAD17). The percentage of methylated DNA was calculated using the formula

\[ \%A^6\text{methylated} = 2^{-(\Delta\text{Ct}^{\text{+ fraction}} - \Delta\text{Ct}^{\text{- fraction}})} \]
4. RESULTS

4.1. Establishment of DTPs and DTPPs

For the first experiment, we tested M026x1, a BRAF-mutant cell line established from a patient-derived xenograft. We choose this cell line that has been maintained in mice and only shortly exposed to in vitro conditions in order to minimize the artefacts resulting from long-term culturing. In previous studies, DTPs and DTPPs had been generated using a vemurafenib concentration ranging from IC$_{70}$ and IC$_{90}$ [12, 40]. Based on our short-term response curves to vemurafenib (FIG.2A), we decided to treat the cells with a dose of 10 μM, which corresponds to the IC$_{70}$ value. However, on day 17 the vemurafenib concentration was lowered to 3 μM in order to exclude possible high-dose off-target effects that could interfere with the development of resistant clones (FIG.2B). We followed the dynamics of viable cell numbers during vemurafenib treatment by counting the cells twice a week. Within a few days, the cell number started to decline and then stabilized between day 11 and day 18, corresponding to the emergence of DTPs (FIG.2C). We noted that the DTPs adopted an altered morphology, becoming larger and flatter (FIG.2D). Despite the dose reduction, no proliferative clones emerged. On the contrary, the cell number started to decline again after day 20 (FIG.2C). We attribute the inability of M026X1 to form DTPPs to their initial exposure to an exceedingly high drug concentration and the resulting cellular stress. As a result, the experiment was stopped on day 42.

FIG.2 Establishment of DTPs from cell line M026x1. (A) M026x1 vemurafenib dose-response curve based on three biological replicates. (B) Timeline of the experiment. (C) Proliferation curve of vemurafenib–treated M026X1. Until day 21, cells were counted twice per week. Then, cells were counted once per week up until day 42. (D) Microphotographs of M026X1 taken at day 28 and day 14 after vemurafenib addition.
For the second experiment, we chose SK-MEL-28, one of the most commonly used BRAF-mutant melanoma cell lines. A derivative cell line from SK-MEL-28 that is resistant to PLX4720, a precursor of vemurafenib, has been previously generated in the lab, suggesting the suitability of SK-MEL-28 for establishment of both DTPs and DTPPs. SK-MEL-28 cells were plated and treated with vemurafenib or left untreated. Cells were harvested for assays at different time points (FIG.3A). Based on short-term response curves to vemurafenib (FIG.3B), we initially applied a dose of 6 µM (corresponding to IC<sub>70</sub>) to the treated cells. Similar to the first experiment, the dose
was lowered to 3 µM on day 17 in order to exclude possible high-dose off-target effects that could interfere with the development of resistant clones (FIG.3A). We followed the dynamics of viable cell numbers during vemurafenib treatment by counting the cells once a week. We observed an initial decrease in cell number followed by a plateau, corresponding to the emergence of DTPs (FIG.3C). Eventually, on day 26, we started to observe the first proliferative colonies (FIG.3D). In order to generate a homogenous DTPP population, we then propagated these cells for several more weeks, carrying out the final harvest and DamID infection after 53 days of treatment. In order to ascertain the resistance of the DTPPs to vemurafenib, we performed a dose response curve for vemurafenib. As shown in FIG.3E, DTPPs were clearly resistant to vemurafenib as manifested by a 65-fold increase in the IC50.

### 4.2. Characterization of DTPs and DTPPs

Since little is known about the features of cells in the drug-tolerant state, we carried out a series of experiments in order to better characterize these subpopulations. We first probed the MAPK pathway activation status by WB using phospho-specific antibodies to MEK1/2 and ERK1/2 (FIG.4A). As expected, vemurafenib induced a decrease in p-MEK1/2 in both cell lines. p-ERK1/2 was clearly decreased in M026x1, however, for SK-MEL-28 the results are inconclusive due to absence of signal from total ERK in DTPs. It should be noted that a rebound

![FIG.4 Characterization of DTPs of M026X1 and SK-MEL-28.](image-url) (A) Western Blot analysis for the components of the MAPK pathway on lysates from M026X1 (left panel) and SK-MEL-28 (right panel) in the presence or absence of vemurafenib harvested at indicated time points. Hsp90 and β-actin used as loading controls. (B) BrdU incorporation analysis of M026X1 (left panel) and SK-MEL-28 (right panel) in the presence or absence of vemurafenib labeled and fixed at indicated time points (n = 10000). (C) Western Blot analysis for apoptotic markers on lysates from M026X1 (left panel) and SK-MEL-28 (right panel) in the presence or absence of vemurafenib at indicated time points. Hsp90 and β-actin used as loading controls.
of both p-ERK and p-MEK was observed at day 21 in the M026x1, which could be explained by the vemurafenib dose reduction. In SK-MEL-28, the emergence of DTPPs was associated with reactivation of p-MEK and p-ERK. In order to determine the cause of the decrease in cell number, we performed cell cycle analysis by BrdU/PI staining followed by FACS. In both M026X1 and SK-MEL-28, vemurafenib led to a proliferative arrest as manifested by a drop in BrdU incorporation (FIG.4B) and an accumulation in G1 (data not shown). Immunoblotting for the apoptotic markers revealed no apparent induction of cleaved PARP in M026x1, however, we observed a signal for the SK-MEL-28 sample of day 4 after treatment (FIG.4C). We therefore conclude that the main response of M026x1 to MAPK-pathway inhibition is cell cycle arrest, while as in SK-MEL-28, both cell cycle arrest and apoptosis take place.

In order to characterize further the nature of cell cycle arrest induced by vemurafenib, we assessed several established senescence markers. We noted a marked increase in SA-β-GAL activity in both M026x1 and SK-MEL-28 upon vemurafenib treatment (FIG.5A). However, Western Blot analysis showed no induction of the tumour suppressors p21 and p15 (FIG.5B). On the contrary, their expression is reduced upon vemurafenib treatment in DTPs and returns to the level of before treatment in SK-MEL-28 DTPPs. We didn’t detect any signal for tumour suppressor p16, which we attribute to the frequent loss of the coding locus in melanoma. In addition, RT-qPCR analysis failed to detect a consistent induction of the inflammatory factors IL-6, IL-8 and CEBPβ (FIG.5C). SA-β-GAL activity can also be induced under conditions that result in quiescence, such as contact inhibition and

![FIG.5 Evaluation of Senescence Biomarkers in M026X1 and SK-MEL-28 DTPs.](image)

A) SA-B-GAL staining of M026X1 (left images) and SK-MEL-28 (right images) at day 4 after vemurafenib addition. Numbers indicate percentages of SA-B-GAL positive cells (n = 300). (B) Western Blot for p21, p16, p15 on whole cell lysates of M026X1 (left panel) and SK-MEL-28 (right panel) at indicated time points after vemurafenib additions. Hsp90 and β-actin used as loading controls. The absence of signal from p16 can be explained by the loss of the coding INK4A locus, which is frequent in melanoma. (C) Inflammatory network genes RT-qPCR for M026x1 samples. Fold change indicated in relation to non-treated samples of the same day.
serum starvation [20]. Therefore, our results suggest that vemurafenib DTPs cells are in quiescent state.

4.3. The drug-tolerant state-associated cell cycle arrest is reversible

To further confirm that the observed cell cycle arrest in DTPs corresponded to quiescence, we asked whether it could be reversed upon removal of vemurafenib. For this, SK-MEL-28 DTPs that had been exposed to vemurafenib for 19 days were subjected to drug holiday (FIG.6A), and cells were harvested 6 and 13 days after drug removal. The proliferation curve (FIG.6B) showed that DTPs started to resume proliferation a week after drug removal, with the proliferation rate further increasing by day 15 of DH. Accordingly, BrdU incorporation analysis showed an increased in the percentage of S-phase cells in DH cells when compared to cells maintained on vemurafenib (FIG.6C). This was associated with a morphological change - for the first six days of drug holiday, the cells adopted a fibroblast-like morphology, eventually becoming more morphologically similar to the parental cell line after two weeks of drug holiday (FIG.6D). These results confirm that treatment with vemurafenib induces quiescence in cells that can be reversed upon withdrawal of the drug.

![FIG.6 The drug tolerant state associated cell cycle arrest is reversible.](image)

(A) Timeline of the experiment. In brackets, the amount of days after initial vemurafenib addition is indicated. (B) Proliferation curve of SK-MEL-28 DTPs over two weeks after vemurafenib removal. (C) BrdU incorporation analysis of SK-MEL-28 DTPs labeled and fixed at indicated time points (n = 10000). In brackets, the day after drug removal is indicated. (D) Microphotographs of SK-MEL-28 DTPs taken at the indicated time points after drug removal.
4.4. Nuclear lamina characterization and Test of DamID Infection and Dam-fusion protein expression

We first assessed the expression status of lamins and the inner nuclear membrane proteins. Compared to the parental cells, M026x1 DTPs displayed an increased expression of lamin B1, lamin A/C and emerin, while LBR was downregulated (FIG. 7A, left panel). In contrast, SK-MEL-28 DTPs displayed a downregulation of lamin A/C, emerin and LBR when compared to the parental cell line, while as lamin B1 levels appeared constant throughout the course of the treatment (FIG.7A, right panel). SK-MEL-28 DTPs showed NE protein expression levels comparable to those of the parental cells (FIG.7A, right panel). Currently, we do not know the causal mechanism for these changes and cannot explain why the direction of these changes differs between cell lines. For M026x1, we have also performed immunofluorescence staining followed by confocal microscopy in order to assess the localization of lamin B1. In comparison to the non-treated cells, we didn’t observe any increase in nuclear morphology alterations and no apparent change in lamin B1 localization in the DTPs on day 4 of treatment (FIG.7B). We did see increase in nuclear malformations on samples from the following time points. However, we attribute these findings to the high level of stress experienced by the cells rather than the drug-tolerant state.

![Western Blot analysis](image)

**FIG. 7 Characterization of the Nuclear Lamina** (A) Western Blot analysis for nuclear envelope proteins on lysates from M026X1 (left panel) and SK-MEL-28 (right panel) in the presence or absence of vemurafenib and harvested at indicated time points. Hsp90 and β-actin used as loading controls. (B) Immunofluorescence staining for lamin B1 on M026x1 samples fixed at day 4 after vemurafenib addition.
Then, we tested the DamID viral transduction and induction. The infections

**FIG. 8** Test of DamID lentiviral infection and expression of Dam proteins (A) Scheme displaying timeline and conditions of DamID infections. The virus dilution is stated in brackets. (B) Principle of the DpnII assay. (C) Assessment of the amount of proviral integration by qPCR for the hPGK promoter. Values are normalised to those of non-infected samples. Data from sample of day 20 vemurafenib Dam with Shield is missing. (D) qPCR results of the DpnII assay for SK-MEL-28 samples in presence or absence of vemurafenib harvested on the indicated days after treatment. Data from sample of day 20 vemurafenib Dam with Shield is missing.
Then, we tested the DamID viral transduction and induction. The infections were carried out in parallel with both non-treated and vemurafenib treated SK-MEL-28 cells on the same days as the harvests. For the first two time-points, cells were infected with DD-Dam and DD-Dam-LamB1 both at a dilution of 1:4 (FIG.8A). After harvesting the DNA from samples of the second time-point, we quantified the amount of methylation at a few loci by performing a DpnII assay (FIG.8B) for 2 LADs (LAD2, LAD8,) and 1 iLAD (UBE2B) (FIG.8D). We could detect significant amount of methylation in uninduced conditions, indicating leaky expression of the Dam proteins. However, the levels of methylation were further increased in the presence of Shield, indicating that the proteotuner system works. In Dam-LamB1 infected cells, the levels of methylation at the tested LADS were higher than at the UBE2 iLAD (FIG.8D). We saw a significant decrease in the activity of both Dam and Dam-LamB1 constructs in vemurafenib treated cells. In order to see whether this was attributable to a difference in infection efficiency, we quantified the amount of proviral integration by qPCR for the hPGK promoter of the construct (FIG.8C). We saw that the DD-Dam virus had around ten times more copies integrated than DD-Dam-LamB1 and that the non-treated cells had exceedingly more copies of both viruses when compared to vemurafenib cells. It is clear that although lentiviruses are able to infect non-dividing cells, they are less effective in doing so. We conclude from this that we need to optimize the amount of virus used to infect the DTPs in order to achieve levels of methylation comparable to non-treated cells. For this, we will test several virus dilutions in the biological replicate of the experiment.
5. DISCUSSION

In this study, we have achieved temporarily drug-tolerant state in M026x1 and failed to generate resistant proliferating clones from this cell line. It is possible that the cells could not proliferate due to undergoing stress from a too sparse seeding resulting from the toxicity of vemurafenib. Perhaps, a lower dose of vemurafenib and a higher initial cell density could improve the outcome, should the experiment be repeated. However, we were able to establish DTPs and DTPPs for SK-MEL-28. The first colonies of DTPPPs were observed on day 26 and the last harvest was done four weeks later, once we were able to split the cells twice per week and to document an increase in resistance to vemurafenib. It should be noted, however, that there are no certain rules on when to claim a cell line resistant. In other studies [12, 40], DTPPs were obtained after around one and a half month and, in the case of one cell line [12], even 15 days. However, the exact criteria by which these cells were claimed resistant were not elaborated upon. Upon further culturing of the SK-MEL-28 DTP cells, we observed a further increase in their vemurafenib IC50 and proliferation rate. We hypothesised that this may be due to DTPPPs initially consisting of several subpopulations arising from the various colonies that have developed differing resistance mechanisms. Eventually one subpopulation, which has the highest growth advantage due to its genetic makeup and expression profile, will take over the other subpopulations. Due to the potential complication of studying the heterogeneous and changing population of DTPPPs, for future experiments we have decided to concentrate on the difference between the parental cell line and DTPs, assuming that the early resistance mechanism, epigenetic in origin, unlike mutations will be more or less uniform in the whole DTPP cell population.

In this study, we showed that the DTPs undergo cell cycle arrest and display increased SA-β-GAL activity, but failed to induce all the other senescence biomarkers [20]. A drug holiday experiment revealed that the cell cycle arrest was reversible, demonstrating that DTPs cells are in a quiescent state. Our findings contrast with the conclusions of Meierjohann and colleagues [41] that vemurafenib induces senescence features in melanoma cells. To study senescence, the research group mainly relied SA-β-GAL staining and other non-established markers of senescence. It is known that SA-β-GAL activity does not have a causal relationship with senescence and can be induced in other situations in which lysosomal activity is increased [20], including quiescence [42]. They also present data that cells on drug holiday display decreased proliferative capacity when compared to non-treated cells, however, no comparison of DH cells with cells on vemurafenib is shown. In a recent paper by René Bernards and collaborators, they have shown that drug removal
results in senescence, which apparently contrasts our finding. However, these experiments were performed on a fully established resistant melanoma cell line that likely corresponds to DTPPs. DTPs, unlike DTPPs cells, do not enter OIS upon drug withdrawal, which could probably be explained by them not having yet selected the mutations leading to reactivation of the MAPK pathway.

Another goal of the project was to test the DamID technique in parental, DTP and DTPP cells. For this, we performed a pilot DamID experiment with lamin B1 in parental and DTPs SK-MEL-28 cells at days 4 and 18 of the treatment. Our results show that GATC methylation can be detected and induced in both cell lines. However, we found that the viral titer of the Dam virus is much higher than that of the Dam-LamB1 virus. Moreover, although lentiviruses are able to infect non-dividing cells, the infection efficiency of DTP cells was much lower than in parental cells. We conclude that amount of virus needs to be optimized for each virus and cell line. Therefore, we have decided to test various viral dilutions for both Dam and Lamin B1 for the subsequent experiments (SK-MEL-28 DTPPs of the first replicate and DTPs of the second replicate) The DpnII assay for these samples is currently on-going. Once we have determined the correct virus dilution, we will process the corresponding samples for deep sequencing.
6. CONCLUSIONS

In this study, we have successfully established a model of DTPs and DTPPs melanoma in the context of vemurafenib treatment. In addition, we showed that DTPs cells are quiescent and the cell cycle arrest can be reversed upon vemurafenib removal. We also tested the DamID technique in parental and DTP SK-MEL-28 cells and identified the virus dilution as a parameter required to optimize in further experiments.

In this work, we address for the first time the role of genome-NL interactions in the establishment of the drug-tolerant state. As such, our project has the potential to bring novel insights into this phenomenon and, in the future can serve as a basis for establishing novel therapeutic strategies for the treatment of melanoma.
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