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A POU3F2-MITF-SHC4 Axis in Phenotype Switching of Melanoma Cells

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1. Introduction

Malignant melanoma is one of the most aggressive human cancers. Metastatic melanoma is highly resistant to genotoxic radiotherapy and chemotherapeutic treatments and patients have a median survival of under a year from diagnosis. As primary melanoma tumours can metastasise very early in tumour development (Chin et al., 2006), rapid diagnosis and curative surgery remain the best hopes for control of the disease. Early surgery that removes radial growth phase tumours that have not yet initiated vertical growth phase can be very effective and prevent further development. However, once the primary tumour has begun to invade the local epidermal and dermal environment treatment becomes much more complicated.

Why melanoma has such a high propensity to invade and metastasize is not well understood, but may be related to the developmental characteristics of the melanocyte lineage. Melanocytes derive from pluripotent neural crest cells as non-pigmented melanoblasts (Dupin et al., 2007; Dupin et al., 2006; Thomas and Erickson, 2008). During embryogenesis melanoblasts migrate via the dorso-lateral pathway to populate the basal layer of the epidermis and hair follicles, as well as a number of other sites including the inner ear and the heart (Yajima and Larue, 2008). The ability of transformed melanoma cells to rapidly invade and migrate away from the primary tumour perhaps reflects an inherent characteristic inherited from their embryonic state.

Neural crest-derived cells are specified as melanoblasts by expression of Microphthalmia-associated Transcription Factor (MITF), a basic helix-loop-helix transcription factor belonging to the MYC superfamily (Goding, 2000a; Goding, 2000b; Hemesath et al., 1994). The *MITF* locus encodes multiple isoforms generated by alternate splicing and use of internal promoters (Steingrimsson, 2008). The MITF-M isoform (hereafter designated simply as MITF) is produced specifically in the melanocyte lineage from an intronic promoter. MITF is required for melanoblast survival and differentiation of the retinal pigment epithelium (RPE) (Hou and Pavan, 2008; McGill et al., 2002). Consequently, MITF-null mice exhibit a white coat colour due to the loss of the melanocyte lineage and a small (microphthalmic) eye phenotype due to loss of the RPE (Hodgkinson et al., 1993; Hughes et al., 1993; Moore, 1995; Steingrimsson et al., 1994). In humans, mutation of the *MITF* gene is responsible for Waardenburg syndrome type 2 (WS2) ((Tassabehji et al., 1994), a syndrome characterised by pigmentary defects and hearing loss highlighting the important role of the

melanocytes located in the inner ear. MITF is thus the master regulator specifying the identity and properties of the melanocyte lineage.

It is now commonly accepted that exposure to solar UV irradiation and consequent DNA damage and mutation, especially during childhood, is the primary cause of most forms of melanoma (Bennett, 2008b). Melanoma can arise spontaneously, but often result from a stepwise process via accumulations of genetic changes leading first to formation of a benign naevus (mole) which can remain stable throughout the individual's life. If one of the cells in the naevus then acquires additional mutations it can go on and form the malignant tumour. In contrast to many other tumours where a diversity of mutations can be found, melanoma formation is tightly associated with specific genetic changes. Activating mutations in BRAF especially the *BRAF^{V600E}* mutation or mutations in NRAS are found in more than 60% of primary melanomas. (Bennett, 2008a). The resulting constitutive activation of the MAP kinase pathway promotes melanocyte proliferation, but cells with activated BRAF or NRAS eventually arrest proliferation in what is assumed to be a senescent state in the form of benign nevi. Escape from this arrested state involves additional mutations one of the most frequent of which is inactivation of the CDKN2A locus encoding the cyclin dependent kinase inhibitor p16INK4 (Larue and Beermann, 2007). This inactivation can occur through genetic lesions (Bennett, 2008a) or DNA hypermethylation and epigenetic silencing (Richards and Medrano, 2009; Rothhammer and Bosserhoff, 2007), or repression via activation of Wnt/beta-catenin signalling (Delmas et al., 2007). The accumulation of these genetic and epigenetic changes allows normal melanocytes to become immortal, escape senescence and go on to form malignant tumours.

2. Phenotype switching versus genetic changes in tumour formation

Following the genetic and epigenetic changes described above, transformed melanoma cells enter the radial then the vertical growth phase where they acquire invasive properties. This is a critical event in tumour formation and two models have been put forward to account for how cells acquire the ability to invade neighbouring tissues, the lymphatic system and eventually form distant metastasis. Cells in the primary tumour may acquire further genetic changes that promote invasion and metastasis. This would be an essentially irreversible genetic event that, unless additional mutations arise, would fix the properties of these cells. An alternate model that has been put forward in the case of melanoma is 'phenotype switching' which postulates that cells can reversibly switch from proliferative to invasive states (Hoek and Goding, 2010). This process involves a specific set of changes in gene expression and hence altered transcription factor activity and can take place dynamically within a population of cells in response to signals from the tumour microenvironment.

The 'phenotype switching' model is based on two sets of observations. Firstly, analysis of 86 cultured melanoma cell lines showed that they could be divided into two classes; highly proliferative cells with low invasive potential and slowly dividing cells with much higher migratory and invasive potential (Hoek et al., 2008; Hoek et al., 2006). Gene expression profiling of these different cell types defined two distinct expression signatures in which a collection of 105 genes show the highest differential expression. The invasive signature is characterised by up regulation of genes such as *INHBA*, *COL5A1*, and *SERPINE1* that are involved in modifying the extracellular environment and are often known targets of TGF β signalling. In contrast, many of the proliferative signature genes are targets of Wnt signaling. Importantly however, a key feature of the two profiles revealed that invasive

phenotype cells exhibited low levels of MITF, while proliferative phenotype cells expressed high levels of MITF (Hoek et al., 2008; Hoek et al., 2006). These observations define at least two cell populations that differ in proliferative and invasive properties and are defined by distinct gene expression profiles including differential MITF expression.

The second set of observations supporting this model are derived from experiments showing that when MITF high and MITF low cells are used to make xenografts, the resulting tumours comprise cells expressing both high and low MITF expression levels (Hoek et al., 2008). Hence, while the original cells were rather homogenous with respect to MITF expression, the resulting tumours are heterogenous. The tumour microenvironment may therefore modify the gene expression profile allowing cells to switch from one state to the other in a dynamic way.

This model is complicated by the observation that a low abundance subpopulation of cells that have strongly reduced MITF expression arise spontaneously in *in vitro* cultures of high-MITF expressing cell lines (Cheli et al., 2011). Depletion of these cells from the population leads to a strong reduction in tumour formation, whereas the purified low-MITF expressing population has high tumour formation potential and rapidly recovers higher MITF expression after subcutaneous injection. These observations confirm that cells can dynamically and even spontaneously switch their MITF expression and that low-MITF expressing cells have much higher tumour initiating capacity.

These observations made from xenografted tumour cell lines have been confirmed in primary human tumours. Staining of primary tumours with antibodies against MITF showed a heterogenous pattern with cells that exhibited high levels of MITF and others that showed lower levels or were completely negative (Goodall et al., 2008). These results highlight the heterogeneity of cell populations within human tumours. This idea is reinforced by the observation that cells in human tumours with no MITF staining expressed high levels of a second transcription factor POU3F2 (also known as N-OCT3 or BRN2), while cells expressing high MITF showed low levels of POU3F2 (Goodall et al., 2008). Thus, these two transcription factors can act as markers for different cell populations within tumours. In an elegant approach, Pinner et al. (Pinner et al., 2009) used intravital imaging to follow the fate of xenografted human melanoma cells engineered to express green fluorescent protein under the control of the POU3F2 promoter. They were clearly able to show that the POU3F2 promoter was upregulated in motile cells and that these cells exhibited a de-differentiated phenotype with diminished pigmentation.

Together the above results are consistent with the idea that cells expressing high levels of MITF are proliferative, but poorly motile and invasive, while those expressing low levels of MITF, but high levels of POU3F2 are highly motile and invasive and that this phenotype is acquired in a dynamic and reversible fashion in tumours. Spontaneous switching can also take place to a certain extent in cell culture, however, the low frequency of this event makes it a difficult process to follow. More recently however, Thurber et al. (Thurber et al., 2011), showed that growth of high MITF expressing cells as non-adherent 'melanospheres' dramatically enhanced the frequency of low MITF/high POU3F2 expressing cells. Similarly, when we grew 501Mel cells expressing high levels of MITF in human embryonic stem cell medium under non-adherent conditions they readily form melanospheres, where we observe the appearance of a significant fraction of low MITF expressing cells in the spheres (Fig. 1). Thus, the change from monolayer to 3 dimensional culture in stem cells medium strongly influences MITF expression. Thurber et al. (Thurber et al., 2011) also showed using

siRNA silencing that POU3F2 was an activator and MITF a repressor of the Notch pathway. Loss of POU3F2-MITF signaling resulted in decreased capacity to form melanospheres and invasion through a collagen matrix. This *in vitro* model appears to recapitulate some of the characteristics of MITF and POU3F2 signaling seen in tumours and may therefore facilitate the identification of the signals and mechanisms that regulate melanoma cell heterogeneity in tumours.

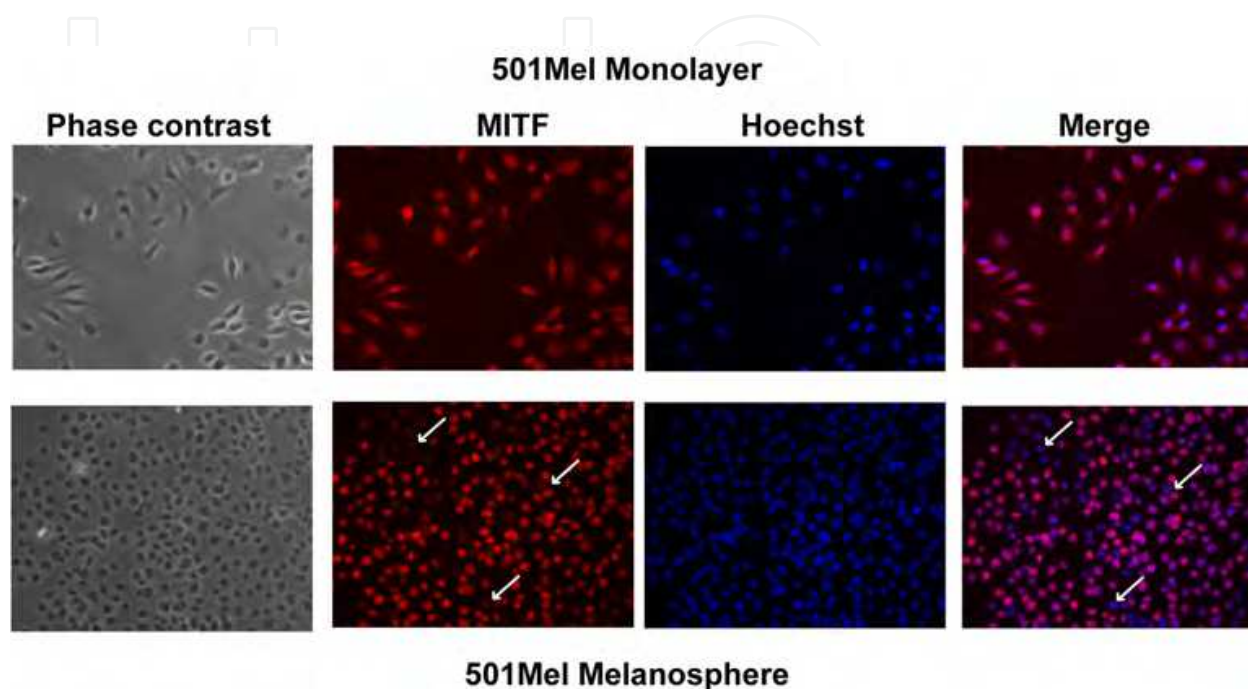


Fig. 1. Heterogenous MITF expression in 501Mel cells grown as melanospheres. The upper panels show phase contrast, MITF-labelling, nuclear Hoechst staining and the merge between the MITF and Hoechst signals of 501Mel cells grown as adherent monolayers under standard conditions. Almost all nuclei are strongly stained with MITF antibody. In the lower panels, the cells were grown as melanospheres in human stem cell medium and a significant number of low-MITF expressing cells can now be seen. Representative low-MITF expressing cells are indicated by arrows.

3. The MITF-POU3F2 axis in melanoma

The results described above show that high levels of MITF promote melanoma cell proliferation and that MITF and POU3F2 show reciprocal levels of expression. Key questions are therefore how does MITF promote proliferation and how does POU3F2 regulate MITF expression, proliferation and motility.

i. POU3F2 an activator and a repressor of critical target genes in melanoma.

POU (Pit, Oct1, Unc86) domain transcription factors play diverse functions in many physiological processes. The POU family factors have a bipartite DNA binding domain formed by the conserved POU-specific domain (POUs) and the POU homeodomain (POUh) that are joined by a linker region of variable length (Phillips and Luisi, 2000; Ryan and Rosenfeld, 1997). The POU and POUh domains each comprise a helix-turn-helix structure of which the third helix recognises the DNA and provides sequence specificity (Cook and Sturm, 2008; Klemm et al., 1994).

POU3F2 and POU4F1 (BRN3) are involved in development of the central nervous system (Schonemann et al., 1998). Genetic studies in mice have demonstrated that POU3F2 is required for generation of specific neuronal lineages in the endocrine hypothalamus and the posterior pituitary gland (Nakai et al., 1995; Schonemann et al., 1995). POU3F2 also plays a role in the production and positioning of neocortical neurons (Sugitani et al., 2002). In addition to the nervous system, several studies have reported that POU3F2 is expressed in normal melanocytes and is upregulated in malignant melanoma cells where its expression is regulated by the Wnt/ β -catenin and activated BRAF signalling pathways (Cook et al., 2003; Eisen et al., 1995; Goodall et al., 2004a; Goodall et al., 2004b; Thomson et al., 1995). However, POU3F2 has not yet been selectively inactivated in the melanocyte lineage and genetic dissection of its role in normal melanocyte physiology and in melanoma will be a welcome and important addition to the field.

POU3F2 directly represses MITF in melanoma cells through binding directly to the proximal internal promoter driving expression of the MITF-M isoform (Goodall et al., 2008). At least 3 POU3F2 binding sites have been identified at the MITF-M locus, two in the proximal promoter region upstream of the transcription start site (TSS) and a third intronic site downstream of the TSS (Kobi et al., 2010). Wellbrock et al., (Wellbrock et al., 2008) have shown that in some melanoma cell types POU3F2 mediates the regulatory effect of the BRAF^{V600E} mutation and induces MITF expression. As discussed above, this suggestion is at odds with what is observed in tumours and melanospheres. To reconcile what may appear to be contradictory observations, we have suggested that the POU3F2 binding to the site closest to the TATA element may repress *MITF-M* transcription through steric hindrance of the basal transcription machinery, for example TFIID, while POU3F2 may activate *MITF-M* transcription via binding to the other sites in the proximal promoter and downstream intron (Kobi et al., 2010). The ability to activate or repress may then be modulated by POU3F2 concentration being able to efficiently compete with TFIID binding and repress MITF expression only at high concentrations. This model would reconcile the observation that POU3F2 is a transcription activator with the reciprocal MITF/POU3F2 expression seen in melanoma tumours (Goodall et al., 2008; Pinner et al., 2009).

Another means by which POU3F2 and MITF may regulate each others expression is through control by miR-211. Boyle et al., (Boyle et al., 2011) have compared miRNA expression profiles and identified miR-211 as down-regulated in melanoma cell lines compared to normal melanocytes. MiR-211 is derived from the TRPM1 gene whose expression is directly regulated by MITF and it targets POU3F2 expression. Thus MITF driven expression of TRPM1/miR-211 provides a mechanism by which high MITF levels maintain low POU3F2 levels. MiR-211 may also have other targets in melanoma. Levy et al., (Levy et al., 2010) have shown that expression of miR-211 reduced migration and invasion in human melanomas with low miR-211 levels. They propose IGF2R, TGFBR2, and NFAT5 as miR-211 targets that mediate the phenotypic effects of its overexpression. In contrast, Mazar et al., (Mazar et al., 2010) identified KCNMA1, encoding a calcium ion-regulated potassium channel protein, as a miR-211 target that influences melanoma cell aggressivity. MiR-211 has therefore many potential targets that influence melanoma cell properties.

Can all of the effects of POU3F2 in melanoma be ascribed to its ability to repress MITF or does it regulate other target genes involved in melanoma development? Genome wide chromatin immunoprecipitation (ChIP) coupled to array hybridisation (ChIP-chip) profiling of POU3F2 promoter occupancy identified target genes that may be involved in modulating the properties of melanoma cells (Kobi et al., 2010). For example, siRNA-mediated

knockdown and transfection of reporter genes indicates that POU3F2 regulates expression of stem cell factor (Kit ligand, *Kitl* or *Steel*) via a cluster of 4 closely spaced binding sites located in the proximal promoter. Amplification and/or activating mutations of the KIT gene, encoding the KITL receptor, have been frequently found in mucosal, acral, and chronic sun-damaged melanomas (Garrido and Bastian, 2009), where it appears to promote melanoma development. The KITL/KIT pathway is also critical for promoting melanocyte migration during embryogenesis and hence may also be important in regulating the migratory/invasive properties of melanoma cells (Wehrle-Haller, 2003). POU3F2 may therefore modulate the properties of melanoma cells via autocrine KITL signalling.

A second mechanism by which POU3F2 may enhance the motile and malignant properties of melanoma cells is through regulation of the cGMP-specific phosphodiesterase PDE5A. Arozarena et al (Arozarena et al., 2011) have shown that POU3F2 binds to two sites in the PDE5A proximal promoter upstream of the TSS to repress its expression. PDE5A down-regulation has only a minor effect on proliferation, but potently increases melanoma cell invasion. Diminished PDE5A levels lead to an increase in cGMP and in cytosolic Ca²⁺, stimulating contractility and inducing invasion. In agreement with this idea, PDE5A levels are also down-regulated in human melanoma tumours. Hence, this model postulates that in melanoma cells, oncogenic BRAF promotes invasion through POU3F2-mediated repression of PDE5A. The ability of POU3F2 to act as a repressor at the PDE5A promoter is nevertheless surprising considering that it acts as a potent transcriptional activator in many other promoter contexts (Kobi et al., 2010).

ii. MITF activates genes required for proliferation and represses genes involved in invasion.

As described above, evidence from cell based models and human tumours suggest that MITF is a major regulator of melanoma cell proliferation. SiRNA-mediated silencing of MITF in proliferative melanoma cells leads to a rapid growth arrest confirming that it is indeed a critical regulator of proliferation (Carreira et al., 2006). Acute loss of MITF through siRNA mediated silencing has been reported to arrest cells at the G1/S phase transition through indirect regulation of CDKN1B (p27^{Kip1}) via direct regulation of Diaphanous related formin *DIAPH1*. *DIAPH1* promotes actin polymerization and coordinates the actin cytoskeleton and microtubule networks at the cell periphery (Carreira et al., 2006). MITF directly regulates *DIAPH1* expression leading to reorganization of the actin cytoskeleton, accounting at least in part for the dramatic change in cell morphology seen upon MITF silencing, and increased ROCK-dependent invasiveness. MITF regulation of *DIAPH1* also indirectly controls CDKN1B degradation leading to a G1 cell cycle arrest.

While the above model accounts for many of the changes seen upon MITF silencing, it has been shown more recently that loss of MITF also leads to defects in mitosis and induces cell senescence (Giuliano et al., 2010; Strub et al., 2011). MITF silenced cells exhibit many characteristics of senescence such as enlarged and flattened cell morphology and senescence-associated β -galactosidase staining and heterochromatin foci. Giuliano et al (Giuliano et al., 2010) showed that acute MITF silencing induced the DNA damage response leading to activation of ATM and CHK2 resulting in p53 phosphorylation and stabilization. SiRNA depletion and pharmacologic inhibition showed that the DNA-damage response is required for entry into senescence.

How does MITF regulate both the proliferative and invasive properties of melanoma cells, and why does loss of MITF result in DNA damage? To answer these questions, Strub et al (Strub et al., 2011) used ChIP coupled to high throughput sequencing (ChIP-seq) to profile genomic MITF occupancy and RNA-seq following MITF silencing to identify MITF

regulated genes. As currently available antibodies do not efficiently ChIP MITF, 501Mel cells expressing 3HA-tagged MITF were used. ChIP-seq was performed not only for tagged MITF, but also for RNA polymerase II (Pol II) and trimethylation of lysine 4 of histone H3 (H3K4me3), a covalent histone modification found at the 5'-end of transcribed genes (Ruthenburg et al., 2007). An example of MITF occupancy of the highly expressed Tyrosinase locus is shown in Fig. 2, where at least 5 MITF-occupied sites can be detected both upstream and downstream of the TSS along with transcribing RNA Pol II. Overall, 12139 MITF occupied loci were identified, 9447 of which could be annotated to more than 5000 potential target genes.

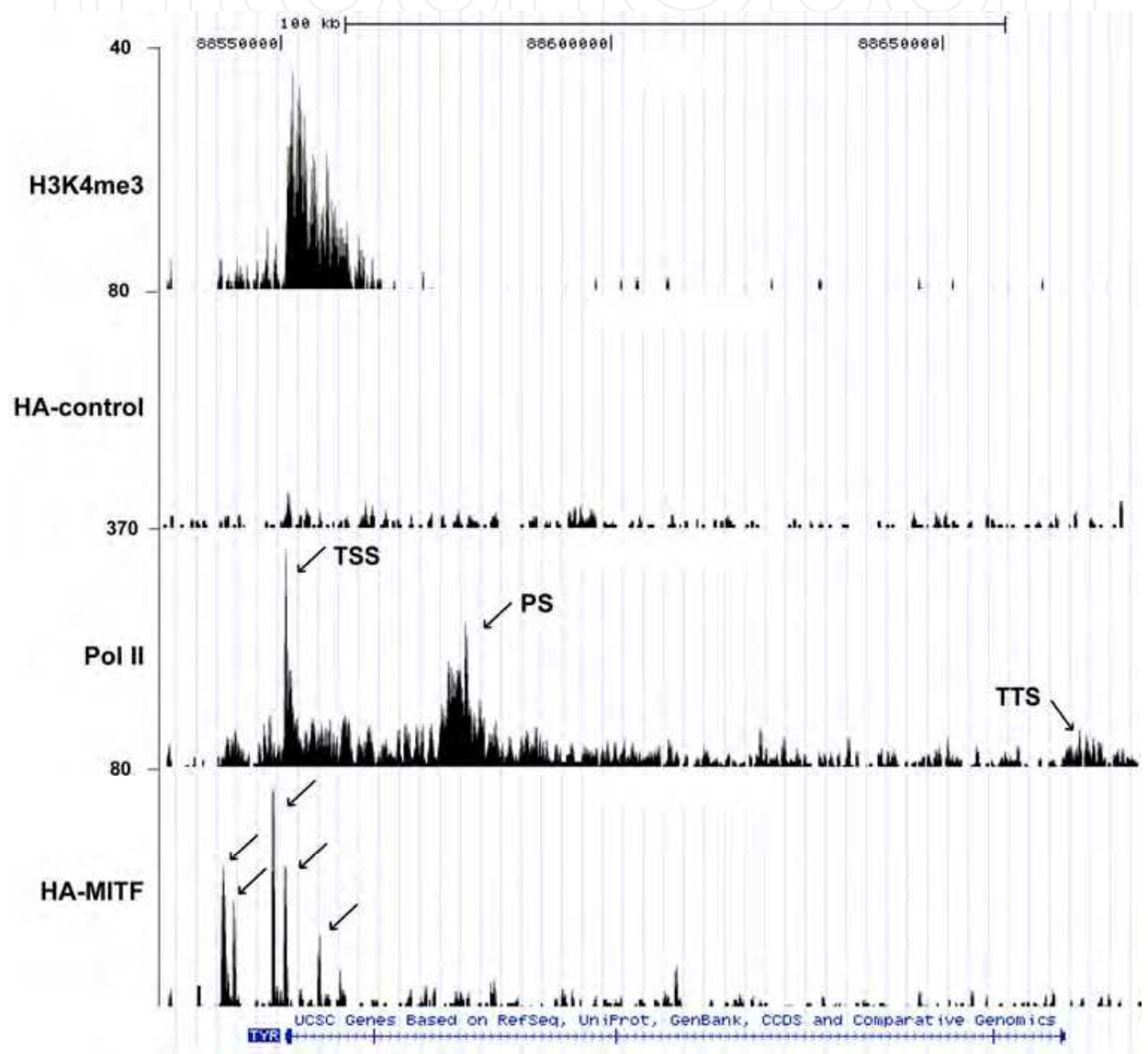


Fig. 2. Example of MITF-occupancy at the Tyrosinase locus. UCSC genome browser view of MITF and Pol II-occupancy and H3K4me3 at the *TYR* locus. The principal MITF occupied sites are indicated by arrows. TSS shows the engaged Pol II at the transcription start site, PS, enhanced Pol II occupancy at an intronic pause site, and TTS, the transcription termination site downstream of the *TYR* polyadenylation site. The data are adapted from the data set of Strub et al, (Strub et al., 2011).

To determine which of these potential targets are regulated by MITF in 501Mel cells, RNA-seq was performed following MITF silencing identifying a large collection of both up and down-regulated genes (Strub et al., 2011). Amongst the genes that appear positively regulated by MITF are at least 31 genes involved in DNA replication, recombination and repair including *LIG1* encoding DNA ligase 1, involved in the religation of Okazaki fragments during DNA replication, as well as religation during DNA repair and maintenance of genome stability (Bentley et al., 2002; Tomkinson and Mackey, 1998), and *TERT* (Telomerase reverse transcriptase) required for the proper replication of telomeres and frequently expressed in human tumours, (Artandi and DePinho, 2000; Artandi and DePinho, ; Kim et al., 1994; Rudolph et al., 1999). In addition, MITF regulates a set of 39 genes involved in centromere organisation and mitosis including the G2-specific cyclins B1 and F (*CCNB1*, *CCNF*) that promote entry into mitosis, as well as polo-like kinase 1 (*PLK1*) (Schmit et al., 2009), the CENPA-nucleosome associated and CENPA-nucleosome distal complexes and the chromosomal passenger complex. Consequently, siMITF silencing leads to mitotic defects with a high frequency of binucleate and micronuclei containing cells.

Together these results provide a more comprehensive picture of how MITF promotes proliferation by activating genes involved in DNA replication and repair as well as mitosis. Loss of expression of genes like *LIG1* and *TERT* leads to incomplete DNA replication and telomere exposition activating the DNA damage and the senescence responses. We propose that, acute siMITF silencing in rapidly proliferating cells induces replication and mitotic defects too severe to be repaired and cells enter in senescence. However, the existence of senescent cells in human melanoma tumours has not yet been investigated and our results do not exclude the possibility that *in vivo*, decreased MITF expression could be offset by up-regulation of other pathways that suppress senescence to produce slow-cycling invasive cells. Alternatively, a more gradual reduction of MITF expression such as may take place in tumours would allow cells to adapt progressively to slower growth due to diminished levels of replication and mitosis factors and not accumulate the high levels of DNA damage that trigger the senescence response.

In contrast to the above, genomic profiling of MITF occupancy revealed genes that appear to be directly repressed by MITF. Amongst these are genes influencing metastasis such as *MCAM* (Ouhtit et al., 2009), *SHC4* (Fagiani et al., 2007). Comparison of the ChIP-seq and mRNA-seq data shows the presence of at least 3 upstream and two intronic MITF occupied sites at the *SHC4* locus and enhanced mRNA expression upon MITF knockdown (Figs. 3A and B). Increased *SHC4* protein expression can also be seen upon MITF silencing (Fig 3C). *SHC4* therefore appears to be directly repressed by MITF suggesting that MITF both activates and represses transcription in a promoter context-dependent manner similar to *POU3F2* discussed above.

SHC4 (also known as *SHCD* (Hawley et al., 2011), or RaLP, retinoic acid-like inducible like protein) belongs to the family of SRC homology and collagen (Shc) signal transduction adaptor proteins. These proteins share a common organisation with an N-terminal phosphotyrosine-binding (PTB) domain, a central region rich in proline and glycine residues (CH1) and a C-terminal SRC homologue 2 (SH2) domain (Pasini et al., 2009). A second N-terminal collagen homology (CH2) region is present in the longest isoform of *SHC4*. *SHC4* is of particular interest in melanoma as its expression is tightly correlated with tumour stage. *SHC4* expression is low or undetectable in benign nevi and in early radial growth phase tumours, but is strongly expressed in the vertical growth phase and in metastases. *SHC4* functions as a substrate of activated IGF-1 and EGF receptors to enhance MAPK signaling and cell migration (Fagiani et al., 2007; Pasini et al., 2009). In contrast, *SHC4* silencing in metastatic cells reduces their migration *in vitro* and inhibits tumour growth *in vivo*.

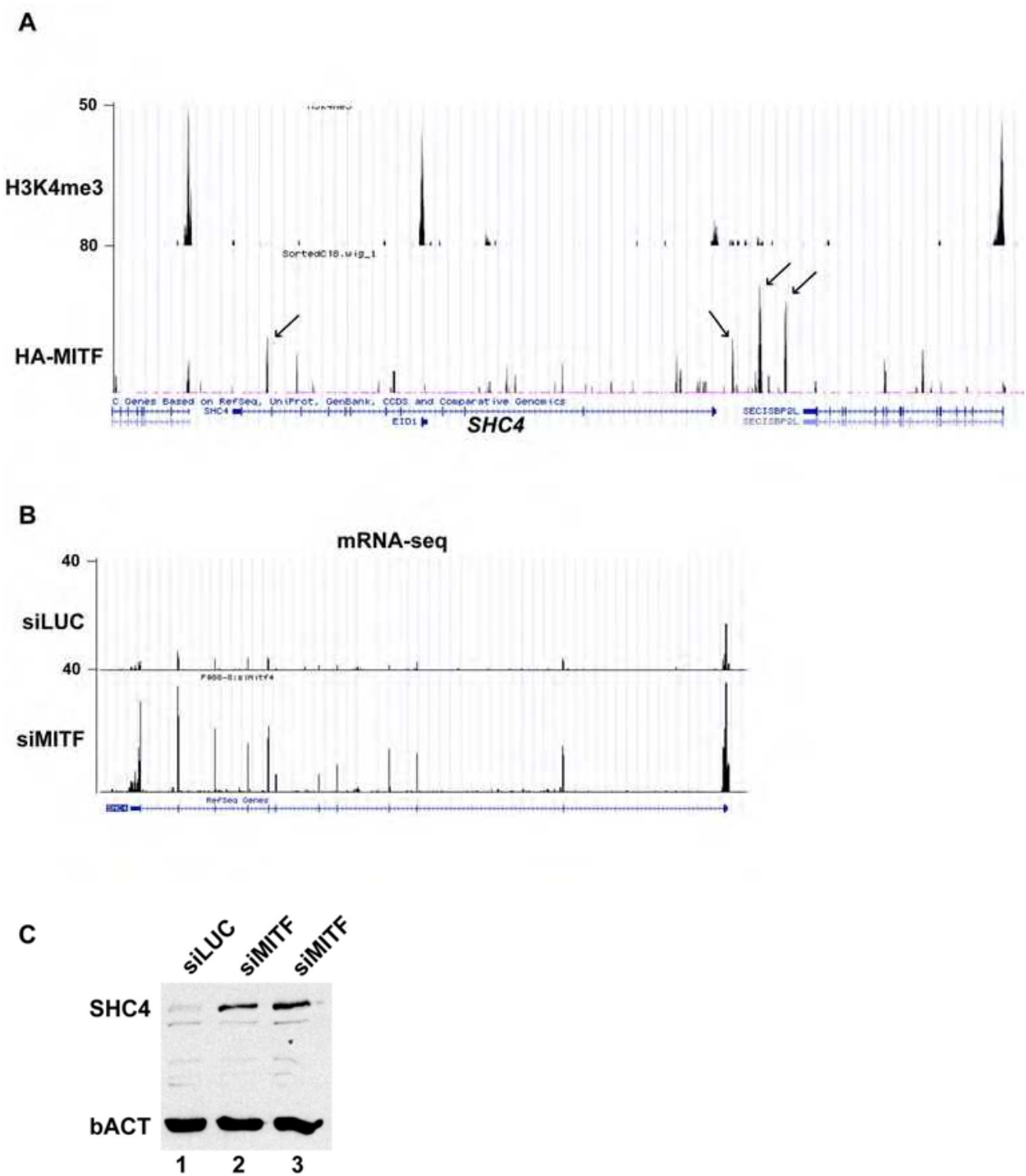


Fig. 3. MITF occupies sites at SHC4 locus and represses its expression. **A.** UCSC genome browser view of MITF occupancy and H3K4me3 at the *SHC4* locus. The principal MITF occupied sites are indicated by arrows. **B.** UCSC genome browser view of mRNA-seq data from 501Mel cells treated with control Luciferase or MITF siRNAs. Increased expression of the *SHC4* gene can be clearly observed in the siMITF cells. **C.** Immunoblot showing increased SHC4 protein expression in total extracts from 501Mel cells following MITF silencing with 20 or 50 nM of siRNA (lanes 2 and 3, respectively). ChIP- and mRNA-seq data are adapted from Strub et al, (Strub et al., 2011).

Together with the results of the ChIP- and RNA-seq, these observations suggest a novel MITF-SHC4 axis in the control of melanoma cell invasion. Proliferating cells express high levels of MITF that repress SHC4 expression, but the loss of MITF that takes place during phenotype switching de-represses SHC4 expression to promote migration and metastasis (summarised in Fig. 4).

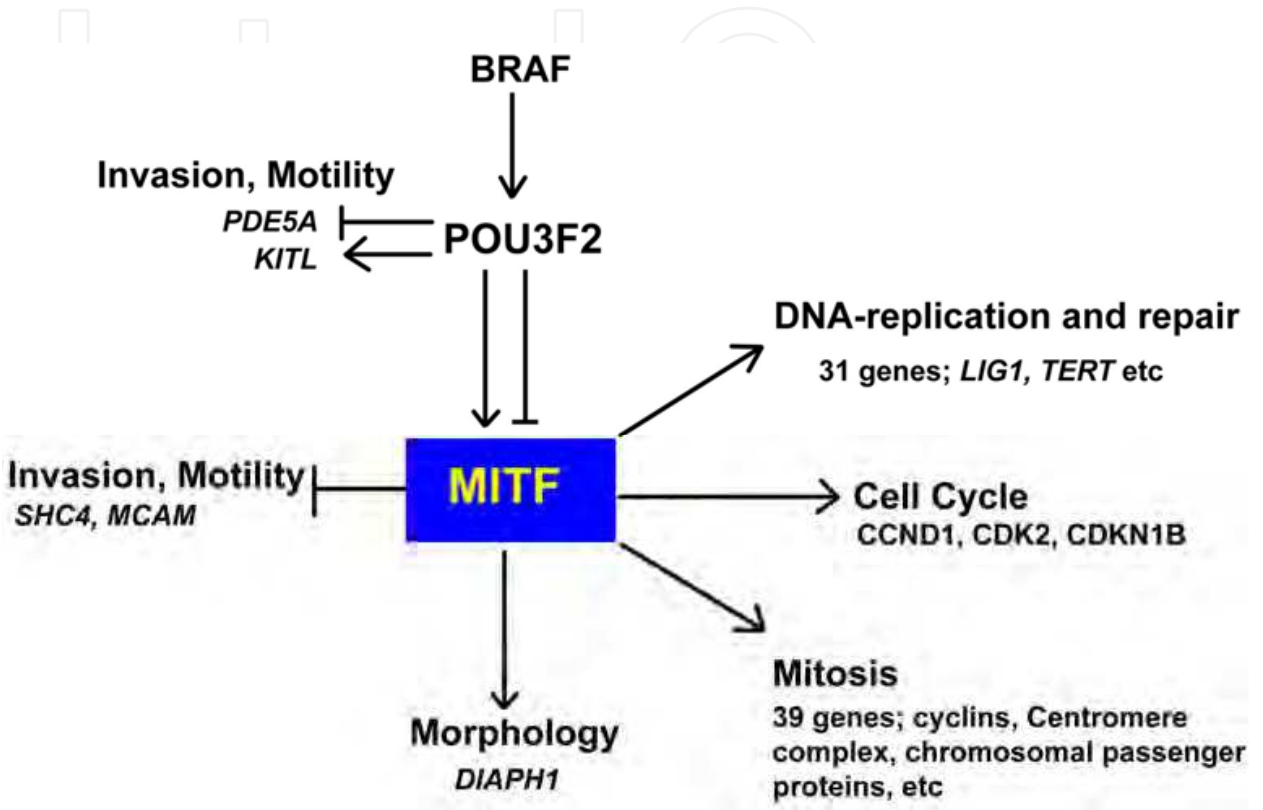


Fig. 4. Summary of regulatory interactions. in the POU3F2-MITF-SHC4 axis. As described in the main text, activated BRAF induces POU3F2 expression which in turn has been described to either activate or repress MITF expression. POU3F2 also activates *KITL* expression but represses *PDE5A* to promote motility and migration. MITF activates a series of genes involved in DNA replication and repair, mitosis and critical cyclin genes, but represses expression of SHC4 and MCAM that promote invasion and metastasis.

4. Future perspectives

In this review, we have limited our discussion to the hypothesis that POU3F2, MITF and SHC4 form a regulatory axis that controls the proliferative and invasive properties of melanoma cells. These are of course not the only transcription factors that regulate the properties of melanoma cells. For example, recent studies have shown that GLI2 and ATF2 modulate the invasive properties of melanoma cells. GLI2 is a transcription factor normally associated with Sonic Hedgehog (SHH) signaling is present in melanoma cells where its expression is rather controlled by TGFbeta and SMAD signaling (Alexaki et al., 2010; Dennler et al., 2009; Santiago-Walker and Herlyn, 2010). GLI2 is overexpressed in

subpopulations of cells and these GLI2-high cells metastasise to bone more readily than GLI2-low cells. GLI2 appears to promote invasion and metastasis through suppression of E-cadherin expression leading to a mesenchymal-like phenotype. Also, the transcription factor ATF2 has been shown to attenuate melanoma susceptibility to apoptosis and to promote its ability to form tumors in xenografts (Bhoumik and Ronai, 2008; Shah et al., 2011). In mouse melanoma models, expression of mutant ATF2 in melanocytes strongly attenuated tumour formation. ATF2 appears to modulate MITF expression through ATF2-JunB-dependent suppression of expression of SOX10 a factor that binds to and activates the MITF-M promoter. Moreover, on melanoma tissue microarrays, a high nuclear ATF2 to MITF ratio was associated with metastatic disease and poor prognosis. In the future it will be important to develop more complex mouse melanoma models in which POU3F2 and/or MITF, GLI2 and ATF2 are (in)activated or mutated to study combinatorial control by multiple transcription factors in melanoma. This will be a challenging, but important task.

We have discussed the proposition that the regulatory interactions between POU3F2-MITF-SHC4 explain in part the phenotype switching of melanoma cells. However, one important and outstanding question concerns the signal(s) in the tumour microenvironment responsible for modulating the expression of POU3F2 and MITF. Are these diffusible signals emanating from cells within or outside the tumour or are cell-cell contacts between the tumour cells and the stroma or amongst tumour cells involved? An alternative possibility is that phenotype switching may take place spontaneously through transient stochastic changes in the expression of POU3F2 or MITF in single cells that set up a reinforcing positive feedback loop to establish a novel stable state. For example, down-regulation of MITF leads to up-regulation of POU3F2 (Strub et al., 2011), but elevated levels of POU3F2 repress MITF. Given this situation, a transient reduction in MITF could set up a reinforcing loop of POU3F2-mediated MITF repression. This would explain the spontaneous appearance of cells low-MITF expressing cells in *in vitro* cultures of otherwise high-MITF expressing cell types. It remains possible that in the tumour environment, extracellular signals may influence the expression of MITF or POU3F2, but the important point is that the effect of these signals can be amplified by the positive and negative feedback loops between these factors. The role that stochastic changes in transcriptional 'noise' may play in biological processes is only just beginning to be considered (Kashyap et al., 2009; Leisner et al., 2008). Whatever the mechanism involved, key features of melanoma cells are their plasticity and heterogeneity. As discussed above, Cheli et al (Cheli et al., 2011) identified low-MITF expression as a criteria for selecting cells with enhanced tumour initiating properties. This fits with the previously proposed 'rheostat' model of MITF expression where high levels of MITF drive a G1/S arrest and differentiation, while low levels are associated with slow cycling tumour initiating cells, and intermediate levels are optimum for proliferation (Carreira et al., 2006; Hoek and Goding, 2010).

However, MITF is not the only biomarker that has been used to select slow cycling tumour initiating cells. Roesch et al., (Roesch et al., 2010) have used the JARID1B histone demethylase as a marker to identify a population of slow cycling cells within the tumour population. Similar to purified low-MITF cells, purified JARID1B-positive cells give rise to a highly proliferative progeny showing the dynamics of their expression. Moreover, JARID1B silencing promotes a transient acceleration of tumor growth that is followed by exhaustion.

This observation suggests that the continuous dynamic appearance of a JARID1B-positive subpopulation is essential to maintain tumor growth. There are thus a number of parallels between JARID1B and MITF expression both in terms of the dynamics of their expression and the properties they confer to tumour cells. Unfortunately, Roesch et al, did not specifically assess MITF expression in the slow cycling JARID1B-positive cells, that one would predict to have low MITF expression or at least low levels of 'active' MITF. Cheli et al, (Cheli et al., 2011) however, did not observe a consistent increase in JARID1B expression in the MITF low population. Previously other markers such as CD133 (Monzani et al., 2007) or ABCB5 (Schatten et al., 2008; Zabierowski and Herlyn, 2008), have been used to characterise stem-like subpopulations in melanomas. Does this mean that there are multiple distinct slow cycling tumour cell populations characterised by different transcriptional programmes? Future studies should answer these questions.

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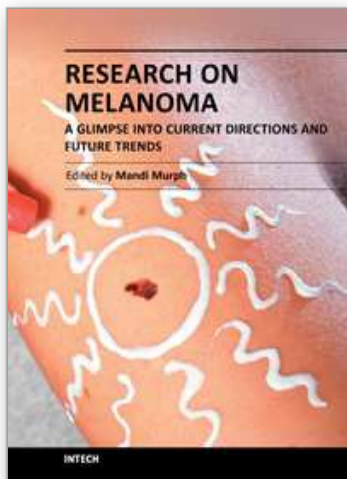
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The book *Research on Melanoma: A Glimpse into Current Directions and Future Trends*, is divided into sections to represent the most cutting-edge topics in melanoma from around the world. The emerging epigenetics of disease, novel therapeutics under development and the molecular signaling aberrations are explained in detail. Since there are a number of areas in which unknowns exist surrounding the complex development of melanoma and its response to therapy, this book illuminates and comprehensively discusses such aspects. It is relevant for teaching the novice researcher who wants to initiate projects in melanoma and the more senior researcher seeking to polish their existing knowledge in this area. Many chapters include visuals and illustrations designed to easily guide the reader through the ideas presented.

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