Author Accepted Version. Accepted: 15-Sep-2015. Published: 29-Mar-2016
In: Pigment Cell and Melanoma Research
Article type: Invited Review
Manuscript Category: Signaling & Cell biology (SCB)

Genetics of melanoma progression: the rise and fall of cell senescence

Dorothy C Bennett

Molecular Cell Sciences Research Centre, St George's, University of London, Cranmer Terrace, London SW17 0RE, UK.

Address correspondence to D. C. Bennett at same address.

Tel. +44 (0)20 8725 5202

e-mail: dbennett@sgul.ac.uk

Please cite this article as doi: 10.1111/pcmr.12422

Summary

There are many links between cell senescence and the genetics of melanoma, meaning both familial susceptibility and somatic-genetic changes in sporadic melanoma. For example *CDKN2A*, the best-known melanoma susceptibility gene, encodes two effectors of cell senescence, while other familial melanoma genes are related to telomeres and their maintenance. This article aims to analyze our current knowledge of the genetic or epigenetic driver changes necessary to generate a cutaneous metastatic melanoma, the commonest order in which these occur and the relation of these changes to the biology and pathology of melanoma progression. Emphasis is laid on the role of cell senescence and the escape from senescence leading to cellular immortality, the ability to divide indefinitely.

Keywords: melanoma, nevus, senescence, p16, TERT, immortalization, metamortal.

Running title: Cell senescence and genetics of melanoma progression

Introduction

Cell senescence is defined as a permanent cell-cycle arrest brought about by either extensive cell proliferation or certain cellular stresses such as oncogene overexpression, radiation or reactive oxygen species (Chandler and Peters, 2013; Muñoz-Espín and Serrano, 2014; Salama et al., 2014). The first signs of the now extensive connection between cell senescence and melanoma genetics arose from discoveries about *CDKN2A* in the 1990s (Hussussian et al., 1994). *CDKN2A* is the commonest known high-penetrance human familial melanoma locus (Aoude et al., 2014; Cust et al., 2011; Goldstein et al., 2006), also very commonly disrupted in sporadic melanoma (Bastian, 2014; Bennett, 2008). Its two main protein products, p16 (INK4A, CDKN2A) and ARF (p14, p19), are both important effectors of cell senescence, although with some interspecies variation. In melanocytes and other cells, ARF appears more important for senescence in mice, but p16 in humans (Chandler and Peters, 2013; Ha et al., 2008; Herbig and Sedivy, 2006; Sviderskaya et al., 2003). Since 1994, a remarkable number of other genes connected with senescence have also been found to be altered in familial and sporadic melanoma (Aoude et al., 2015; Iles et al., 2014). In this review I will discuss reasons for this connection, in the broader context of current knowledge about the genetic and/or epigenetic steps required to generate a metastatic cutaneous melanoma. There is much new information since a related review on this topic (Bennett, 2008), emerging especially from the application of deep sequencing to melanomas. The present focus will largely be on sporadic (human) melanoma, because of the escalating amount of literature; an excellent review on familial melanoma appeared recently (Aoude et al., 2014). However the two fields overlap, and evasion of cell senescence is a major theme in both cases. Another focus here will be on metastatic melanoma, since that is the form that kills and that we most urgently need to understand to inform advances in diagnosis and therapy.

It is illuminating to consider at which stage in melanoma progression a given genetic change tends to occur. This discussion will be based on the four increasingly progressed types of cutaneous pigmented lesion described by W. Clark et al. (Clark, 1994). In summary these are: (1) the benign (or banal) nevus or mole: usually small, symmetrical and static; (2) the dysplastic nevus: larger with some cellular atypia by histopathology; (3) radial growth-phase or RGP melanoma, an expanding but thin lesion growing only in or close to the epidermis, and (4) vertical growth-phase or VGP melanoma, which has larger nests of cells dividing in the dermis and invading into the deep, reticular dermis (Clark, 1994; Mooi and Krausz, 2007). VGP melanoma is thought to be already competent for lymphatic invasion and metastasis. Another common system of classification includes superficial spreading melanoma (SSM), composed of RGP only or RGP plus VGP melanoma; and nodular melanoma (NM), a raised nodule of VGP with no RGP detected. This system also includes acral

lentiginous melanoma, lentigo maligna melanoma and mucosal melanoma (Mooi and Krausz, 2007). These last three subtypes are less common than SSM and NM in Caucasian populations, and their genetic aberrations seem to follow different patterns (Bastian, 2014; Curtin et al., 2005); they are mostly not discussed here for reasons of space. Likewise primary melanomas of other organs are not discussed here; the commonest is ocular melanoma, which again shows different patterns of genetic change from cutaneous melanoma (Bastian, 2014; Dono et al., 2014).

The commonest genetic and epigenetic driver changes in melanoma

High-throughput sequencing has now revealed many genes to be commonly mutated in melanomas. For example Hodis et al. (2012) found 515 genes that were each mutated in at least 10% of melanomas. However, not all of these mutations prove to be relevant for malignancy. Melanomas have a median of 13-17 mutations per Mbp of DNA, the highest frequency among 21 cancer types analyzed (The Cancer Genome Atlas Network, 2015; Hodis et al., 2012; Lawrence et al., 2014), even without considering rearrangements, copy-number alterations and epigenetic changes. These mutations predominantly carry the signature of ultraviolet light mutagenesis (Hodis et al., 2012). Every melanoma specimen has a different set of genetic and epigenetic changes. Many of these appear to be random or "passenger" events, while fewer comprise the key "drivers" (Stratton et al., 2009), the primary events selected for in the clonal evolution of the developing lesion. At 13 mutations per Mbp, around 2% of copies of a middle-sized coding sequence of 1,500 bases would contain a mutation by chance. [13 per 10^6 bp = 1 per 76,923 bp, = 1 per (51.3 x 1,500 bp). 1 in 51.3 copies ≈ 2%.] Moreover genes poorly expressed or not vital in a given cell type can accumulate many more harmful mutations than others. It is therefore necessary to use careful statistical and functional screening to identify real driver mutations, though the screening criteria vary somewhat between studies and the identified gene set varies in part (Hodis et al., 2012; The Cancer Genome Atlas Network, 2015; Krauthammer et al., 2015). Overall, even the validated drivers are quite numerous. However we can make some sense of this morass of molecular malfunction, since many of the common drivers affect the same cellular signalling pathways. Genetic manipulation of cells or animals then adds powerful tools to distinguish the important from the accidental.

Based on such criteria, Table 1 presents the 20 commonest driver changes currently known, with both similarities and differences from those previously listed in a similar table (Bennett, 2008). Since 2008 the numbers of samples tested have increased greatly, and uncultured lesions rather than cell lines are coming to predominate, giving more accurate estimates of mutation frequencies in clinical melanoma as opposed to cell culture (which can select for properties such as cell

immortality and thus distort the picture). There are various interesting new arrivals in the table, like *TERT*, *PREX2* and *ARID2*, to be discussed. Note: HGNC-approved symbols will be used throughout.

The table aims to combine the different ways a gene is reported to be altered, where known. Thus *CDKN2A* is often homozygously deleted, but also sometimes mutated or epigenetically silenced by methylation. If we assume that usually only one of these defects would arise in a given melanoma, we can add the frequencies to estimate the total frequency of disruption. Two genes in the table, *ARID2* and *PPP6C*, do not yet have clear biological functions in melanoma apart from predicted tumour suppression (since loss of function mutations are seen), but their biochemical functions are known. ARID2 is an epigenetic regulator, part of the SWI/SNF chromatin remodeling complex. Rarer mutations in other members of this complex were also identified (Hodis et al., 2012; Krauthammer et al., 2015). *PPP6C* encodes the catalytic subunit of protein phosphatase 6 (PP6), which can negatively regulate both cyclin D1 (CCND1, discussed under Step 2 below), and the mitotic kinase Aurora kinase A (AURKA) (Hodis et al., 2012).

A genetic model was proposed for the minimal biological changes required to make a melanoma in relation to progression (Bennett, 2003) and this, with an update (Soo et al., 2011), seems to help make sense of most currently known changes. Figure 1 shows the current four-step model. In a technical tour de force, Khavari's group (Chudnovsky et al., 2005) showed that a combination of overexpressed genes adding the same four changes to diploid human melanocytes – mitogenic driver ($NRAS^{G12V}$), senescence evasion ($CDK4^{R24C}$), antiapoptotic ($p53^{R248W}$) and TERT expression – were indeed sufficient to produce invasive melanoma-like lesions in human skin xenografts. This model (Figure 1) will therefore be used as a framework to discuss the genetic changes in melanoma. A key point to appreciate is that the "mutations that make cancer cells divide" fall into two very different classes: transforming or mitogenic mutations (Step 1), and immortalizing mutations (Steps 2 and 4). Mutations to suppress apoptosis make Step 3.

Step 1. Mitogenic driver mutations and nevus growth

A mitogenic or transforming mutation is one that stimulates cell proliferation, by mimicking growth-factor (mitogen) signalling. Natural mitogenic mutations in cancers most frequently affect the RAS-RAF and PI3K pathways, which normally signal the activation of tyrosine kinase growth-factor receptors (RTKs) by their ligands, and also activation of certain G-protein-coupled and other types of receptors (Figure 2). A genomic classification of melanomas according to the mitogenic driver has recently been proposed (Cancer Genome Atlas Network, 2015).

RAS and RAF mutations

The commonest mitogenic mutations in nevi are shared with melanomas. These include oncogenic mutations of *BRAF* and *NRAS* (Table 1), which cause these intermediates to signal autonomously, without their upstream activators. As in melanoma, these mutations usually seem to be mutually exclusive at the cell level; cases have been detected of both *BRAF* and *NRAS* mutations, or different *BRAF* mutations, within apparently the same nevus or melanoma but in different subclones (Lin et al., 2009; Shitara et al., 2015). Induced co-expression of both was reported to lead to senescence of melanoma cells (Petti et al., 2006). Activating *BRAF* mutations are reported in nearly half of all human cutaneous melanomas (Table 1) (Forbes et al., 2015), and one now-famous mutation accounts for the great majority of activating *BRAF* mutations in both nevi and melanomas: T to A at nucleotide 1799, producing a valine to glutamic acid substitution (V600E) in the protein, and providing a major therapeutic target for metastatic melanoma (e.g. Robert et al., 2015). Interestingly the prevalence of $BRAF^{V600E}$ mutations was recently reported to vary between different populations, at 43% of 60 melanoma cases in Denmark, similar to global levels, but only 24% of 689 cases in Ireland (van den Hurk et al., 2015).

A mitogenic driver mutation is thought usually to be the first step in any neoplasia, since it could stimulate an otherwise normal cell to proliferate into a clonal mass. While this greatly increases the probability of further driver mutations, the clone usually arrests as a benign growth, a nevus in the case of a melanocyte clone (Figure 1). Both mice and zebrafish with oncogenic *BRAF* expression targeted to melanocytes develop skin nevi (Dhomen et al., 2009; Patton et al., 2005). Actually, since the skin in these strains with all melanocytes expressing oncogenic *BRAF* does not become one big nevus, this hints at the need for a further unknown event to form a nevus. Also reported in nevi (but uncommon in melanoma) are activating mutations of *KRAS* (13%), and *HRAS* (3%) (Forbes et al., 2015). With *BRAF* activation in 63% and *NRAS* in 8% of nevi (Table 1), the remaining 13% of naevi presumably have other mitogenic mutations.

We do not know what proportion of melanomas arise from a nevus; only a minority are associated with detectable nevus tissue (Bevona et al., 2003), but a precursor nevus might become obscured by melanoma. Figure 1 does not imply that all melanomas arise from nevi, but rather that melanomas have additional mutations not found in nevi. Melanomas also have a somewhat different distribution of mitogenic driver mutations from those in nevi. They have many fewer *KRAS* (1%), more *NRAS* (18%) and somewhat fewer *BRAF* (47%) mutations than nevi (Forbes et al., 2015). Perhaps some oncogenic mutations are better than others at inducing malignant as opposed to benign melanocytic growth. Where melanomas do have a detectable contiguous nevus, the majority

of tested cases (though not all) show the same *RAF* or *RAS* mutation in both the nevus and the melanoma (Shitara et al., 2015; Vredeveld et al., 2012), implying origin from the nevus.

PTKs and PTPs

Various other mitogenic drivers can be found in melanomas. To start at the top of the pathway (Figure 2), some of these are activating mutations of protein tyrosine kinases (PTKs). PTKs include both receptors (RTKs) like KIT and nonreceptor PTKs like SRC, FAK and PTK2 – both of which classes function upstream of the RAS and RAF protein families. Also reported in melanoma are disabling mutations of at least one of the protein tyrosine phosphatases, opponents of PTKs (PTPs) (Table 1; Figure 2). We reviewed PTKs and PTPs previously in relation to melanoma (Easty et al., 2011), so here the focus will be on updates and common alterations. RTK ERBB4 may be the most commonly activated PTK in cutaneous melanoma (Table 1), with evidence for frequent expression and a functional role (Easty et al., 2011; Prickett et al., 2009), although notably none of the recent sequencing studies confirmed its mutation rate to be significant, so physiological rather than genetic upregulation may be involved. ERBB4 is a receptor for neuregulins (NRGs, heregulin). It is involved in neural development and can promote melanoma cell proliferation (Prickett et al., 2009), while again NRG1 can stimulate glial cell (Sviderskaya et al., 2009) and melanoma cell proliferation (Zhang et al., 2012).

A long "tail" of less common PTK gene mutations has been reported in melanomas (Forbes et al., 2015; Hodis et al., 2012; Prickett et al., 2009; Ruhe et al., 2007); however these are large genes and the mutation rate did not reach significance for any PTK, except that *KIT* showed amplifications (Hodis et al., 2012; Krauthammer et al., 2015; The Cancer Genome Atlas, 2015). KIT, the PTK receptor for stem cell factor (KIT ligand, a melanocyte growth factor) is a special case. While it is amplified rarely in cutaneous melanoma, and its expression is often downregulated instead (Easty et al., 2011), the mutation rate for *KIT* is much higher for mucosal, acral and non-sun-exposed melanoma subtypes, with copy-number increases also (Curtin et al., 2006; Garrido and Bastian, 2010). KIT has thus become a valuable drug target in those subtypes (Carvajal, 2013). Apart from genetic change, expression levels of both EPHs and EFNs (among other PTKs and ligands) are commonly upregulated in melanoma (Easty et al., 2011). The EPH family are receptors for the ephrins (EFNs) and are involved in development, cell migration and cell-cell interactions, especially of the nervous system (Poliakov et al., 2004).

PTPs attenuate PTK signalling by removing phosphate from tyrosine in proteins, so PTP-inactivating mutations can be mitogenic drivers in cancer. *PTPRD*, encoding a receptor PTP, may be the PTP gene most commonly defective in melanoma, with both mutations and deletions reported (Table 1) (Ding et al., 2014; Forbes et al., 2015). Others are frequently mutated but mostly at rates

attributable to chance, again being large genes, with the possible exception of PTPRK (Krauthammer et al., 2012). The total rate for *PTPRD* (band 9p23) includes 9% deletions. This might be a side-effect of somatic selection for deletion of *CDKN2A* at nearby band 9p21 (more in next section), but a study of 9p in lung cancer found that 9p21 and 9p23 deletions were not contiguous nor correlated (Sato et al., 2005). See Easty et al. (2011) for further discussion of PTPs and PTKs in melanoma.

Glutamate receptors

Following the striking finding that overexpression of the metabotropic glutamate receptor GRM1 (a G-protein-coupled receptor) could strongly promote melanoma development in mice (Pollock et al., 2003), the related *GRM3* was reported to show activating mutations in melanoma at a significant frequency (Krauthammer et al., 2012) (Table 1). There is evidence for both MAPK and AKT signalling from GRM1 (Wen et al., 2014) (see GPCR in Figure 2). However GRM3 is equivocal as a driver at present, as its mutation rate did not reach significance in other studies (Hodis et al., 2012; Krauthammer et al., 2015; The Cancer Genome Atlas Network, 2015).

Downstream mitogenic signalling intermediates

Another recently identified locus for somatic melanoma driver mutations is *NF1* (Hodis et al., 2012; Xia et al., 2014), the gene responsible for familial neurofibromatosis (Schwann cell tumors) (Table 1). NF1 opposes RAS activity (Figure 2) by acting as a GTPase-activating protein (GAP), so it can function as a tumor suppressor and its inactivation can drive melanoma. Cells with oncogenic RAS or RAF would be insensitive to this, explaining why *NF1* inactivation is found mostly in melanomas without *NRAS* or *BRAF* mutations (Hodis et al., 2012). Two other elements in the MAPK signalling pathway (Figure 2), RAC1 and MAP2K1 (MEK) also showed significant although rarer activating mutations in all three large sequencing studies. (MAP2K1 with around 4% mutations is not in Table 1.)

Further down the pathway is the transcription factor and core cell-cycle activator MYC, commonly activated in cancers by gene amplification. While it was not detected in some earlier studies, several reports now seem to agree that *MYC* amplification is also widespread in cutaneous melanoma, at over 30% (Table 1). This is the figure for specific local amplification, as opposed to extra whole chromosomes 8, which are very common in melanoma (Treszl et al., 2004).

Overall, it is interesting to notice a recurrent neural theme among the driver molecules signalling nevus or melanoma proliferation; perhaps not surprising since melanocytes develop from the neural crest. NRAS is neuroblastoma RAS, NF1 is a neurofibroma gene, many EPHs/ephrins regulate neurons, and others of the above-mentioned receptors (ERBB4, GRMs) have neural roles.

One might wonder whether the known mitogenic driver classes can now account fully for the set of melanomas that do not have *BRAF, NRAS* or *NF1* mutations. Hodis et al. (2012) showed in their Figure 6 that they do seem to cover the great majority of cutaneous melanomas (97% of their samples), even without including *MYC*.

Consequent to step 1: cell senescence in nevi

Cell senescence is now understood as a major mechanism of tumor suppression (Kuilman et al., 2010; Muñoz-Espín and Serrano, 2014; Salama et al., 2014), whereby mitogenic driver mutations initially generate a small benign tumor such as a nevus, cyst or polyp, but these become arrested through senescence. Cutaneous acquired nevi are viewed as a classic case of this process. These are thousands of times more common than melanomas, an indicator of how many potential melanomas are suppressed through cell senescence. More recently cell senescence has also become strongly implicated in ageing, a topic beyond the present scope but well reviewed by others (Campisi et al., 2011; Chandler and Peters, 2013; Muñoz-Espín and Serrano, 2014). Before going further, a brief molecular explanation of cell senescence may be helpful.

Cell senescence and its effector pathways

Our knowledge of cell senescence pathways is still incomplete, as reviewed in more detail elsewhere (Chandler and Peters, 2013; Herbig and Sedivy, 2006; Salama et al., 2014). Mechanisms of cell senescence vary widely between different animal species; the emphasis here will be on humans.

Two major pathways are well established as signalling arrest in human cell senescence: the p16 pathway and the p53 pathway, with their relative importance varying between cell types (Salama et al., 2014). A simplified version of these and some of their cross-talk is shown in Figure 3. The sequences (*TP53 and CDKN2A*) encoding p53 and p16 are the two genes overall most commonly defective in human cancer (Ben-Porath and Weinberg, 2005). This underlines the importance of senescence in tumor suppression, especially since *CDKN2A* encodes (in different reading frames) two proteins that function as effectors of cell senescence, and one (p16) has no other established function. These two are called p16 (or INK4A, CDKN2A or other names) and ARF (Alternative reading frame), also called $p14^{ARF}$ in human and $p19^{ARF}$ in mouse, reflecting different protein sizes (14 and 19 kDa) (Ha et al., 2007; Ruas and Peters, 1998). Remarkably, families with germline *CDKN2A* defects have a greatly increased risk of melanoma but little change for other cancer types, except some increase for pancreatic cancer (p16 defects) or nervous-system tumors (one family with ARF defects) (Aoude et al., 2014; Cust et al., 2011), or increases in several types including smoking-related cancers with two specific exon 2 defects (de Snoo et al., 2008; Helgadottir et al., 2014). This seems

surprising since somatic defects in p16 are common in many cancer types (Ben-Porath and Weinberg, 2005; Forbes et al., 2015; Ruas and Peters, 1998).

Various different triggers can activate cell senescence (Chandler and Peters, 2013; Salama et al., 2014) (Figure 3). One is extensive cell division, leading in normal somatic cells to "replicative" senescence, the first type discovered (Hayflick and Moorhead, 1961). This involves the fact that human somatic cells express little or none of the enzyme telomerase. In the germline this reverse transcriptase maintains the length of telomeres (chromosome ends); but without telomerase the telomeres shorten at every division (Martínez and Blasco, 2011; Salama et al., 2014; Shay and Wright, 2011). Telomeres have a protective protein cap, the shelterin complex (Martínez and Blasco, 2011), but when a telomere reaches a critically short length, its cap is destabilized and the DNA end becomes exposed, leading to mis-recognition of this end as a DNA double-strand break (Bartkova et al., 2006; Rossiello et al., 2014; Von Zglinicki et al., 2005). This leads to a telomere-induced focus (TIF) of DNA damage signalling, thence activation of p53 through ATM and CHEK2 kinases, and proliferative arrest (Figure 3). This signalling becomes permanent, since such one-ended "breaks" cannot be repaired; a small number of these TIFs is sufficient to establish cell senescence (Rossiello et al., 2014; Von Zglinicki et al., 2005). As shown, p16 is also usually upregulated in replicative senescence, in correlation with TIFs (Herbig et al., 2006). p16 can be activated by induced telomere dysfunction (Jacobs and de Lange, 2005), and given also the genetic correlations of both p16 variants and telomere-related variants with ageing (Jeck et al., 2012) (and with melanoma, as discussed later), it seems increasingly likely that p16-mediated replicative senescence also depends on short telomeres. There is sporadic evidence for a route for p16 upregulation upon DNA damage (as indicated tentatively in Figure 3) (Al-Khalaf et al., 2011; Pavey et al., 2013); but there is no accepted mechanism for this.

Oncogene overexpression (where an experimenter adds an oncogene to a cell such that it is highly expressed from a strong promoter) can also upregulate both p16 and p53, leading to "oncogene-induced" senescence (OIS). Again the pathway to p16 is unclear, whereas p53 is activated through DNA damage signalling, which can be induced by oncogene overexpression (Chandler and Peters, 2013; Rossiello et al., 2014; Salama et al., 2014). In mouse cells, p53 can also be upregulated in OIS through ARF, the alternative product of *CDKN2A*, which inhibits MDM2-mediated p53 degradation, and can also help mediate replicative senescence, including in melanocytes (Ha et al., 2007), but direct evidence is lacking for such a role for human ARF (Chandler and Peters, 2013). Activated RAS or RAF oncoproteins are reported to generate DNA damage foci through (i) premature reinitiation of DNA synthesis and replication fork stalling; and (ii) promoting mitochondrial generation of reactive oxygen species (ROS) (Passos et al., 2010; Rossiello et al.,

2014). Most or all of the other cellular stresses that trigger senescence are known to cause DNA damage too: for example radiation, oxidative stress and sublethal levels of cytotoxic drugs. Accordingly the components of DNA-damage signalling foci, like 53BP1, γH2AX and phospho-CHEK2, are becoming accepted as general markers of cell senescence (Cairney et al., 2012; Herbig et al., 2006; Salama et al., 2014).

p53 can mediate arrest and senescence through various routes (Salama et al., 2014), but a major transcriptional target is the CDK (cyclin-dependent kinase) inhibitor p21 (CDKN1A) (Figure 3), which can inhibit CDKs including CDK1, -2, -4 and -6, and so can inhibit both S-phase and mitosis (Xiong et al., 1993; Chandler and Peters, 2013) (contrary to some descriptions of p21 as acting only through CDK4/6 and RB at the G1/S boundary). p21 can alternatively act as a positive assembly factor for CDK4 and -6 with cyclins D (Chandler and Peters, 2013), possibly depending on stoichiometry. p16 acts more specifically by inhibiting CDK4 and -6. Until recently the only downstream pathway from CDK4 inhibition was thought to be activation of the RB family leading to inactivation of E2F, the family of master transcription factors for S-phase (Chandler and Peters, 2013; Salama et al., 2014). However another major target of CDK4 is now known: CDK4 is also required to phosphorylate and activate the master G2/M transcription factor FOXM1 (Anders et al., 2011) (Figure 3), so that inhibition of CDK4 is also expected to arrest cells in G2, or in a G1-like tetraploid state following a failed mitosis. This may help to explain the occurrence of cells with giant or multiple nuclei, mitotic defects and/or 4N DNA content in senescent cell populations (Gray-Schopfer et al., 2006; Johmura et al., 2014; Laoukili et al., 2007). A FOXM1 transcriptional signature was reported as specifically lost in fibroblast senescence ("growth arrest") as compared to growth or quiescence (Rovillain et al., 2011). Moreover FOXM1 can actively suppress senescence in various cells, at least in part by upregulation of SKP2, a component of a ubiquitin ligase that can mediate degradation of p21 (Figure 3) and another common CDK inhibitor p27 (Anders et al., 2011; Laoukili et al., 2007; Lin et al., 2010; Rovillain et al., 2011).

There are also potential senescence pathways from PTEN, mentioned since a role for PTEN (interactive with p53) in senescence has been reported (Chen et al., 2005) and because of a proposed role in nevi (next section). One through the FOXO family is shown in Figure 3. PTEN is an antagonist of phosphoinositide 3-kinase (PI3K) and hence of AKT activation (Figure 2). FOXO proteins are AKT substrates; they have long been reported as important in ageing, and more recently as antagonists of FOXM1 (Lam et al., 2013; Laoukili et al., 2007). Secondly the AKT family can inhibit GSK3B and thus activate β-catenin (CTNNB) (Figure 2); β-catenin can repress p16 and promote immortalization in mouse melanocytes (Delmas et al., 2007). Moreover, a recent paper reports that

PTEN loss can act through a separate PI3K-independent and caveolin-dependent pathway, to upregulate nuclear β-catenin and impair p16 expression and senescence (Conde-Perez et al., 2015).

Cell senescence in nevi, and relation to melanoma susceptibility genes

Cutaneous benign nevi do not grow (except when initially forming), and express numerous senescence markers including p16, β-galactosidase, DNA damage markers 53BP1 and γH2AX, H2AFY (macroH2A), PML, H3K9Me, lack of MKI67 (Ki67), prominent nucleoli, large cell and nuclear size and multinucleacy (Alonso et al., 2004; Gray-Schopfer et al., 2006; Johmura et al., 2014; MacKenzie Ross et al., 2013; Michaloglou et al., 2005; Suram et al., 2012; Tran et al., 2012). Upon explantation, only a small fraction of melanocytes from a nevus proliferate (Soo et al., 2011). Thus benign nevi seem to be composed very largely of senescent melanocytes. As senescence markers are also commonly expressed heterogeneously in primary melanomas, Tran et al noted that no single senescence marker could reliably distinguish nevi from melanomas (Tran et al., 2012) in line with the consensus that senescence is best identified using more than one marker. Neither benign nevi nor replicatively senescent normal human neonatal melanocytes in culture express appreciable levels of p53 or p21, suggesting a high dependence on the p16 pathway for melanocyte senescence in vitro and in vivo (Bandyopadhyay et al., 2001; Gray-Schopfer et al., 2006; MacKenzie Ross et al., 2013; Stefanaki et al., 2008). This is strongly supported by the greatly extended culture lifespans (impaired senescence) of human melanocytes either homozygously defective in p16 yet ARF-competent (Sviderskaya et al., 2003), or with p16 silenced by shRNA (Fung et al., 2013). Moreover *Cdkn2a*-null mouse melanocytes do not senesce at all (Sviderskaya et al., 2002), nor do p16-null, ARF-competent mouse melanocytes, although these require extra growth factors (Ha et al., 2007).

However, not all cells in typical nevi express immunoreactive p16 (Gray-Schopfer et al., 2006; Michaloglou et al., 2005). This may reflect an admixture of some non-senescent but quiescent melanocytes, or of cells with senescence maintained by stable gene silencing (Bandyopadhyay et al., 2007; Narita et al., 2003), or there may be (an)other effector(s) able to mediate senescence without p16. Peeper's group have proposed AKT inhibition by PTEN for a maintenance role in nevus senescence (Vredeveld et al., 2012), as discussed under Step 2 below. They did not propose a pathway for this, but there are theoretical proliferative arrest pathways from PTEN both through FOXO and through AKT, β-catenin and p16 (Figures 2, 3), although the latter would become irrelevant if p16 were already lost. One more candidate for helping to effect arrest in nevus cells is p15INK4B/CDKN2B, a paralogue of p16 that maps close to *CDKN2A* and is reported to be upregulated by $BRAF^{V600E}$ overexpression (Vredeveld et al., 2012).

While we do not yet understand the inactivity of p53 in melanocyte senescence, this does help to explain why *CDKN2A* is a major susceptibility locus for melanoma yet not for other cancer types;

and why *CDK4* is also a familial melanoma locus, although rarer, with susceptibility mutations that impair p16 binding (Aoude et al., 2014; Tsao et al., 2012). If cell senescence arrests benign proliferative lesions, then a subclone must escape from senescence for further proliferation into cancer to occur. If p16 is the main effector, then loss of one copy will substantially increase the probability of such escape; and indeed loss of the p16 pathway is a major genetic feature of sporadic melanoma (Table 1 and "Step 2" below). Moreover, in mouse melanocytes, loss of only one copy of *Cdkn2a* is sufficient to impair replicative senescence (Sviderskaya et al., 2002). If this is also true in humans, such a heterozygous germline loss would lead to melanocytes dividing more times before senescing, and nevi growing larger than normal. Multiple large nevi are indeed seen in some families carrying heterozygous *CDKN2A* defects, although not all (Gruis et al., 1995; Tsao et al., 2012); it is known that other genetic factors also affect nevus size (Bataille et al., 2007; Newton-Bishop et al., 2010).

OIS versus telomeres in nevus senescence

Since nevi express activated oncogenes, their arrest is often referred to as oncogene-induced senescence (OIS). However their arrest is very different from OIS as studied in vitro. When a strong *RAS* or *RAF* oncogene is transduced and overexpressed in cultured cells, including melanocytes, the cells tend to stop dividing almost immediately (e.g. Gray-Schopfer et al., 2006; Michaloglou et al., 2005; Serrano et al., 1997). This is now attributed to activation of DNA damage pathways as discussed above. However the RAS-MAPK pathway is of course mitogenic: its physiological stimulation by growth factors does not induce DNA damage or senescence, but proliferation. The author knows of no evidence that natural oncogenic mutation of one endogenous *BRAF* or *NRAS* gene copy expressed from its own weak promoter would produce high enough levels of activation to generate immediate senescence, and the observed lesions suggest otherwise. Typical acquired benign nevi can often contain 10^6 cells or more, as judged by area and depth, requiring 20 or more cell doublings to produce them. This suggests that in nevi the oncogene (generally one copy, expressed from its own promoter) initially stimulates proliferation, not immediate arrest. It might alternatively be argued that for every nevus there are many other melanocytes that acquire an oncogene by mutation and these do senesce immediately, nevi then only arising when one such cell has/gets another mutation such as a single p16 defect, partially impairing senescence. However this does not seem compatible with the genetic data. This hypothesis would require senescence-related defects such as p16 mutations in all benign nevi, whereas p16 mutations are not detected in benign nevi (Table 1). Moreover we would then expect many small groups of melanocytes with senescence

markers in skin from young people (in whom nevi arise), whereas these are not seen in immunostained sections; only single p16-positive cells in aged epidermis (Ressler et al., 2006).

A person's age when a nevus is initiated has some relation to the number of divisions it undergoes; congenital nevi can be enormous while nevi appearing at later ages are progressively smaller (reviews: Bastian, 2003; Bennett, 2003). This in turn suggests that telomere shortening plays a part in mole size, and there is further evidence that it does: people with long telomeres tend to have more large moles than average (Bataille et al., 2007). The probability of acquisition of further mutations leading to melanoma is presumably proportional to the total number of nevus cells (among other things), and thus to the number and size of moles. Nevus number is a strong risk factor for melanoma (Newton-Bishop et al., 2010; Zhu et al., 1999) and this is one likely reason.

In line with this, several recently reported melanoma susceptibility genes are connected with telomeres (Aoude et al., 2014, 2015). One melanoma-susceptible family had a germline activating promoter mutation of *TERT*, encoding telomerase reverse transcriptase, the catalytic subunit of telomerase (Horn et al., 2013). Multiple nevi were also found in this family. This mutation creates a binding site for ETS-family and TCF-family transcription factors, and can upregulate expression of TERT, predicted to restore telomerase activity (Horn et al., 2013). Another melanoma family with the same mutation has also been identified (J.A. Newton-Bishop, personal communication). Other such families had mutations in one of several shelterin (telomeric cap) components: POT1, ACD and TERF2IP (Aoude et al., 2015; Robles-Espinoza et al., 2014; Shi et al., 2014). Moreover, a study of melanoma risk in relation to single-nucleotide polymorphisms affecting telomere length found a strong positive correlation between predicted telomere length and melanoma risk (Iles et al., 2014); and actual telomere length is also reported as a positive risk factor for melanoma. This contrasts with the finding that longer telomeres are associated with lower risk for various other cancers (Nan et al., 2011); however Rode et al. report that genetic tendency for longer telomeres does predict increased risk of cancer in general, suggesting that the positive association between risk of some cancers and observed short telomeres may be due to common causes of both, such as smoking (Rode et al., 2015).

So do oncogenes in vivo in nevi (not overexpressed) contribute at all to triggering senescence before replicative senescence would normally set in? Despite the evidence from nevus size, genetics and age, it was reported that telomeres in nevus cells are not detectably shorter than those of neighboring stromal cells (Michaloglou et al., 2005). This suggested a role for OIS, although it might also be attributable to stromal cells proliferating reactively during the growth of a nevus, thus also undergoing telomere shortening. More specifically, the great majority of nevus cells were found to exhibit DNA-damage foci associated with telomeres (TIFs), indicating senescence induced by

telomere dysfunction; but interestingly those telomeres with TIFs did not appear noticeably shorter than other telomeres in the same cells (Suram et al., 2012). These findings were potentially reconciled by evidence that DNA damage within telomeric DNA repeats is poorly repaired, so that such damage can generate subterminal DNA-damage signalling foci that are stable, like those at the termini of very short telomeres (Hewitt et al., 2012; Suram et al., 2012). These subterminal TIFs could be mediators of OIS, since oncogene activation can increase reactive oxygen species production and DNA damage (Passos et al., 2010; Rossiello et al., 2014). So the answer to "OIS or short telomeres?" may be both: OIS involving DNA damage or other pathways may add to telomere shortening to determine nevus size at arrest. One last factor to consider is WNT pathway activation. Evidence was reported for WNT signalling in human nevi, especially in or near the epidermis; and that such signalling can delay OIS in cells and mouse models. This suggested that WNT signalling may contribute to the expansion of nevi (Pawlikowski et al., 2013), irrespective of the lower expression level of oncogenes. The pathway was previously shown to repress p16 expression in mice (Delmas et al., 2007) (Figure 3).

Do nevi have a SASP?

An important property of senescent cells is the SASP or Senescence-Associated Secretory Phenotype, in which many kinds of senescent cells (such as fibroblasts, prostatic epithelial cells and drug-treated cancer cells) secrete a large repertoire of cytokines and other inflammation-related factors, such as IL6 and IL1β; also including proteases such as MMP2, angiogenic factors like VEGFs, and other growth factors (Coppé et al., 2008; Kuilman et al., 2008). There is evidence that this process depends on NFκB RELA (p65) activity and on MAPK14 (p38) (Figure 3), and can function to attract immune effector cells and promote clearance of senescent cells from the body (Chien et al., 2011; Freund et al., 2011; Ohanna et al., 2011). There seems to be little or no evidence yet on whether SASP factors are produced by melanocytic nevi in vivo. Benign nevi are not usually cleared but can persist on the skin for decades. A lymphocytic response is more associated with regression of longstanding nevi or with progression to dysplasia (Mooi and Krausz, 2007). This is interesting in the light of findings that a SASP was not detected when cells were induced to senesce with p16 overexpression alone (or p21 alone); it was interpreted that p53 signalling was required (Coppé et al., 2011). As mentioned, p53 activation appears to be absent from nevi, except sporadically in dysplastic nevi, with some impression that lymphocytes and melanophages (macrophages containing melanin, usually as fragments of dead melanocytes) may co-localize with p53 expression (Gray-Schopfer et al., 2006; MacKenzie Ross et al., 2013). However these points are circumstantial and evidence is needed on the presence or absence of SASP factors in nevi.

Step 2: Escaping primary senescence, and radial growth

Genetic requirements for immortality of human melanocytes

Cell immortality, the ability to divide indefinitely, has been described as a hallmark of cancer (Hanahan and Weinberg, 2011) – although actually not all early cancers appear to be immortal (Artandi and DePinho, 2010; Reddel, 2010), as will be discussed. Human cultured melanocytes seem to require two co-operating genetic changes for immortality: disruption of the p16 pathway, and expression of TERT (implying telomere length maintenance) (Gray-Schopfer et al., 2006; Sviderskaya et al., 2003).

Both of these changes are very common in advanced melanomas, but somatic p16 loss or reduced expression is also found early in progression, in dysplastic nevi and RGP (Gray-Schopfer et al., 2006; MacKenzie Ross et al., 2013 and literature cited). p16 pathway disruption may be the only additional change needed to generate a dysplastic rather than benign nevus (Figure 1). Actually full disruption of the p16 pathway will itself often comprise two events: defects in both p16 alleles or else one defective allele and then loss of heterozygosity, or CDK4 mutation etc; it is speculated that the difference between dysplasia and RGP may be the difference between partial and full ablation of the p16 pathway. p16/*CDKN2A* itself is either homozygously deleted, mutated, or silenced by methylation in a total of around 88% of tested melanomas (Table 1), a frequency that agrees well with the fraction of advanced (VGP) melanomas that fail to express nuclear p16 (MacKenzie Ross et al., 2013). [Mutant, inactive p16 can be cytoplasmic (Gray-Schopfer et al., 2006), so that only nuclear or nuclear plus cytoplasmic p16 can reliably be counted as active.] The p16 pathway overall may be defective in all melanomas, since various other pathway defects are also found, including *CDK4* and *RB*-family mutations, and cyclin D1 (*CCND1*) and *CDK4* amplifications (Bastian, 2014; Forbes et al., 2015; Hodis et al., 2012). *PPP6C* inactivation could speculatively be added to these since PPP6C can negatively regulate CCND1 as mentioned previously. *CDK4* is a melanoma susceptibility gene (Aoude et al., 2014), and *CCND1* is tightly linked to a melanoma susceptibility locus (Barrett et al., 2015). The *CDKN2A* neighboring paralogue *CDKN2B,* encoding p15/INK4B which is reported to be another CDK4 inhibitor, is also commonly deleted in melanoma (Table 1), although the lack of familial or sporadic point mutations in p15 suggests that its importance in melanocyte senescence and nevi is lesser, and that *CDKN2B* deletion may sometimes be a side-effect of *CDKN2A* deletion.

It is important to note that disruption of p16 itself is not a mitogenic driver and has no known biological effect on normal melanocytes in vivo. Most of the skin of *CDKN2A* mutation carriers, even homozygotes, appears normal (Gruis et al., 1995). This is because normal cells, including epidermal melanocytes, do not express p16, except sometimes upon ageing (Gray-Schopfer et al., 2006; Herbig

et al., 2006; Ressler et al., 2006). For the same reason, it is rather misleading to describe p16 as a cell-cycle regulator (suggesting a reversible action in normal cycling cells). It is more a stress-response mediator like p53. Thus p16 loss becomes a driver only once p16 is expressed and mediating senescence. Conversely, CDK4 and CCND1 do regulate the normal cell cycle (Figure 2). *CCND1* amplification is seen in some melanomas with no *RAS, RAF, NF1* or *KIT* mutations (Hodis et al., 2012), so this may double as both a mitogenic driver and a senescence defect.

Vredeveld et al (2012) reported that when melanomas are observably contiguous with a nevus, the melanoma showed reduced PTEN and/or increased AKT3 expression compared to the nevus in over half the cases (12/21), and that phospho-AKT (activated) was upregulated in the melanoma in 15/17 cases. They proposed that overactivation of the AKT pathway could independently contribute to escape from senescence by melanoma cells, on the basis that PTEN depletion could abrogate senescence induced by $BRAF^{V600E}$ overexpression in human fibroblasts and in p16-depleted human melanocytes. On the other hand this OIS appeared not to be induced by a rising PTEN level, since the PTEN level did not increase (Vredeveld et al., 2012). The situation is complex since complete loss (as opposed to a rise) of PTEN can apparently also induce senescence (Alimonti et al., 2010). A recent study of 355 melanomas and 37 nevi found no significant difference in prevalence of PTEN expression between nevi and primary melanomas, suggesting that this is not a major route for senescence evasion (Lade-Keller et al., 2014). One might wonder if instead PTEN mutation is a major route for this, but this does not seem possible since, from COSMIC, only about 10% of all melanomas have a PTEN mutation (n>1500; Table 1). Lade-Keller et al. (2014) did however note a correlation among melanomas of low PTEN level with stage, thickness, prognosis, and also with p16 loss, i.e. they associated low PTEN with more advanced melanoma. Perhaps PTEN deficiency is more connected with "Step 3", discussed below.

Another common genetic change in Table 1 probably relevant to evasion of senescence is the amplification of *TBX2* (Jönsson et al., 2007). Actually a broad area around *TBX2* becomes amplified, so that this gene is only speculatively identified as the key player, but there is other evidence for driver status. TBX2 has been described as an "anti-senescence" transcription factor, is able to repress both ARF and p21 transcription, and is frequently overexpressed in melanoma (Jacobs et al., 2000; Wansleben et al., 2014). p21 is not widely upregulated at any point in melanoma progression, even when p53 is expressed (MacKenzie Ross et al., 2013), but it is unclear why. There is evidence that TBX2 overexpression can contribute to that lack of p21 (Wansleben et al., 2014), as can MDM4 overexpression as discussed later. As mentioned, no clear role has been found for ARF in human cell senescence, although it is very important in mouse cell senescence (Ha et al., 2007). Nonetheless, a role for ARF in melanoma suppression would provide an attractive explanation for why its joint locus

with p16 is more commonly deleted or silenced than mutated in melanoma. A recently reported new function for ARF may explain this puzzle. Human melanocyte cultures were found to express ARF protein at all times, with a slight rise at high passage level. Surprisingly, ARF was located entirely in the cytoplasm, not the nucleus (ARF is normally viewed as mainly a nuclear protein), and moved increasingly to mitochondria with passage level, where it functioned to repress ROS production, in interaction with BCL2L2 (BCL-xL) (Christensen et al., 2014). A conserved hexapeptide within ARF was required for this function; interestingly this sequence is often targeted by familial melanoma mutations in *CDKN2A*, suggesting that loss of this ARF function may increase melanoma risk by mutagenicity of ROS (Christensen et al., 2014).

Apoptosis in p16-deficient melanocytes without TERT: relation to DN and RGP

A surprising property of human p16-deficient melanocytes may provide insight into RGP melanoma biology. This property, observed in cell culture, was a high rate of apoptosis, in a basic culture medium in which normal melanocytes could grow without significant apoptosis. The death of the p16-deficient cells could be suppressed by the presence of keratinocytes or two growth factors that keratinocytes secrete: stem cell factor (SCF or KITL) and endothelin 1 (EDN1) (Sviderskaya et al., 2003). There was evidence that this apoptosis was partly p53-dependent (Sviderskaya et al., 2003). In short, the p16-deficient melanocytes were more dependent on keratinocytes than were normal melanocytes. While still unexplained as to mechanism, this finding is striking because it provides a potential explanation for why dysplastic nevi and RGP melanomas are thin: that cells in these early lesions have p16 pathway defects but no other compensating (antiapoptotic) mutations. They would therefore be abnormally dependent on factors from epidermal keratinocytes, and could not survive in the deeper dermis (Figure 1).

This hypothesis was well supported by evidence that explanted RGP cells did indeed similarly show poor growth and high rates of apoptosis in monoculture, and the apoptosis was indeed suppressed by either added keratinocytes or SCF and EDN1 (Soo et al., 2011). There was also some evidence that expression of nuclear p16 in DN and RGP lesions is lower than in benign nevi (Gray-Schopfer et al., 2006; MacKenzie Ross et al., 2013). Again, there is a tendency for patients with germline *CDKN2A* mutations to develop initially-thin (SSM) melanomas (Sargen et al., 2015; van der Rhee et al., 2011). This idea also explains the apparent paradox that benign nevi (expressing p16) often grow in the deeper dermis and become quite thick, whereas the more progressed RGP melanomas do not. It is as if, even when the efficient antitumor barrier of p16 is disrupted, a second barrier appears immediately, that the cells now die if they migrate too far from the epidermis.

Step 3: Overcoming apoptosis, and vertical growth

Benign nevi may "invade" into the dermis and become very thick, as mentioned above. They can even apparently "metastasize" to form small, static deposits elsewhere: a percentage of lymph nodes biopsied for melanoma contain an area of benign melanocytic nevus (Mooi and Krausz, 2007; Topping et al., 2004). However, most RGP cells (p16-deficient) apparently die in the deeper dermis. There will then be selection pressure on surviving cells for genetic and epigenetic change(s) that reduce or suppress apoptosis, permitting invasion of the deeper dermis to form a VGP melanoma (Figure 1). This nicely explains why a wide range of antiapoptotic changes are seen in advanced, thick melanomas.

The first of these in Table 1 is loss or silencing of *APAF1*, an apoptotic effector and a transcriptional target of E2F factors (Figure 2) – activated downstream of RB-family inactivation, giving one potential pathway for the apoptosis in p16-deficient cells (Figure 3). Of note, groups assessing homozygous deletion of genes in melanoma did not detect *APAF1* as affected (Jönsson et al., 2007; Stark and Hayward, 2007), whereas the locus did feature frequently when hemizygous deletion was studied (Fujimoto et al., 2004; Moore et al., 2008), suggesting this is more common. The locus can also be methylated (Soengas et al., 2001) (Table 1). These findings are puzzling: if *APAF1* deletion promotes cell survival, why would homozygous deletion of it be rare? It is also puzzling that the sequencing studies rarely detected inactivating mutation or partial deletions of *APAF1*.

More interpretable is the common loss of *PTEN*, deleted (often homozygously) or mutated in around a quarter of melanomas (Table 1). PTEN is an inhibitor of the powerful cell survival mediator and protein kinase AKT, so PTEN loss upregulates activity of AKT and several consequent anti-apoptotic pathways, as well as growth (protein synthesis) through MTOR, and indirectly proliferation (Figure 2). PTEN can also maintain cell senescence under some conditions (Vredeveld et al., 2012) (previous section). Given these several potential antitumor effects, it is not surprising that *Pten* deletion within melanocytes in mice greatly accelerates the development of melanomas induced by activated *Braf* and (human) *NRAS* transgenes (Dankort et al., 2009; Nogueira et al., 2010). Since NRAS is expected to activate the AKT pathway (Figure 2), the oncogenic effect of *Pten* ablation additional to activated NRAS suggests that either there is room for further AKT activation, or that PTEN may have additional cellular targets. A related player is PREX2, an inhibitor of PTEN (Figure 2; Table 1), recently reported to become a melanoma driver by common activating mutations (16% of melanomas) (Berger et al., 2012); thus *PREX2* activation would be broadly equivalent to *PTEN* loss.

Since AKT is also activated by RAS but not RAF signalling (Figure 2), RAS-driven lesions may have a lesser requirement for a separate antiapoptotic change for invasion and would thus be less likely

than BRAF-driven ones to have an RGP stage. This is consistent with data that among nodular melanomas (no visible RGP), only 33% have *BRAF* activation compared to 47% of all melanomas, while 30% have *NRAS* and 4% have *KRAS* mutations compared to 18% and 1% of all melanomas (Forbes et al., 2015; Table 1). With *BRAF*-mutated melanomas this further change may occasionally be a *RAS* activation or mutation, but various other mutations in Table 1 are also capable of upregulating RAS and thus AKT signalling: PTK activation (*ERBB4*); PTP defects (*PTPRD*), and *NF1* defects.

Last in this category are *TP53* (p53) pathway mutations. Since 87% of melanomas do not have mutant p53 (Forbes et al., 2015) (Table 1), unlike many other kinds of cancer, this mutation is clearly not required in melanocytes for escape from senescence. However, this is not to say that p53 impairment is unimportant: it can reduce the apoptosis in p16-deficient human melanocytes (Sviderskaya et al., 2003), and likewise restoration of ARF [activator of p53] to *Cdkn2a*-deleted mouse melanocytes induced apoptosis rather than arrest, whereas restoration of p16 induced arrest (Sviderskaya et al., 2002). Accordingly p53 pathway defects may rate here more as anti-apoptotic than anti-senescence changes; although if ARF is largely mitochondrial in senescent melanocytes as discussed earlier (Christensen et al., 2014), then it may not be available anyway to inhibit MDM2 and activate p53 in these cells. MDM2 can be amplified in cancers, though rarely (3.5%) in melanoma (Cancer Genome Atlas Network, 2015). However we also note one very common anti-apoptotic change that is not known to be genetic, namely the upregulation (apparently post-translational) of expression of the p53 antagonist MDM4 (MDMX) (Gembarska et al., 2012). This upregulation is found in about 65% of uncultured melanomas, primary and metastatic, though not in nevi. This seems attractive as an answer to the longstanding puzzle of why many melanomas seem to express high levels of wild-type p53 (Albino et al., 1994), even p53 activated by phosphorylation, yet without associated senescence, apoptosis or expression of p21 (MacKenzie Ross et al., 2013). Unlike MDM2, MDM4 inactivates p53 by simply binding to it rather than by inducing its degradation.

Metamortality and telomeric crisis: evasion of senescence without telomere maintenance

Since TERT is apparently not yet upregulated in the majority of primary and thin melanomas (Griewank et al., 2014; Horn et al., 2013) (see also next section), then many of these melanomas will be in a state where they have evaded primary senescence and are growing, but are not yet fully immortal. Their telomeres will continue to shorten. A name for this intermediate state seems helpful, so in Figure 1 these RGP and VGP tumors are termed "**metamortal**" (meta = beyond). Markers of metamortality would include little or no p16 combined with short to absent telomeres, DNA damage signalling foci, phospho-p53 (MacKenzie Ross et al., 2013), proliferative markers such as Ki67 (MKI67), and eventually markers of telomeric crisis including anaphase bridges, abnormal

mitoses and multinucleate cells (Soo et al., 2011). This term telomeric crisis also requires explanation.

Crisis occurs in normal-cell-derived cultures when cell-senescence effector pathways are disrupted without expressing TERT, for example in fibroblasts with the p16 and p53 pathways abrogated (Bond et al., 1999), or in human melanocytes lacking functional p16 (Sviderskaya et al., 2003). Normal senescence is bypassed and cells now proliferate for many extra divisions, but their telomeres continue to shorten. Eventually a second state of net growth-arrest is reached, called telomeric crisis (Artandi and DePinho, 2010; DePinho and Polyak, 2004; Shay and Wright, 2011). Crisis also used to be called M2 or "mortality stage 2", where M1 is cell senescence (Bond et al., 1999; Shay and Wright, 2011) (see Figure 1 of Bennett and Medrano, 2002). In crisis, some telomeres become lost altogether and the chromosome ends become susceptible to being joined to one another by DNA repair, yielding dicentric chromosomes. These are unstable, generating telomeric bridge-break cycles, abnormal or aborted mitoses, frequent cell death, multiple chromosomal translocations and polyploidy, as commonly seen in developing cancers (Artandi and DePinho, 2010; DePinho and Polyak, 2004; Gisselsson and Höglund, 2005). Cell populations in crisis are typically proliferating but also dying (Bond et al., 1999). We described such crisis markers, including frequent anaphase bridges, in primary melanomas (Soo et al., 2011). Most explants from primary melanomas were not immortal: these would grow for a period but later arrest, while the few explants that did grow indefinitely were expressing TERT (Soo et al., 2011). Combining these findings with the *TERT* data (Step 4 below), it seems likely that many primary melanomas are metamortal rather than immortal: they are approaching or in crisis.

At this point there is strong selection pressure for any change that can repair the telomeres and allow escape from crisis and resumed growth. Only two such mechanisms are known: either re-expression of *TERT* or another pathway called ALT, for Alternative Lengthening of Telomeres (Artandi and DePinho, 2010; Londono-Vallejo et al., 2004). ALT is observed rarely in melanomas (7%; Heaphy et al., 2011), but *TERT* upregulation has emerged as common.

Step 4: Immortality, TERT and metastasis

Escape from crisis; immortality

In principle a melanoma could metastasize as soon as it generates cells able to survive away from the epidermis (Figure 1 step 3; VGP), and "metastasis" by benign nevi has already been mentioned. However to form a metastatic colony that will grow to produce clinical symptoms, a cell very likely needs to be immortal, or to become so within a metastasis (Figure 1 step 4). Telomerase activity has been known for some time to rise with melanoma progression (Carvalho et al., 2006;

Glaessl et al., 1999), and this has now been explained by the finding of common *TERT* promoter mutations in sporadic melanoma (like the familial mutation already mentioned but further 5' of the start site), all of which mutations generate a new binding site for ETS and TCF-family transcription factors, and can upregulate TERT expression (Horn et al., 2013; Huang et al., 2013). The upregulation was assessed only in cell culture; but given such a mutation, TERT expression should be expected in cells or tissues expressing ETS and/or TCF factors. ETS activity is upregulated by the MAPK pathway (Figure 2), and hence by mutant BRAF and the other mitogenic drivers discussed under Step 1. On the other hand, an atypical ETS family member, GABP, was recently reported to be preferentially recruited to this mutant site in various cells – similar mutations having also now been found in a range of different cancer types (Bell et al., 2015). These *TERT* mutations are reported in 70% of unspecified melanomas (Huang et al., 2013), but only 33% of primary melanomas, and none were found yet in nevi or RGP melanomas (Griewank et al., 2014; Horn et al., 2013) (Table 1). They were also reported in a striking 85% of melanoma metastases (Horn et al., 2013), although these lesions were possibly preselected for immortality, being the sources for existing cell lines. As they can upregulate TERT, it seems highly likely (although not yet proven) that these common mutations can confer immortality to lesions with p16 pathway defects. In line with this, they were found in 74% of immortal melanoma lines (Horn et al., 2013). Such promoter mutations would then be the commonest pathway to restore telomere maintenance in melanoma, though rare cases of ALT have been found, as mentioned (Heaphy et al., 2011). Occasional *TERT* gene amplifications have also been seen (Hodis et al., 2012), though it is uncertain whether these could restore sufficient TERT expression alone or would combine with a promoter mutation to increase its effect. *TERT* reactivation by translocation has also been reported, in leukemias (Nagel et al., 2010). Overall, *TERT* promoter mutation is expected to be the main route to immortalization. This has already been reported as an unfavorable prognostic marker in melanoma (Griewank et al., 2014).

Phenotype switching versus genetics in progression

Evidence has been accumulating for "phenotype switching" also playing a part in metastasis. This means an ability of melanoma cells to switch epigenetically – no mutation involved – between two fairly well-defined gene expression programs discovered through microarray analysis and called "proliferative" and "invasive" (Hoek and Goding, 2010; Widmer et al., 2012), with distinct cell behaviors as the names suggest. Proliferative cells divide more, express the master melanocytic transcription factor MITF, and have other gene expression related to pigmentary differentiation. Invasive cells express POU3F2 (also called BRN2), as well as WNT5A and AXL (Wellbrock and Arozarena, 2015), proliferate less, have more tendency to migrate and potentially metastasize, but later they can switch back to being proliferative. Their gene expression may somewhat resemble

that of melanoblasts, the migratory precursors of melanocytes. This model has been well reviewed elsewhere (Hoek and Goding, 2010; Widmer et al., 2012; Wellbrock and Arozarena, 2015) and will not be detailed here, except to mention that it is entirely compatible with the genetic progression model in Figure 1, and that there is evidence for a role of miR-211 in the switch (Boyle et al., 2011). These switching events might occur at almost any stage in genetic progression, and the invasive epigenetic phenotype might substitute for antiapoptotic mutations via expression of different growth-factor receptors. However, extensive proliferation after metastasis would still likely depend on genetic immortalization. Also certain genetic lesions are likely to influence the switching; for example around 4% of melanomas have focal amplification of *MITF* (Hodis et al., 2012), with broader amplification in further cases (Wellbrock and Arozarena, 2015); the 11.8% figure in Table 1 is from Stark and Hayward (2007). This amplification could predispose towards the proliferative phenotype. See Wellbrock and Arozarena (2015) for discussion of the role of MITF in melanoma, and its interactions with $BRAF^{V600E}$ and other melanoma-associated mutations.

Genetic steps in a different order

The genetic changes in metastatic melanoma have been described in a specific order (Figure 1), because the changing selection pressures make this likely to be the commonest sequence. However the changes could also occasionally occur by chance in a different order, helping to explain some alternative types of lesion. Heterozygous *CDKN2A* defects can occur in the germline, and then a mitogenic driver will be the second event, tending to yield large nevi with an abnormally high risk of further progression, through weakened senescence.

Melanoma genesis with no observed previous nevus may suggest that here senescence defects develop fully in a melanocyte before any mitogenic driver, or develop in a small growing nevus before it senesces. Antiapoptotic changes may occur before evasion of senescence, or simultaneously (for example with a 9p deletion that includes *PTPRD* as well as *CDKN2A*); and then the lesion could be invasive immediately – a nodular melanoma. Perhaps *PTPRD* loss is also a mitogenic driver, and/or there are other combined mitogenic and antiapoptotic driver(s) that quite often arise before senescence evasion, since in real life most nodular melanomas are reported to lack any visible associated nevus (Bevona et al., 2003).

Perspectives

This article began with the puzzle of why so many melanoma genes are connected with cell senescence defects. The whole answer is probably not yet known, but part of it seems to be that senescence evasion is relevant to two of the four key clonal steps needed to generate a metastatic

melanoma (Figure 1); and that the p16 pathway is especially important for melanoma because p53 does not noticeably contribute to melanocyte senescence (rather to apoptosis), unlike the case with other cell types. Nonetheless there is surely much still to learn about the repertoire of ways to achieve these biological changes. The model in Figure 1 may evolve on the basis of new data. Reports continue to emerge of further driver mutations and copy-number changes found in melanomas. Whole new fields of research may enter the picture: for example information is now emerging steadily about the roles of miRNAs and other noncoding RNAs in melanoma or in senescence, including p16-mediated senescence (Overhoff et al., 2014), miRNAs being another field omitted here for reasons of space. Then there are important recurrent changes in gene and protein expression in the disease for which no cell-heritable mechanism is yet known. We have the stunning example of the *TERT* promoter mutation – so common and such an obvious mechanism with hindsight – yet nobody sequenced the promoter before. This example will surely inspire many studies of promoters of other genes that are dysregulated in melanoma (and cancers generally) in mysterious ways, such as *MDM4* and various PTKs. An integrated understanding of the pathology, biology and genetics of melanoma, including the role of senescence, promises to be a powerful tool-set in the quest for more effective single and combined therapies.

Acknowledgements

The author thanks many colleagues for valuable discussions and apologizes to the many whose relevant work was not cited for reasons of space. Particular thanks to Nick Hayward, Julia Newton-Bishop, Rob Brooks and all the reviewers, for valuable comments on the article. Recent work in the author's laboratory on these topics was supported by Cancer Research UK, St George's Graduate School, Age UK, the Wellcome Trust and the Comitato Emme Rouge Onlus.

References

Al-Khalaf, H.H., Hendrayani, S.F., and Aboussekhra, A. (2011). The Atr protein kinase controls UV-dependent upregulation of $p16^{INK4A}$ through inhibition of Skp-2 related polyubiquitination/degradation. Mol. Cancer Res. *9*, 311-319.

Albino, A.P., Vidal, M.J., McNutt, N.S., Shea, C.R., Prieto, V.G., Nanus, D.M., Palmer, J.M., and Hayward, N.K. (1994). Mutation and expression of the p53 gene in human malignant melanoma. Melanoma Res. *4*, 35-45.

Alimonti, A., Nardella, C., Chen, Z., Clohessy, J.G., Carracedo, A., Trotman, L.C., Cheng, K., Varmeh, S., Kozma, S.C., Thomas, G. et al. (2010). A novel type of cellular senescence that can be enhanced in mouse models and human tumor xenografts to suppress prostate tumorigenesis. J. Clin. Invest *120*, 681-693.

Alonso, S.R., Ortiz, P., Pollan, M., Perez-Gomez, B., Sanchez, L., Acuna, M.J., Pajares, R., Martinez-Tello, F.J., Hortelano, C.M., Piris, M.A. et al. (2004). Progression in cutaneous malignant melanoma is associated with distinct expression profiles - A tissue microarray-based study. Am. J. Pathol. *164*, 193-203.

Anders, L., Ke, N., Hydbring, P., Choi, Y.J., Widlund, H.R., Chick, J.M., Zhai, H.L., Vidal, M., Gygi, S.P., Braun, P. et al. (2011). A systematic screen for CDK4/6 substrates links FOXM1 phosphorylation to senescence suppression in cancer cells. Cancer Cell *20*, 620-634.

Aoude, L.G., Pritchard, A.L., Robles-Espinoza, C.D., Wadt, K., Harland, M., Choi, J., Gartside, M., Quesada, V., Johansson, P., Palmer, J.M. et al. (2015). Nonsense Mutations in the Shelterin Complex Genes ACD and TERF2IP in Familial Melanoma. J. Natl. Cancer Inst. *107*.

Aoude, L.G., Wadt, K.A., Pritchard, A.L., and Hayward, N.K. (2014). Genetics of familial melanoma: 20 years after *CDKN2A*. Pigment Cell Melanoma Res. 28, 148-160.

Artandi, S.E., and DePinho, R.A. (2010). Telomeres and telomerase in cancer. Carcinogenesis *31*, 9-18.

Bandyopadhyay, D., Curry, J.L., Lin, Q., Richards, H.W., Chen, D., Hornsby, P.J., Timchenko, N.A., and Medrano, E.E. (2007). Dynamic assembly of chromatin complexes during cellular senescence: implications for the growth arrest of human melanocytic nevi. Aging Cell *6*, 577-591.

Bandyopadhyay, D., Timchenko, N., Suwa, T., Hornsby, P.J., Campisi, J., and Medrano, E.E. (2001). The human melanocyte: a model system to study the complexity of cellular aging and transformation in non-fibroblastic cells. Exp. Gerontol. *36*, 1265-1275.

Barrett, J.H., Taylor, J.C., Bright, C., Harland, M., Dunning, A.M., Akslen, L.A., Andresen, P.A., Avril, M.F., Azizi, E., Bianchi, S.G. et al. (2015). Fine mapping of genetic susceptibility loci for melanoma reveals a mixture of single variant and multiple variant regions. Int. J. Cancer *136*, 1351-1360.

Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L.V., Kolettas, E., Niforou, K., Zoumpourlis, V.C. et al. (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature *444*, 633-637.

Bastian, B.C. (2003). The longer your telomeres, the larger your nevus? Am. J. Dermatopathol. *25*, 83-84.

Bastian, B.C. (2014). The molecular pathology of melanoma: an integrated taxonomy of melanocytic neoplasia. Annu. Rev. Pathol. *9*, 239-271.

Bataille, V., Kato, B.S., Falchi, M., Gardner, J., Kimura, M., Lens, M., Perks, U., Valdes, A.M., Bennett, D.C., Aviv, A. et al. (2007). Nevus size and number are associated with telomere length and represent potential markers of a decreased senescence in vivo. Cancer Epidemiol. Biomarkers Prev. *16*, 1499-1502.

Bell, R.J.A., Rube, H.T., Kreig, A., Mancini, A., Fouse, S.D., Nagarajan, R.P., Choi, S., Hong, C., He, D., Pekmezci, M. et al. (2015). The transcription factor GABP selectively binds and activates the mutant TERT promoter in cancer. Science *348*, 1036-1039.

Ben-Porath, I., and Weinberg, R.A. (2005). The signals and pathways activating cellular senescence. Int. J. Biochem. Cell Biol. *37*, 961-976.

Bennett, D.C. (2003). Human melanocyte senescence and melanoma susceptibility genes. Oncogene *22*, 3063-3069.

Bennett, D.C. (2008). How to make a melanoma: what do we know of the primary clonal events? Pigment Cell Melanoma Res. *21*, 27-38.

Bennett, D.C., and Medrano, E.E. (2002). Molecular regulation of melanocyte senescence. Pigment Cell Res. *15*, 242-250.

Berger, M.F., Hodis, E., Heffernan, T.P., Deribe, Y.L., Lawrence, M.S., Protopopov, A., Ivanova, E., Watson, I.R., Nickerson, E., Ghosh, P. et al. (2012). Melanoma genome sequencing reveals frequent *PREX2* mutations. Nature *485*, 502-506.

Bevona, C., Goggins, W., Quinn, T., Fullerton, J., and Tsao, H. (2003). Cutaneous melanomas associated with nevi. Arch. Dermatol. *139*, 1620-1624.

Bond, J.A., Haughton, M.F., Rowson, J.M., Smith, P.J., Gire, V., Wynford-Thomas, D., and Wyllie, F.S. (1999). Control of replicative life span in human cells: barriers to clonal expansion intermediate between M1 senescence and M2 crisis. Mol. Cell. Biol. *19*, 3103-3114.

Boyle, G.M., Woods, S.L., Bonazzi, V.F., Stark, M.S., Hacker, E., Aoude, L.G., Dutton-Regester, K., Cook, A.L., Sturm, R.A., and Hayward, N.K. (2011). Melanoma cell invasiveness is regulated by miR-211 suppression of the BRN2 transcription factor. Pigment Cell Melanoma Res. *24*, 525-537.

Cairney, C.J., Bilsland, A.E., Evans, T.R., Roffey, J., Bennett, D.C., Narita, M., Torrance, C.J., and Keith, W.N. (2012). Cancer cell senescence: a new frontier in drug development. Drug Discov. Today *17*, 269-276.

Campisi, J., Andersen, J.K., Kapahi, P., and Melov, S. (2011). Cellular senescence: A link between cancer and age-related degenerative disease? Semin. Cancer Biol. *21*, 354-359.

Cao, J., Wan, L., Hacker, E., Dai, X., Lenna, S., Jimenez-Cervantes, C., Wang, Y., Leslie, N.R., Xu, G.X., Widlund, H.R. et al. (2013). MC1R is a potent regulator of PTEN after UV exposure in melanocytes. Mol. Cell *51*, 409-422.

Carvajal, R.D. (2013). Another option in our KIT of effective therapies for advanced melanoma. J. Clin. Oncol. *31*, 3173-3175.

Carvalho, L., Lipay, M., Belfort, F., Santos, I., Andrade, J., Haddad, A., Brunstein, F., and Ferreira, L. (2006). Telomerase activity in prognostic histopathologic features of melanoma. J. Plast. Reconstr. Aesthet. Surg. *59*, 961-968.

Chandler, H., and Peters, G. (2013). Stressing the cell cycle in senescence and aging. Curr. Opin. Cell Biol. *25*, 765-771.

Chen, Z., Trotman, L.C., Shaffer, D., Lin, H.K., Dotan, Z.A., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W. et al. (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature *436*, 725-730.

Chien, Y., Scuoppo, C., Wang, X., Fang, X., Balgley, B., Bolden, J.E., Premsrirut, P., Luo, W., Chicas, A., Lee, C.S. et al. (2011). Control of the senescence-associated secretory phenotype by NF-κB promotes senescence and enhances chemosensitivity. Genes Dev. *25*, 2125-2136.

Christensen, C., Bartkova, J., Mistrik, M., Hall, A., Lange, M.K., Ralfkiaer, U., Bartek, J., and Guldberg, P. (2014). A short acidic motif in ARF guards against mitochondrial dysfunction and melanoma susceptibility. Nat. Commun. *5*, 5348.

Chudnovsky, Y., Adams, A.E., Robbins, P.B., Lin, Q., and Khavari, P.A. (2005). Use of human tissue to assess the oncogenic activity of melanoma-associated mutations. Nat. Genet. *37*, 745-749.

Clark, W.H. (1994). From the melanocyte to melanoma to tumor biology. Adv. Cancer Res. *65*, 113-140.

Conde-Perez, A., Gros, G., Longvert, C., Pedersen, M., Petit, V., Aktary, Z., Viros, A., Gesbert, F., Delmas, V., Rambow, F. et al. (2015). A caveolin-dependent and PI3K/AKT-independent role of PTEN in β-catenin transcriptional activity. Nat. Commun. *6*, 8093.

Coppé, J.P., Patil, C.K., Rodier, F., Sun, Y., Munoz, D.P., Goldstein, J., Nelson, P.S., Desprez, P.Y., and Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS. Biol. *6*, 2853-2868.

Coppé, J.P., Rodier, F., Patil, C.K., Freund, A., Desprez, P.Y., and Campisi, J. (2011). Tumor suppressor and aging biomarker $p16^{INK4a}$ induces cellular senescence without the associated inflammatory secretory phenotype. J. Biol. Chem. *286*, 36396-36403.

Corre, S., Primot, A., Sviderskaya, E., Bennett, D.C., Vaulont, S., Goding, C.R., and Galibert, M.D. (2004). UV-induced expression of key component of the tanning process, the POMC and MC1R genes, is dependent on the p-38-activated upstream stimulating factor-1 (USF-1). J. Biol. Chem. 279, 51226-51233.

Curtin, J.A., Busam, K., Pinkel, D., and Bastian, B.C. (2006). Somatic activation of KIT in distinct subtypes of melanoma. J. Clin. Oncol. *24*, 4340-4346.

Curtin, J.A., Fridlyand, J., Kageshita, T., Patel, H.N., Busam, K.J., Kutzner, H., Cho, K.H., Aiba, S., Brocker, E.B., LeBoit, P.E. et al. (2005). Distinct sets of genetic alterations in melanoma. N. Engl. J. Med. *353*, 2135-2147.

Cust, A.E., Harland, M., Makalic, E., Schmidt, D., Dowty, J.G., Aitken, J.F., Agha-Hamilton, C., Armstrong, B.K., Barrett, J.H., Chan, M. et al. (2011). Melanoma risk for *CDKN2A* mutation carriers who are relatives of population-based case carriers in Australia and the UK. J. Med. Genet. *48*, 266-272.

Dankort, D., Curley, D.P., Cartlidge, R.A., Nelson, B., Karnezis, A.N., Damsky, W.E., Jr., You, M.J., DePinho, R.A., McMahon, M., and Bosenberg, M. (2009). $Braf^{V600E}$ cooperates with *Pten* loss to induce metastatic melanoma. Nat. Genet. *41*, 544-552.

Delmas, V., Beermann, F., Martinozzi, S., Carreira, S., Ackermann, J., Kumasaka, M., Denat, L., Goodall, J., Luciani, F., Viros, A. et al. (2007). β-catenin induces immortalisation of melanocytes by suppressing p16INK4a expression and co-operates with N-Ras in melanoma development. Genes Dev. *21*, 2923-2935.

DePinho, R.A., and Polyak, K. (2004). Cancer chromosomes in crisis. Nat. Genet. *36*, 932-934.

de Snoo, F.A., Bishop, D.T., Bergman, W., van Leeuwen, I., van der Drift, C., van Nieuwpoort, F.A., Out-Luiting, C.J., Vasen, H.F., ter Huurne, J.A., Frants, R.R. et al. (2008). Increased risk of cancer other than melanoma in CDKN2A founder mutation (p16-Leiden)-positive melanoma families. Clin. Cancer Res. *14*, 7151-7157.

Dhomen, N., Reis-Filho, J.S., Da Rocha, D.S., Hayward, R., Savage, K., Delmas, V., Larue, L., Pritchard, C., and Marais, R. (2009). Oncogenic Braf induces melanocyte senescence and melanoma in mice. Cancer Cell *15*, 294-303.

Ding, L., Kim, M., Kanchi, K.L., Dees, N.D., Lu, C., Griffith, M., Fenstermacher, D., Sung, H., Miller, C.A., Goetz, B. et al. (2014). Clonal architectures and driver mutations in metastatic melanomas. PLoS. One. *9*, e111153.

Dono, M., Angelini, G., Cecconi, M., Amaro, A., Esposito, A.I., Mirisola, V., Maric, I., Lanza, F., Nasciuti, F., Viaggi, S. et al. (2014). Mutation frequencies of *GNAQ, GNA11, BAP1, SF3B1, EIF1AX* and *TERT* in uveal melanoma: detection of an activating mutation in the *TERT* gene promoter in a single case of uveal melanoma. Br. J. Cancer *110*, 1058-1065.

Easty, D.J., Gray, S.G., O'Byrne, K.J., O'Donnell, D., and Bennett, D.C. (2011). Receptor tyrosine kinases and their activation in melanoma. Pigment Cell Melanoma Res. *24*, 446-461.

Forbes, S.A., Beare, D., Gunasekaran, P., Leung, K., Bindal, N., Boutselakis, H., Ding, M., Bamford, S., Cole, C., Ward, S. et al. (2015). COSMIC: exploring the world's knowledge of somatic mutations in human cancer. Nucleic Acids Res. *43*, D805-D811.

Freund, A., Patil, C.K., and Campisi, J. (2011). p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. EMBO J. *30*, 1536-1548.

Fujimoto, A., Takeuchi, H., Taback, B., Hsueh, E.C., Elashoff, D., Morton, D.L., and Hoon, D.S. (2004). Allelic imbalance of 12q22-23 associated with APAF-1 locus correlates with poor disease outcome in cutaneous melanoma. Cancer Res. *64*, 2245-2250.

Fung, C., Pupo, G.M., Scolyer, R.A., Kefford, R.F., and Rizos, H. (2013). p16INK4a deficiency promotes DNA hyper-replication and genetic instability in melanocytes. Pigment Cell Melanoma Res. *26*, 236-246.

Garrido, M.C., and Bastian, B.C. (2010). KIT as a therapeutic target in melanoma. J. Invest Dermatol. *130*, 20-27.

Gembarska, A., Luciani, F., Fedele, C., Russell, E.A., Dewaele, M., Villar, S., Zwolinska, A., Haupt, S., de, L.J., Yip, D. et al. (2012). MDM4 is a key therapeutic target in cutaneous melanoma. Nat. Med. *18*, 1239-1247.

Gisselsson, D., and Höglund, M. (2005). Connecting mitotic instability and chromosome aberrations in cancer--can telomeres bridge the gap? Semin. Cancer Biol. *15*, 13-23.

Glaessl, A., Bosserhoff, A.K., Buettner, R., Hohenleutner, U., Landthaler, M., and Stolz, W. (1999). Increase in telomerase activity during progression of melanocytic cells from melanocytic naevi to malignant melanomas. Arch. Dermatol. Res. *291*, 81-87.

Goldstein, A.M., Chan, M., Harland, M., Gillanders, E.M., Hayward, N.K., Avril, M.F., Azizi, E., Bianchi-Scarra, G., Bishop, D.T., Bressac-de Paillerets, B. et al. (2006). High-risk melanoma susceptibility genes and pancreatic cancer, neural system tumors, and uveal melanoma across GenoMEL. Cancer Res. *66*, 9818-9828.

Gray-Schopfer, V.C., Cheong, S.C., Chow, J., Moss, A., Abdel-Malek, Z.A., Marais, R., Wynford-Thomas, D., and Bennett, D.C. (2006). Cellular senescence in naevi and immortalisation in melanoma: a role for p16? Br. J. Cancer *95*, 496-505.

Griewank, K.G., Murali, R., Puig-Butille, J.A., Schilling, B., Livingstone, E., Potrony, M., Carrera, C., Schimming, T., Moller, I., Schwamborn, M. et al. (2014). *TERT* promoter mutation status as an independent prognostic factor in cutaneous melanoma. J. Natl. Cancer Inst. *106*.

Gruis, N.A., van der Velden, P.A., Sandkuijl, L.A., Prins, D.E., Weaver-Feldhaus, J., Kamb, A., Bergman, W., and Frants, R.R. (1995). Homozygotes for *CDKN2* (p16) germline mutation in Dutch familial melanoma kindreds. Nat. Genet. *10*, 351-353.

Ha, L., Ichikawa, T., Anver, M., Dickins, R., Lowe, S., Sharpless, N.E., Krimpenfort, P., DePinho, R.A., Bennett, D.C., Sviderskaya, E.V. et al. (2007). ARF functions as a melanoma tumor suppressor by inducing p53-independent senescence. Proc. Natl. Acad. Sci. USA *104*, 10968-10973.

Ha, L., Merlino, G., and Sviderskaya, E.V. (2008). Melanomagenesis: overcoming the barrier of melanocyte senescence. Cell Cycle *7*, 1944-1948.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of Cancer: The Next Generation. Cell *144*, 646-674.

Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. Exp. Cell Res. *25*, 585-621.

Heaphy, C.M., Subhawong, A.P., Hong, S.M., Goggins, M.G., Montgomery, E.A., Gabrielson, E., Netto, G.J., Epstein, J.I., Lotan, T.L., Westra, W.H. et al. (2011). Prevalence of the alternative lengthening of telomeres telomere maintenance mechanism in human cancer subtypes. Am. J. Pathol. *179*, 1608-1615.

Helgadottir, H., Hoiom, V., Jonsson, G., Tuominen, R., Ingvar, C., Borg, A., Olsson, H., and Hansson, J. (2014). High risk of tobacco-related cancers in *CDKN2A* mutation-positive melanoma families. J. Med. Genet. *51*, 545-552.

Herbig, U., Ferreira, M., Condel, L., Carey, D., and Sedivy, J.M. (2006). Cellular senescence in aging primates. Science *311*, 1257.

Herbig, U., and Sedivy, J.M. (2006). Regulation of growth arrest in senescence: telomere damage is not the end of the story. Mech. Ageing Dev. *127*, 16-24.

Hewitt, G., Jurk, D., Marques, F.D., Correia-Melo, C., Hardy, T., Gackowska, A., Anderson, R., Taschuk, M., Mann, J., and Passos, J.F. (2012). Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. Nat. Commun. *3*, 708.

Hodis, E., Watson, I.R., Kryukov, G.V., Arold, S.T., Imielinski, M., Theurillat, J.P., Nickerson, E., Auclair, D., Li, L., Place, C. et al. (2012). A landscape of driver mutations in melanoma. Cell *150*, 251-263.

Hoek, K.S., and Goding, C.R. (2010). Cancer stem cells versus phenotype-switching in melanoma. Pigment Cell Melanoma Res.

Horn, S., Figl, A., Rachakonda, P.S., Fischer, C., Sucker, A., Gast, A., Kadel, S., Moll, I., Nagore, E., Hemminki, K. et al. (2013). TERT promoter mutations in familial and sporadic melanoma. Science *339*, 959-961.

Huang, F.W., Hodis, E., Xu, M.J., Kryukov, G.V., Chin, L., and Garraway, L.A. (2013). Highly recurrent TERT promoter mutations in human melanoma. Science *339*, 957-959.

Hussussian, C.J., Struewing, J.P., Goldstein, A.M., Higgins, P.A.T., Ally, D.S., Sheahan, M.D., Clark, W.H., Tucker, M.A., and Dracopoli, N.C. (1994). Germline p16 mutations in familial melanoma. Nat. Genet. *8*, 15-21.

Iles, M.M., Bishop, D.T., Taylor, J.C., Hayward, N.K., Brossard, M., Cust, A.E., Dunning, A.M., Lee, J.E., Moses, E.K., Akslen, L.A. et al. (2014). The effect on melanoma risk of genes previously associated with telomere length. J. Natl. Cancer Inst. *106*.

Jacobs, J.J., Keblusek, P., Robanus-Maandag, E., Kristel, P., Lingbeek, M., Nederlof, P.M., van, W.T., van de Vijver, M.J., Koh, E.Y., Daley, G.Q. et al. (2000). Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. Nat. Genet. *26*, 291-299.

Jacobs, J.J.L., and de Lange, T. (2005). p16INK4a as a second effector of the telomere damage pathway. Cell Cycle *4*, 1364-1368.

Jeck, W.R., Siebold, A.P., and Sharpless, N.E. (2012). Review: a meta-analysis of GWAS and age-associated diseases. Aging Cell *11*, 727-731.

Johmura, Y., Shimada, M., Misaki, T., Naiki-Ito, A., Miyoshi, H., Motoyama, N., Ohtani, N., Hara, E., Nakamura, M., Morita, A. et al. (2014). Necessary and sufficient role for a mitosis skip in senescence induction. Mol. Cell *55*, 73-84.

Jönsson, G., Dahl, C., Staaf, J., Sandberg, T., Bendahl, P.O., Ringnér, M., Guldberg, P., and Borg, Å. (2007). Genomic profiling of malignant melanoma using tiling-resolution arrayCGH. Oncogene *26*, 4738-4748.

Kraehn, G.M., Utikal, J., Udart, M., Greulich, K.M., Bezold, G., Kaskel, P., Leiter, U., and Peter, R.U. (2001). Extra c-myc oncogene copies in high risk cutaneous malignant melanoma and melanoma metastases. Br. J. Cancer *84*, 72-79.

Krauthammer, M., Kong, Y., Bacchiocchi, A., Evans, P., Pornputtapong, N., Wu, C., McCusker, J.P., Ma, S., Cheng, E., Straub, R. et al. (2015). Exome sequencing identifies recurrent mutations in *NF1* and RASopathy genes in sun-exposed melanomas. Nat. Genet. *47*, 996-1002.

Krauthammer, M., Kong, Y., Ha, B.H., Evans, P., Bacchiocchi, A., McCusker, J.P., Cheng, E., Davis, M.J., Goh, G., Choi, M. et al. (2012). Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. Nat. Genet. 44, 1006-1014.

Kuilman, T., Michaloglou, C., Mooi, W.J., and Peeper, D.S. (2010). The essence of senescence. Genes Dev. *24*, 2463-2479.

Kuilman, T., Michaloglou, C., Vredeveld, L.C., Douma, S., van, D.R., Desmet, C.J., Aarden, L.A., Mooi, W.J., and Peeper, D.S. (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell *133*, 1019-1031.

Lade-Keller, J., Riber-Hansen, R., Guldberg, P., Schmidt, H., Hamilton-Dutoit, S.J., and Steiniche, T. (2014). Immunohistochemical analysis of molecular drivers in melanoma identifies p16 as an independent prognostic biomarker. J. Clin. Pathol. *67*, 520-528.

Lam, E.W., Brosens, J.J., Gomes, A.R., and Koo, C.Y. (2013). Forkhead box proteins: tuning forks for transcriptional harmony. Nat. Rev. Cancer *13*, 482-495.

Laoukili, J., Stahl, M., and Medema, R.H. (2007). FoxM1: at the crossroads of ageing and cancer. Biochim. Biophys. Acta *1775*, 92-102.

Lawrence, M.S., Stojanov, P., Mermel, C.H., Robinson, J.T., Garraway, L.A., Golub, T.R., Meyerson, M., Gabriel, S.B., Lander, E.S., and Getz, G. (2014). Discovery and saturation analysis of cancer genes across 21 tumour types. Nature *505*, 495-501.

Lin, H.K., Chen, Z., Wang, G., Nardella, C., Lee, S.W., Chan, C.H., Yang, W.L., Wang, J., Egia, A., Nakayama, K.I. et al. (2010). Skp2 targeting suppresses tumorigenesis by Arf-p53-independent cellular senescence. Nature *464*, 374-379.

Lin, J., Takata, M., Murata, H., Goto, Y., Kido, K., Ferrone, S., and Saida, T. (2009). Polyclonality of BRAF mutations in acquired melanocytic nevi. J. Natl. Cancer Inst. *101*, 1423-1427.

Londono-Vallejo, J.A., Der-Sarkissian, H., Cazes, L., Bacchetti, S., and Reddel, R.R. (2004). Alternative lengthening of telomeres is characterized by high rates of telomeric exchange. Cancer Res. *64*, 2324-2327.

MacKenzie Ross, A.D., Cook, M.G., Chong, H., Hossain, M., Pandha, H.S., and Bennett, D.C. (2013). Senescence evasion in melanoma progression: uncoupling of DNA-damage signaling from p53 activation and p21 expression. Pigment Cell Melanoma Res. *26*, 226-235.

Martínez, P., and Blasco, M.A. (2011). Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. Nat. Rev. Cancer *11*, 161-176.

Michaloglou, C., Vredeveld, L.C., Soengas, M.S., Denoyelle, C., Kuilman, T., van der Horst, C.M., Majoor, D.M., Shay, J.W., Mooi, W.J., and Peeper, D.S. (2005). $BRAF^{E600}$-associated senescence-like cell cycle arrest of human naevi. Nature *436*, 720-724.

Mooi, W.J. and Krausz, T. (2007). Pathology of Melanocytic Disorders, 2 edn (London: Hodder Arnold).

Moore, S.R., Persons, D.L., Sosman, J.A., Bobadilla, D., Bedell, V., Smith, D.D., Wolman, S.R., Tuthill, R.J., Moon, J., Sondak, V.K. et al. (2008). Detection of copy number alterations in metastatic melanoma by a DNA fluorescence in situ hybridization probe panel and array comparative genomic hybridization: a southwest oncology group study (S9431). Clin. Cancer Res. *14*, 2927-2935.

Muñoz-Espín, D., and Serrano, M. (2014). Cellular senescence: from physiology to pathology. Nat. Rev. Mol. Cell Biol. *15*, 482-496.

Nagel, I., Szczepanowski, M., Martin-Subero, J.I., Harder, L., Akasaka, T., Ammerpohl, O., Callet-Bauchu, E., Gascoyne, R.D., Gesk, S., Horsman, D. et al. (2010). Deregulation of the telomerase reverse transcriptase (*TERT*) gene by chromosomal translocations in B-cell malignancies. Blood *116*, 1317-1320.

Nan, H., Du, M., De, V., I, Manson, J.E., Liu, S., McTiernan, A., Curb, J.D., Lessin, L.S., Bonner, M.R., Guo, Q. et al. (2011). Shorter telomeres associate with a reduced risk of melanoma development. Cancer Res. *71*, 6758-6763.

Narita, M., Nunez, S., Heard, E., Narita, M., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., and Lowe, S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell *113*, 703-716.

Newton-Bishop, J.A., Chang, Y.M., Iles, M.M., Taylor, J.C., Bakker, B., Chan, M., Leake, S., Karpavicius, B., Haynes, S., Fitzgibbon, E. et al. (2010). Melanocytic nevi, nevus genes, and melanoma risk in a large case-control study in the United Kingdom. Cancer Epidemiol. Biomarkers Prev. *19*, 2043-2054.

Nogueira, C., Kim, K.H., Sung, H., Paraiso, K.H., Dannenberg, J.H., Bosenberg, M., Chin, L., and Kim, M. (2010). Cooperative interactions of PTEN deficiency and RAS activation in melanoma metastasis. Oncogene *29*, 6222-6232.

Ogbah, Z., Puig-Butille, J.A., Simonetta, F., Badenas, C., Cervera, R., Mila, J., Benitez, D., Malvehy, J., Vilella, R., and Puig, S. (2012). Molecular characterization of human cutaneous melanoma-derived cell lines. Anticancer Res. *32*, 1245-1251.

Ohanna, M., Giuliano, S., Bonet, C., Imbert, V., Hofman, V., Zangari, J., Bille, K., Robert, C., Bressac-de, P.B., Hofman, P. et al. (2011). Senescent cells develop a PARP-1 and nuclear factor-κB-associated secretome (PNAS). Genes Dev. *25*, 1245-1261.

Overhoff, M.G., Garbe, J.C., Koh, J., Stampfer, M.R., Beach, D.H., and Bishop, C.L. (2014). Cellular senescence mediated by $p16^{INK4A}$-coupled miRNA pathways. Nucleic Acids Res. *42*, 1606-1618.

Passos, J.F., Nelson, G., Wang, C., Richter, T., Simillion, C., Proctor, C.J., Miwa, S., Olijslagers, S., Hallinan, J., Wipat, A. et al. (2010). Feedback between p21 and reactive oxygen production is necessary for cell senescence. Mol. Syst. Biol. *6*, 347.

Patton, E.E., Widlund, H.R., Kutok, J.L., Kopani, K.R., Amatruda, J.F., Murphey, R.D., Berghmans, S., Mayhall, E.A., Traver, D., Fletcher, C.D. et al. (2005). BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma. Curr. Biol. *15*, 249-254.

Pavey, S., Spoerri, L., Haass, N.K., and Gabrielli, B. (2013). DNA repair and cell cycle checkpoint defects as drivers and therapeutic targets in melanoma. Pigment Cell Melanoma Res. *26*, 805-816.

Pawlikowski, J.S., McBryan, T., van, T.J., Drotar, M.E., Hewitt, R.N., Maier, A.B., King, A., Blyth, K., Wu, H., and Adams, P.D. (2013). Wnt signaling potentiates nevogenesis. Proc. Natl. Acad. Sci. U. S. A *110*, 16009-16014.

Petti, C., Molla, A., Vegetti, C., Ferrone, S., Anichini, A., and Sensi, M. (2006). Coexpression of NRASQ61R and BRAFV600E in human melanoma cells activates senescence and increases susceptibility to cell-mediated cytotoxicity. Cancer Res. *66*, 6503-6511.

Poliakov, A., Cotrina, M., and Wilkinson, D.G. (2004). Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. Dev. Cell *7*, 465-480.

Pollock, P.M., Cohen-Solal, K., Sood, R., Namkoong, J., Martino, J.J., Koganti, A., Zhu, H., Robbins, C., Makalowska, I., Shin, S.S. et al. (2003). Melanoma mouse model implicates metabotropic glutamate signaling in melanocytic neoplasia. Nat. Genet. *34*, 108-112.

Prickett, T.D., Agrawal, N.S., Wei, X., Yates, K.E., Lin, J.C., Wunderlich, J.R., Cronin, J.C., Cruz, P., Rosenberg, S.A., and Samuels, Y. (2009). Analysis of the tyrosine kinome in melanoma reveals recurrent mutations in ERBB4. Nat. Genet. *41*, 1127-1132.

Reddel, R.R. (2010). Senescence: an antiviral defense that is tumor suppressive? Carcinogenesis *31*, 19-26.

Ressler, S., Bartkova, J., Niederegger, H., Bartek, J., Scharffetter-Kochanek, K., Jansen-Durr, P., and Wlaschek, M. (2006). $p16^{INK4A}$ is a robust *in vivo* biomarker of cellular aging in human skin. Aging Cell *5*, 379-389.

Robert, C., Karaszewska, B., Schachter, J., Rutkowski, P., Mackiewicz, A., Stroiakovski, D., Lichinitser, M., Dummer, R., Grange, F., Mortier, L. et al. (2015). Improved overall survival in melanoma with combined dabrafenib and trametinib. N. Engl. J. Med. *372*, 30-39.

Rode, L., Nordestgaard, B.G., and Bojesen, S.E. (2015). Peripheral blood leukocyte telomere length and mortality among 64,637 individuals from the general population. J. Natl. Cancer Inst. *107*, djv074.

Robles-Espinoza, C.D., Harland, M., Ramsay, A.J., Aoude, L.G., Quesada, V., Ding, Z., Pooley, K.A., Pritchard, A.L., Tiffen, J.C., Petljak, M. et al. (2014). POT1 loss-of-function variants predispose to familial melanoma. Nat. Genet. *46*, 478-481.

Rossiello, F., Herbig, U., Longhese, M.P., Fumagalli, M., and d'Adda di, F.F. (2014). Irreparable telomeric DNA damage and persistent DDR signalling as a shared causative mechanism of cellular senescence and ageing. Curr. Opin. Genet. Dev. *26C*, 89-95.

Rovillain, E., Mansfield, L., Caetano, C., Alvarez-Fernandez, M., Caballero, O.L., Medema, R.H., Hummerich, H., and Jat, P.S. (2011). Activation of nuclear factor-kappa B signalling promotes cellular senescence. Oncogene *30*, 2356-2366.

Ruas, M., and Peters, G. (1998). The $p16^{INK4a}$/CDKN2A tumor suppressor and its relatives. Biochim. Biophys. Acta *1378*, F115-F177.

Ruhe, J.E., Streit, S., Hart, S., Wong, C.H., Specht, K., Knyazev, P., Knyazeva, T., Tay, L.S., Loo, H.L., Foo, P. et al. (2007). Genetic alterations in the tyrosine kinase transcriptome of human cancer cell lines. Cancer Res. *67*, 11368-11376.

Salama, R., Sadaie, M., Hoare, M., and Narita, M. (2014). Cellular senescence and its effector programs. Genes Dev. *28*, 99-114.

Sargen, M.R., Kanetsky, P.A., Newton-Bishop, J., Hayward, N.K., Mann, G.J., Gruis, N.A., Tucker, M.A., Goldstein, A.M., Bianchi-Scarra, G., Puig, S. et al. (2015). Histologic features of melanoma associated with *CDKN2A* genotype. J. Am. Acad. Dermatol. *72*, 496-507.

Sato, M., Takahashi, K., Nagayama, K., Arai, Y., Ito, N., Okada, M., Minna, J.D., Yokota, J., and Kohno, T. (2005). Identification of chromosome arm 9p as the most frequent target of homozygous deletions in lung cancer. Genes Chromosomes. Cancer *44*, 405-414.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and $p16^{INK4a}$. Cell *88*, 593-602.

Shay, J.W., and Wright, W.E. (2011). Role of telomeres and telomerase in cancer. Semin. Cancer Biol. *21*, 349-353.

Shi, J., Yang, X.R., Ballew, B., Rotunno, M., Calista, D., Fargnoli, M.C., Ghiorzo, P., Bressac-de, P.B., Nagore, E., Avril, M.F. et al. (2014). Rare missense variants in POT1 predispose to familial cutaneous malignant melanoma. Nat. Genet. *46*, 482-486.

Shitara, D., Tell-Marti, G., Badenas, C., Enohikara, M.M., Alós, L., Larque, A.B., Michalany, N., Puig-Butille, J., Carrera, C., Malvehy, J. et al. (2015). Mutational status of nevus associated-melanomas. Br. J. Dermatol.

Soengas, M.S., Capodieci, P., Polsky, D., Mora, J., Esteller, M., Opitz-Araya, X., McCombie, R., Herman, J.G., Gerald, W.L., Lazebnik, Y.A. et al. (2001). Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature *409*, 207-211.

Soo, J.K., MacKenzie Ross, A.D., Kallenberg, D.M., Milagre, C., Chong, H., Chow, J., Hill, L., Hoare, S., Collinson, R.S., Hossain, M. et al. (2011). Malignancy without immortality? Cellular immortalization as a possible late event in melanoma progression. Pigment Cell Melanoma Res. *24*, 490-503.

Stark, M., and Hayward, N. (2007). Genome-wide loss of heterozygosity and copy number analysis in melanoma using high-density single-nucleotide polymorphism arrays. Cancer Res. *67*, 2632-2642.

Stefanaki, C., Stefanaki, K., Antoniou, C., Argyrakos, T., Stratigos, A., Patereli, A., and Katsambas, A. (2008). G1 cell cycle regulators in congenital melanocytic nevi. Comparison with acquired nevi and melanomas. J. Cutan. Pathol. *35*, 799-808.

Stratton, M.R., Campbell, P.J., and Futreal, P.A. (2009). The cancer genome. Nature *458*, 719-724.

Straume, O., Sviland, L., and Akslen, L.A. (2000). Loss of nuclear p16 protein expression correlates with increased tumor cell proliferation (Ki-67) and poor prognosis in patients with vertical growth phase melanoma. Clin. Cancer Res. *6*, 1845-1853.

Suram, A., Kaplunov, J., Patel, P.L., Ruan, H., Cerutti, A., Boccardi, V., Fumagalli, M., Di, M.R., Mirani, N., Gurung, R.L. et al. (2012). Oncogene-induced telomere dysfunction enforces cellular senescence in human cancer precursor lesions. EMBO J. *31*, 2839-2851.

Sviderskaya, E.V., Easty, D.J., Lawrence, M.A., Sanchez, D.P., Negulyaev, Y.A., Patel, R.H., Anand, P., Korchev, Y.E., and Bennett, D.C. (2009). Functional neurons and melanocytes induced from immortal lines of postnatal neural crest-like stem cells. FASEB J. *23*, 3179-3192.

Sviderskaya, E.V., Gray-Schopfer, V.C., Hill, S.P., Smit, N.P., Evans-Whipp, T.J., Bond, J., Hill, L., Bataille, V., Peters, G., Kipling, D. et al. (2003). p16/cyclin-dependent kinase inhibitor 2A deficiency in human melanocyte senescence, apoptosis, and immortalization: possible implications for melanoma progression. J. Natl. Cancer Inst. *95*, 723-732.

Sviderskaya, E.V., Hill, S.P., Evans-Whipp, T.J., Chin, L., Orlow, S.J., Easty, D.J., Cheong, S.C., Beach, D., DePinho, R.A., and Bennett, D.C. (2002). $p16^{Ink4a}$ in melanocyte senescence and differentiation. J. Natl. Cancer Inst. *94*, 446-454.

The Cancer Genome Atlas Network (2015). Genomic Classification of Cutaneous Melanoma. Cell *161*, 1681-1696.

Topping, A., Dewar, D., Rose, V., Cavale, N., Allen, R., Cook, M., and Powell, B. (2004). Five years of sentinel node biopsy for melanoma: the St George's Melanoma Unit experience. Br. J. Plast. Surg. *57*, 97-104.

Tran, S.L., Haferkamp, S., Scurr, L.L., Gowrishankar, K., Becker, T.M., Desilva, C., Thompson, J.F., Scolyer, R.A., Kefford, R.F., and Rizos, H. (2012). Absence of distinguishing senescence traits in human melanocytic nevi. J. Invest Dermatol. *132*, 2226-2234.

Treszl, A., Adány, R., Rákosy, Z., Kardos, L., Bégány, A., Gilde, K., and Balázs, M. (2004). Extra copies of c-myc are more pronounced in nodular melanomas than in superficial spreading melanomas as revealed by fluorescence in situ hybridisation. Cytometry Part B-Clinical Cytometry *60B*, 37-46.

Tsao, H., Chin, L., Garraway, L.A., and Fisher, D.E. (2012). Melanoma: from mutations to medicine. Genes Dev. *26*, 1131-1155.

van den Hurk, K., Balint, B., Toomey, S., O'Leary, P.C., Unwin, L., Sheahan, K., McDermott, E.W., Murphy, I., van den Oord, J.J., Rafferty, M. et al. (2015). High-throughput oncogene mutation profiling shows demographic differences in BRAF mutation rates among melanoma patients. Melanoma Res. *25*, 189-199.

van der Rhee, J.I., Krijnen, P., Gruis, N.A., de Snoo, F.A., Vasen, H.F., Putter, H., Kukutsch, N.A., and Bergman, W. (2011). Clinical and histologic characteristics of malignant melanoma in families with a germline mutation in CDKN2A. J. Am. Acad. Dermatol. *65*, 281-288.

Von Zglinicki, T., Saretzki, G., Ladhoff, J., d'Adda di Fagagna, F., and Jackson, S.P. (2005). Human cell senescence as a DNA damage response. Mech. Ageing Dev. *126*, 111-117.

Vredeveld, L.C., Possik, P.A., Meissl, K., Michaloglou, C., Horlings, H.M., Ajouaou, A., Kortman, P.C., Dankort, D., McMahon, M., Mooi, W.J. et al. (2012). Abrogation of BRAFV600E-induced senescence by PI3K pathway activation contributes to melanomagenesis. Genes & Development *26*, 1055-1069.

Wansleben, S., Peres, J., Hare, S., Goding, C.R., and Prince, S. (2014). T-box transcription factors in cancer biology. Biochim. Biophys. Acta *1846*, 380-391.

Wellbrock, C., and Arozarena, I. (2015). Microphthalmia-associated transcription factor in melanoma development and MAP-kinase pathway targeted therapy. Pigment Cell Melanoma Res. *28*, 390-406.

Wen, Y., Li, J., Koo, J., Shin, S.S., Lin, Y., Jeong, B.S., Mehnert, J.M., Chen, S., Cohen-Sola, K.A., and Goydos, J.S. (2014). Activation of the glutamate receptor GRM1 enhances angiogenic signaling to drive melanoma progression. Cancer Res. *74*, 2499-2509.

Widmer, D.S., Cheng, P.F., Eichhoff, O.M., Belloni, B.C., Zipser, M.C., Schlegel, N.C., Javelaud, D., Mauviel, A., Dummer, R., and Hoek, K.S. (2012). Systematic classification of melanoma cells by phenotype-specific gene expression mapping. Pigment Cell Melanoma Res. *25*, 343-353.

Worm, J., Christensen, C., Gronbaek, K., Tulchinsky, E., and Guldberg, P. (2004). Genetic and epigenetic alterations of the APC gene in malignant melanoma. Oncogene *23*, 5215-5226.

Xia, J., Jia, P., Hutchinson, K.E., Dahlman, K.B., Johnson, D., Sosman, J., Pao, W., and Zhao, Z. (2014). A meta-analysis of somatic mutations from next generation sequencing of 241 melanomas: a road map for the study of genes with potential clinical relevance. Mol. Cancer Ther. *13*, 1918-1928.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. Nature *366*, 701-704.

Zhang, K., Wong, P., Zhang, L., Jacobs, B., Borden, E.C., Aster, J.C., and Bedogni, B. (2012). A Notch1-neuregulin1 autocrine signaling loop contributes to melanoma growth. Oncogene *31*, 4609-4618.

Zhu, G., Duffy, D.L., Eldridge, A., Grace, M., Mayne, C., O'Gorman, L., Aitken, J.F., Neale, M.C., Hayward, N.K., Green, A.C. et al. (1999). A major quantitative-trait locus for mole density is linked to the familial melanoma gene CDKN2A: a maximum-likelihood combined linkage and association analysis in twins and their sibs. Am. J. Hum. Genet. *65*, 483-492.

Table 1: The 20 loci with commonest known genetic/epigenetic "driver" changes in advanced human cutaneous melanoma

Gene	Locat-ion	Type of change[a] (↑,↓ = inferred effect on **activity**)	% melanomas[b] (no. of samples tested)	Data source (cultured or not)	Also earlier in progression?
CDKN2A **(p16, ARF)**	9p21	↓ Total Deletion Methylation Mutation	88[c] 50 (119) 19 (59) 19 (2263)	both	Known only for mutations: 0% (31) in benign nevi, 10% (31) in dysplastic nevi
TERT	5p15	↑ Promoter mutation	71[d] (70)	uncultured	71% in unspecified stages, 33% (77) in primary, 0% (12) in RGP, nevi
BRAF	7q34	↑ Mutation	47 (14519)	both	63% (1315) of benign nevi
TBX2	17q23	↑ Amplification Mutation	43 (46) 1 (747)	cultured both	?
APAF1	12q23	↓ Deletion (≥1 copy) Methylation	37 (98) mets 42 (24)	uncultured uncultured	19% (54) in primary
CDKN2B **(p15)**	9p21	↓ Deletion	36 (74)	cultured	?
MYC	8q24	↑ Amplification	34 (127)	uncultured	Said to be rare in nevi.
PTEN	10q23	↓ Total Mutation Deletion	23 10 (1529) 13 (119)	 both cultured	PTEN abundant in nevi.
PTPRD	9p23	↓ Total Mutation Deletion	23 14 (114) 9 (76)	 both cultured	?
NRAS	1p13	↑ Mutation	18 (7500)	both	8% (382) of benign nevi.
PREX2	8q13	↑ Mutation	16 (843)	both	?
APC	5q21-22	↓ Methylation +1 mutation	16 (94)	both	?
TP53 **(p53)**	17p13	↓ Mutation	12.5 (1299)	both	?
GRM3	7q21	↑ Mutation	12.1 (791)	both	?
ERBB4	2q34	↑ Mutation	12.1 (796)	both	?
MITF	3p14-12	↑ Amplification	11.8 (76)	both	?
NF1	17q11	↓ Mutation	11.2 (768)	both	?
ARID2	12q12	↓ Mutation	10.0 (747)	both	?
PPP6C	9q33.3	↑ Mutation	7.1 (747)	both	?
RAC1	7p22	↑ Mutation	5.8 (1158)	both	?

[a]Data for copy number changes are from Stark and Hayward (2007) and Jönsson et al. (2007), or from Kraehn et al. (2001); Moore et al. (2008); Ogbah et al. (2012) and Treszl et al. (2004) for *MYC* and Curtin et al. (2006) for *KIT*. Deletions in these reports are generally homozygous deletions. "Amplification" here indicates definite copy-number increase relative to the parent chromosome; varying criteria are used however in different studies. Methylation data are from (Straume et al., 2000) for *CDKN2A*, (Soengas et al., 2001) for *APAF1* and (Worm et al., 2004) for *APC*. Deletion of at least 1 copy of *APAF1* was reported (Fujimoto et al., 2004); since single-copy deletion may coexist with methylation this fraction (37%) was not added to the fraction for methylated.

[b]Frequencies and rankings given here are indicative only, as different frequencies are reported in different studies. Mutation data except for TERT/melanoma are from COSMIC (the Catalogue of Somatic Mutations in Cancer database) (Forbes et al., 2015), combining all cutaneous melanoma types (not mucosal) or all benign naevi except congenital (which had 59% *NRAS* mutations). Where mutations in separate splice variants were listed, only longest sequence represented here.

[c]Mutation, methylation and homozygous deletion are assumed to be mutually exclusive, so their frequencies are added. Mutation data from both cultured and uncultured samples are included here for *CDKN2A,* as most samples are now uncultured, and for comparability with other loci shown.

[d]*TERT* data by stage are from Horn et al. (2013) and Griewank et al. (2014), and "unspecified" from Huang et al. (2013).

Figure Legends

Figure 1. Genetic model for melanoma progression. The four steps are suggested to represent the commonest sequence in generation of a metastatic melanoma, where each step adds a further genetic or epigenetic change to those at the previous step. However not all melanomas will have the changes in this order or pass through each of these lesion types. Black spots at step 2 represent a host reaction (lymphocytes and macrophages). The dotted line indicates that some VGP melanomas have telomere maintenance, although most do not. See text for other details.

Figure 2. Proliferative and anti-apoptotic pathways involving genes commonly altered in melanoma. Partly adapted from a figure in Easty et al. (2011). T-bars: inhibition. Dark red and blue: components altered by known driver mutations, changes in copy number or methylation, which activate oncoproteins (red) or cause defects in tumor suppressors (blue). Light red and blue: components/processes that are pro- and anti-tumorigenic respectively, commonly upregulated or downregulated respectively in melanoma, but secondarily rather than by mutation (to our knowledge). Grey: components not thought to influence malignancy. Rectangles: transcriptional regulators or coregulators. Some symbols represent protein families (e.g. RAS, ETS, JUN). GPCR: G-protein-coupled receptors. A few alternative names for proteins are shown where the HGNC term may be less familiar. (TPA): 12-O-tetradecanoyl phorbol 13-acetate, a phorbol ester and protein kinase C agonist commonly used as a mitogen for cultured melanocytes and included for information. Dashed line indicates an uncertain mechanism. Outline of pathways only; many steps are omitted. See text for other details.

Figure 3. Cell senescence pathways involving genes commonly altered in melanoma. Some related apoptotic pathways also shown. Most details as in figure 2. Also, dotted line indicates a pathway used by other cell types, but p21 not usually upregulated in melanocytes or their lesions. Blue T-bars show core inhibitory processes for senescence. Factors upstream of PTEN are poorly understood, but activation of PTEN by MC1R (as shown) in response to ultraviolet light was reported (Cao et al., 2013). This gives potential relevance to the finding that MC1R can be upregulated downstream of MAPK14/p38 signalling via USF1 (Corre et al., 2004). Other signalling pathways (e.g. Notch, Hedgehog) can interact with those shown in Figures 2 and 3 but are omitted to avoid further complexity.

Figure 1

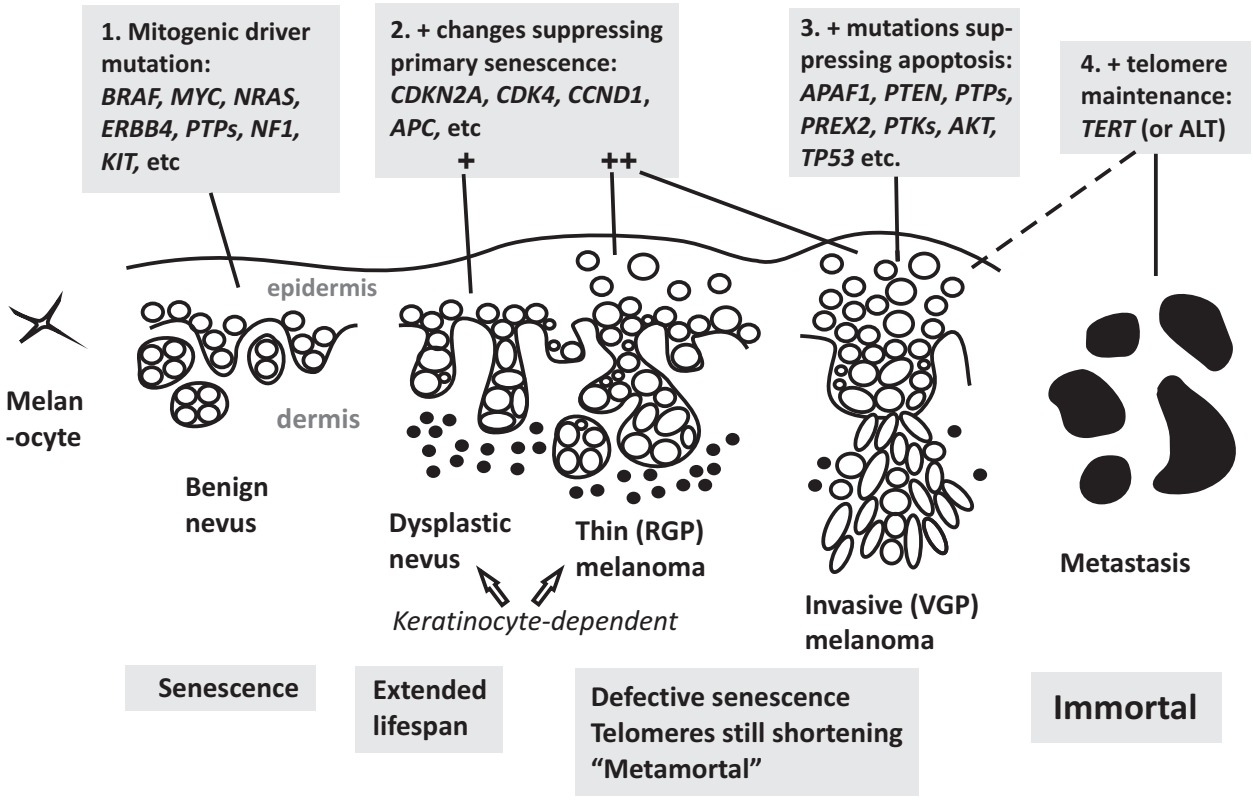

1. Mitogenic driver mutation: *BRAF, MYC, NRAS, ERBB4, PTPs, NF1, KIT,* etc
2. + changes suppressing primary senescence: *CDKN2A, CDK4, CCND1, APC,* etc
+
++
3. + mutations suppressing apoptosis: *APAF1, PTEN, PTPs, PREX2, PTKs, AKT, TP53* etc.
4. + telomere maintenance: *TERT* (or ALT)
epidermis
dermis
Melan -ocyte
Benign nevus
Dysplastic nevus
Thin (RGP) melanoma
Keratinocyte-dependent
Invasive (VGP) melanoma
Metastasis
Senescence
Extended lifespan
Defective senescence
Telomeres still shortening
"Metamortal"
Immortal

Figure 2

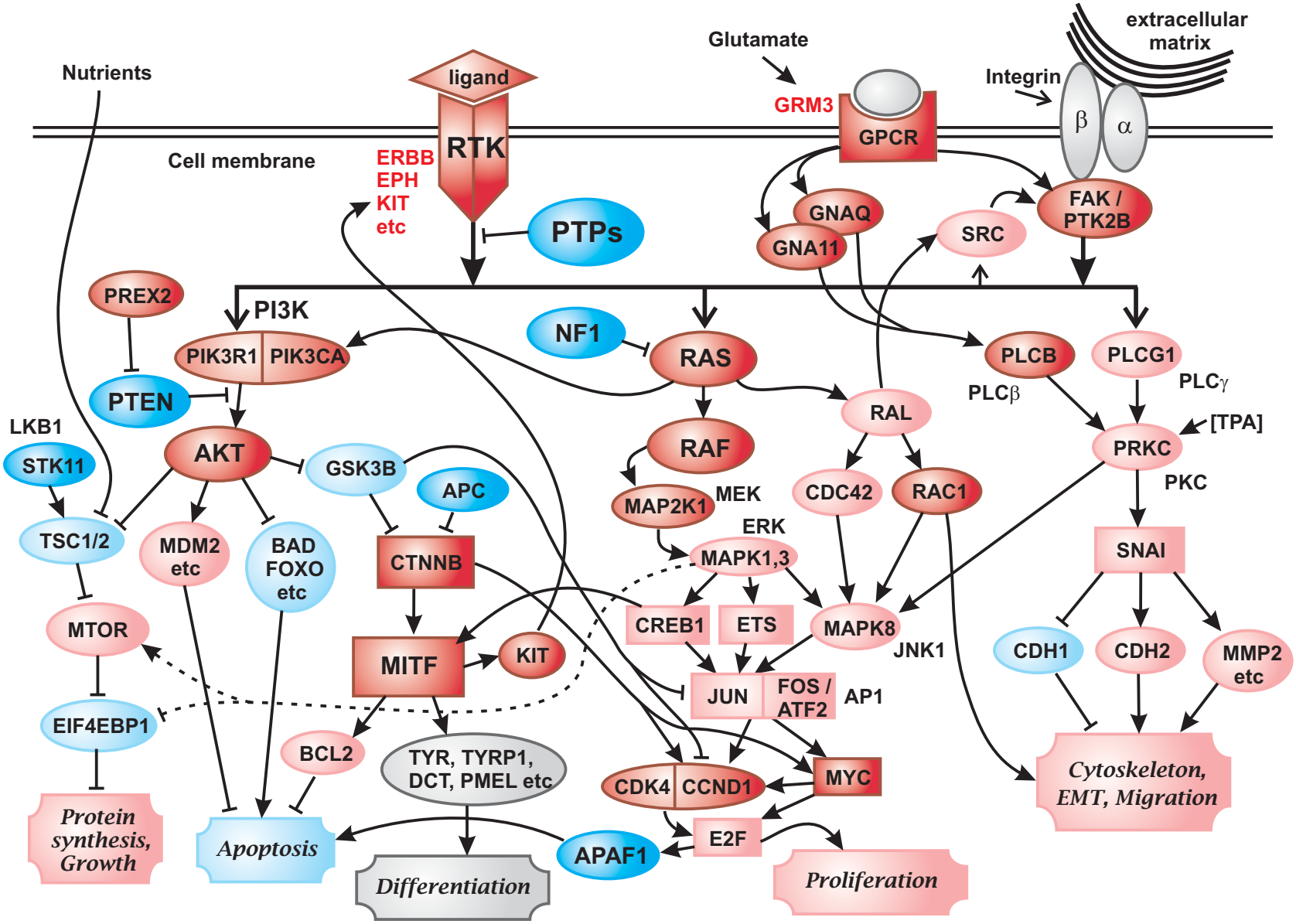
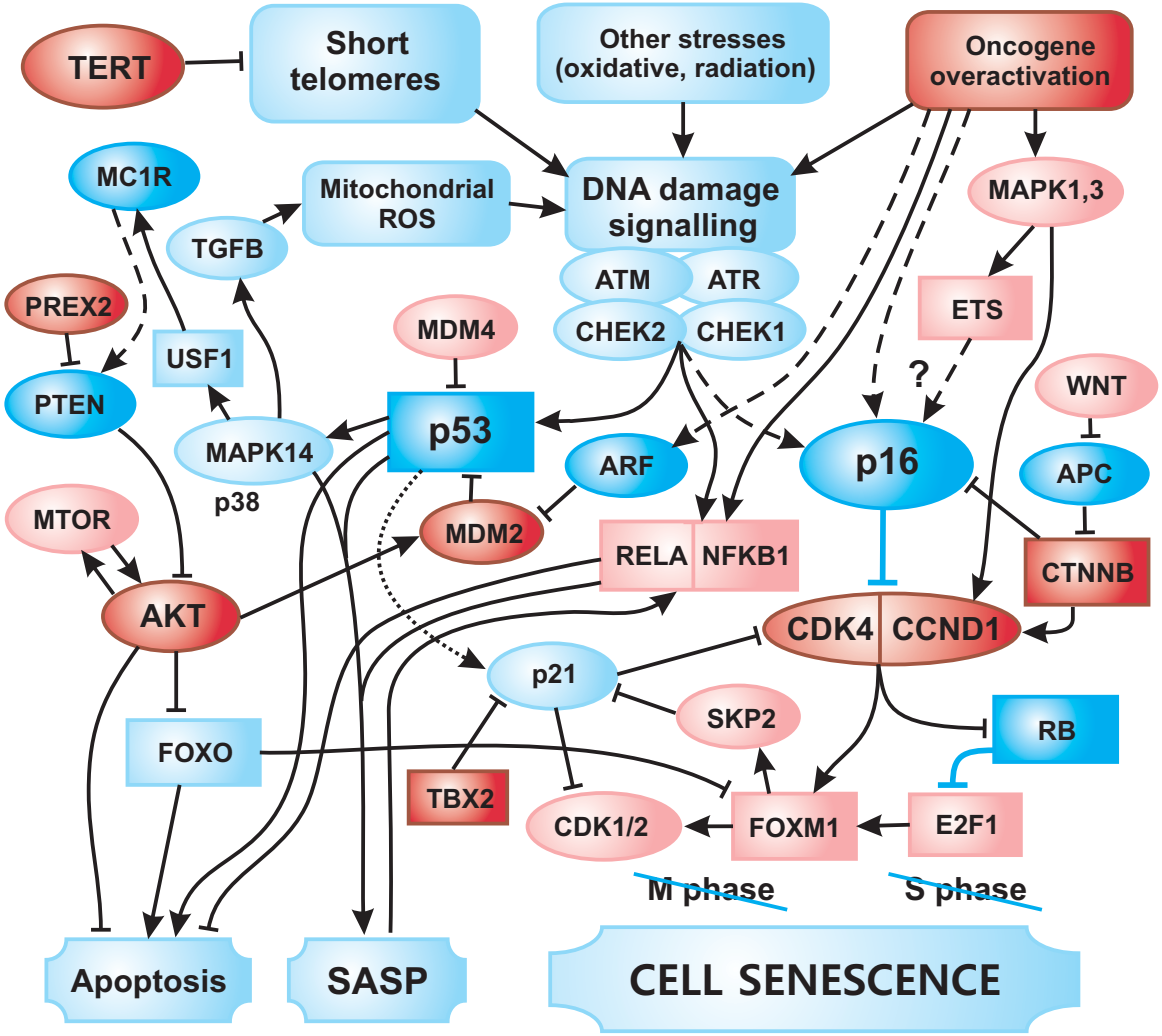

Nutrients
ligand
RTK
ERBB
EPH
KIT
etc
Cell membrane
PTPs
Glutamate
GRM3
GPCR
extracellular matrix
Integrin
β
α
GNAQ
GNA11
SRC
FAK / PTK2B
PREX2
PI3K
PIK3R1
PIK3CA
PTEN
NF1
RAS
PLCB
PLCβ
PLCG1
PLCγ
[TPA]
PRKC
PKC
RAL
RAF
MAP2K1
MEK
ERK
MAPK1,3
CDC42
RAC1
LKB1
STK11
AKT
GSK3B
APC
TSC1/2
MDM2
etc
BAD
FOXO
etc
CTNNB
MTOR
MITF
KIT
CREB1
ETS
MAPK8
JNK1
JUN
FOS /
ATF2
AP1
SNAI
CDH1
CDH2
MMP2
etc
EIF4EBP1
BCL2
TYR, TYRP1,
DCT, PMEL etc
CDK4
CCND1
MYC
E2F
APAF1
Protein
synthesis,
Growth
Apoptosis
Differentiation
Proliferation
Cytoskeleton,
EMT, Migration

Figure 3


TERT
Short telomeres
Other stresses (oxidative, radiation)
Oncogene overactivation
MC1R
Mitochondrial ROS
DNA damage signalling
MAPK1,3
TGFB
ATM
ATR
ETS
PREX2
CHEK2
CHEK1
MDM4
?
WNT
USF1
PTEN
p53
p16
MAPK14
ARF
APC
p38
MTOR
MDM2
RELA
NFKB1
CTNNB
AKT
CDK4
CCND1
p21
SKP2
RB
FOXO
TBX2
CDK1/2
FOXM1
E2F1
M phase
S phase
Apoptosis
SASP
CELL SENESCENCE