Commentary

How to make a melanoma: what do we know of the primary clonal events?

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What is the most critical moment in the development of a melanoma? According to the widely-respected Clark model of tumorigenesis (1, 2), it would be the moment at which clonal evolution generates a cell with "vertical growth-phase" (VGP) properties – a melanoma cell whose progeny can enter the deeper dermis rather than growing only in or near the epidermis. That is because VGP melanomas are believed to be competent for metastasis (1), so the tumor would then proceed to spread and kill the patient if untreated. This commentary is about our current understanding of the key clonally-heritable changes required to convert a normal melanocyte to a VGP or invasive melanoma cell. There is much we do not know, but perhaps some patterns and molecular mechanisms are starting to emerge: patterns that could help us to pick out the most critical therapeutic targets and diagnostic markers.

Primary events versus secondary changes in oncogenesis

Overall we now know of huge numbers of molecular changes that have been described in advanced melanomas compared to melanocytes. Some commoner and well-known changes are, for example, activating mutations of *BRAF* (3), silencing of E-cadherin expression (4) and acquisition of telomerase activity (reviewed in (5)) But hundreds more, mostly unfamiliar, are emerging from comparative gene expression profiling of melanomas from various stages (e.g. (6, 7)). What can we make of all these changes? Can we determine which are central to malignancy itself and thus are candidate therapeutic targets? Certainly we still have much to learn, but as a start, we can seek to distinguish **primary** from **secondary** changes. A primary event in progression would be a cellular change that is clonally inherited, that contributes to the eventual malignancy, and that occurs independently rather than as a secondary result of some other oncogenic change. These events are either genetic (gene mutation, deletion, amplification or translocation), or epigenetic (a heritable change other than in the DNA sequence, generally transcriptional modulation by DNA methylation and/or by chromatin alterations such as histone modification). In clonal evolution of cancer, such a primary event would initiate a new, more progressed, clone with a growth advantage over its neighbors.

All other changes, occurring as consequences of a primary change, can be called "secondary" changes. One can use microarrays to detect hundreds of such changes associated with a single primary change such as *BRAF* activation (8), and not all of these will contribute to malignancy. Identifying primary events is obviously scientifically valuable because they are by definition causal in cancer growth, rather than "bystanders". On the other hand secondary changes can also be causal, as will be discussed.

The commonest primary events in melanoma

Not all melanomas show the same set of primary clonal events, but some such events are more common than others. A good deal of information has been gathered on specific recurrent somatic genetic alterations in melanoma, initially by sequence analysis of candidate genes or their hot-spots, and more recently by the global approach of "CGH", comparative genome hybridization, which reveals deletions and gains in chromosomal

segments in tumors, pointing the way to detection of copy-number changes in single genes. Several recent reports using this method, including two this year, have significantly expanded our understanding of melanoma genomics (9-11). Incorporating this new information, Table 1 lists the genes that now appear to be most frequently clonally altered in sporadic human cutaneous melanoma, to the author's knowledge. This listing does not include acral and mucosal melanomas which seem to have a different genetic pattern (9), but it does combine melanomas from skin with and without chronic sun-exposure, although they also show certain mutual differences (9).

Table 1 is ranked, but this ranking should be interpreted with caution, for the following reasons. First, genes have often been tested only for mutations, rather than other events like deletion or amplification, so frequencies of aberration may be underestimated. Second, some studies use only cell lines while others use only uncultured lesional tissues, and the two can give different results. Data from cell lines (especially long-term cultures) may be unrepresentative because additional changes or selection may occur during the culture process. On the other hand, some data from uncultured lesions can also be questionable, because a proportion of normal stromal or reactive tissue may be present, especially in early, thin lesions, reducing the sensitivity for detection of say gene deletions.

This said, some interesting features emerge. Relative newcomers to the spectrum of genes known to show aberrations in melanoma are *TBX2*, *PTPRD* and *STK11*, discussed individually below. Every gene in Table 1 encodes some kind of regulatory molecule. Some of them have closely related functions, and this is illustrated in Fig. 1, which (however complicated it looks) is a greatly simplified representation of the known signaling pathways in melanoma cells that contain these particular proteins, and some of their interactions. A good many other reported actions and interactions have been omitted, although some are in a separate figure. (Certainly some important pathways and interactions will be missing altogether owing to either the ignorance of the author or the wish not to complicate the diagram further.) Apologies that there are too many symbols to explain everything in detail, but detailed information on any gene can be found in OMIM (12). In general these molecules (Table 1) control either cell proliferation, apoptosis, cell behavior or cell senescence.

1. Proliferative pathways

The products of genes subject to recurrent primary clonal alteration in melanoma (Table 1) are shown in Fig. 1 in bright red for those activated or amplified, and bright blue for those inactivated or deleted. This helps to display certain relationships between the components in Table 1. For example, it has been noted that melanomas tend to contain activating mutations of either *NRAS* or *BRAF* but not both; moreover those with increased copy numbers of cyclin D1 (*CCND1*) tend to have neither *NRAS* nor *BRAF* activation (8-10). All three alterations appear to be powerful stimulators of cell proliferation, and adding their frequencies accounts for over-proliferation in at least about 70% of melanomas. (The figure may be higher, since estimates of the freq uency of *BRAF* mutation alone vary up to around 70%.) Why the lack of overlap? Perhaps it is because too much stimulation of proliferation, which probably always works through E2F1, can lead to increased apoptosis through APAF1 (Fig. 1). This could be countered by silencing of *APAF1,* as seen in a good many advanced melanomas, attributed to promoter methylation (Table 1; (13)), or by other anti-apoptotic changes. It is also possible that the highest levels of stimulation of the MEK-MAPK (mitogen-activated protein kinase) pathway [RAS-RAF-MEK-MAPK] have reduced oncogenicity because of rapid induction of cell senescence, as seen in cultured melanocytes with activated BRAF overexpressed from a viral promoter (14, 15). This would immediately halt clonal expansion *in vivo*. Once a progressing melanoma has developed defects in senescence and apoptosis however (see sections 2 and 4), the inhibitory effects of very high MAPK activity may be lost, and the pathway may become able to be activated further, for example by copy-number increases in mutant *BRAF* and *NRAS*, as sometimes observed (10, 11, 16).

Another element in this pathway, MYC, is the subject of conflicting reports. Studies using FISH (fluorescence in-situ hybridization) concluded that around 40% of cutaneous melanomas had increased *MYC* copy number (17, 18), while CGH analyses found either no such increases around 8q24 (10) or some – number not specified (11). Further work may clarify this; perhaps there is geographical variation, or a difference between melanoma subtypes. For example, one group reported *MYC* copy-number increase in 6/67 melanomas on intermittently sun-exposed skin, but 0 on chronically sun-exposed skin (19). The links between RAF-MAPK signaling, JUN and MYC shown in Fig. 1 have partly been taken from other cell types (20), but have also partly been confirmed in melanoma cells (21). Note that there is a family of different AP1 (JUN/FOS) transcription factors. Among the JUN family, both JUN (c-Jun) and JUND (JunD) are found in melanoma cells (21, 22), with suggestions that JUND may be more mitogenic (22).

A different and less common primary pathway to stimulate cell proliferation, without MAPK activation, seems to be the reduction of RB (retinoblastoma protein family) activity by *CCND1* (cyclin D1) or *CDK4* amplification, or RB1 mutation. Loss of this pathway for E2F1 inactivation (Fig. 1) has been noted as a means of escape from cell senescence (more in section 4). However, the mutual exclusion between *CCND1* amplification, *CDK4* amplification, *BRAF* activation and *NRAS* activation (9) suggests that RB1 activity is also limiting the division of normal, non-senescent epidermal melanocytes. In this case, impaired RB1 activity through increased CDK4/cyclin D1 could substitute for MAPK activation and initiate clonal expansion.

The β-catenin pathway can be upregulated by several kinds of primary and secondary changes in melanoma (Fig. 1, Table 1). These include uncommon activating mutations of β-catenin (*CTNNB1*) itself, methylation or mutation of APC, overexpression of proto-oncoprotein SKI (not shown) (23) and probably increased activity / copy number of AKT3, assuming this can inhibit GSK3 (Fig.1) as can AKT1. Activation of this pathway is oncogenic for several reasons (23-26); β-catenin (complexed with LEF and TCF factors) can transcriptionally upregulate MYC and cyclin D1, can suppress apoptosis, can upregulate MITF (24)(see section 5), and was recently shown to repress p16 transcription (26).

Some types of cancers commonly show primary activation of proliferative pathways "from the top", by amplification or activating mutations of receptor protein tyrosine kinases (RTKs), which would activate both the MAPK and AKT pathways (20) (Fig. 1). This is less common in melanoma. There are amplifications and rare activating mutations of *KIT*, the RTK receptor for stem cell factor, a crucial mitogen and survival factor for normal melanocytes and melanoblasts (27, 28) (Table 1). The *KIT* mutations are found in melanomas that retain KIT expression (27) whereas, paradoxically, KIT mRNA and protein expression are downregulated or lost in most melanomas compared to melanocytes (60-100%), as reviewed previously, and possible reasons discussed (5, 29). KIT activation seemed more common in melanoma subtypes where BRAF and NRAS activation were rare (27). Two activating point mutations of the RTK *FGFR1* were also reported in melanomas that had neither *BRAF* nor *NRAS* mutations (30). Melanomas do show frequent secondary changes in RTK expression, of which the commonest known seem to be overexpression of the ephrin receptors EPHB3 (94%) and EPHA2 (93%) (29). The EPHA2 ligand ephrin A1/EFNA1 (initially called B61) can promote melanoma cell proliferation (31). FGFR4 (fibroblast growth-factor receptor 4) overexpression is also quite common (64%), and others overexpressed were reviewed elsewhere (29).

2. Anti-apoptotic pathways

There are a large number of known primary changes in melanoma that tend to suppress apoptosis (Table 1; Fig. 1), supporting the idea that reduced apoptosis is highly selected or required in advanced melanoma development. Frequent changes of this sort include loss of APAF1, PTEN or APC; NRAS activation acting through PI3K (20); BRAF activation acting through IKBK and NFκB activation (32), and β-catenin activation, as reviewed previously (5, 33). Transcription factor NFκB (nuclear factor κB) is a node of convergence of various

signals (Fig. 1); the NFκB pathway is prominent in mediating cytokine activation of leukocytes in inflammation, but is clearly important in melanoma also. NFκB can be held inactive in the cytoplasm by binding to the "inhibitor of κB", IκB, which in turn is inactivated by phosphorylation by the regulatory kinase IκB kinase (IKBK). NFκB is a family of related heterodimers, of which the p50-RELA dimer seems to be most active, and is found in melanoma cells (24) (RELA is also called p65). This dimer can upregulate a number of anti-apoptotic products, including those in Fig. 1 (24, 34).

Since BRAF but not NRAS oncogenic activation is commonly accompanied by independent PTEN loss in late melanomas (9-11), it seems likely that one copy of activated BRAF does not suppress apoptosis so efficiently as activated NRAS (so that PTEN loss is still selected for). Still, the newly reported activation of NFκB by BRAF (32) may account for some of the frequent overactivity of NFκB in melanoma. There are at least two other ways in which NFκB may become more active and reduce apoptosis in melanoma (Fig. 1). One is through cytokines, which might be produced in a melanoma by reactive white cells or even by the melanoma cells, notably TNF (tumor necrosis factor, TNFα). TNF (despite its name) can promote survival of melanoma cells where BRAF signaling is inhibited, and so has therapeutic implications (35). Another NFκB activation route is through hypoxia-inducible factor 1α (HIF1α), reported to be present and important for AKT transforming activity in melanomas. This was proposed to be because skin is hypoxic (36), but HIF1α has also been identified as a target of the RAF-MAPK pathway (37), and of MITF (38) (section 5).

One potential anti-apoptotic pathway from NRAS is the activation of NFκB by activated AKT, through IKBK, demonstrated at least in carcinoma cells and for AKT1 (39). The somewhat less-studied AKT3 kinase is however the AKT family member commonly upregulated in melanoma; this appears sometimes to be a primary event, since there is a recurrent broad amplification of this chromosomal region (40) (Table 1 legend). The AKT kinases are in any case potent suppressors of apoptosis, downregulating several proapoptotic factors and upregulating antiapoptotic factors including MDM2 and (in melanoma) the RAPTOR-interacting protein PRAS40/AKT1S1 (41) (Fig.1).

Lastly, deletions of receptor-type protein tyrosine phosphatase δ (*PTPRD*) in melanoma were recently pointed out by Stark et al. (10) (Table 1). PTPRD is classed as a "death PTP" (42), although its ligand and how it increases cell death rate are unknown. Accordingly, its deletion seems to provide yet another pathway for suppression of cell death. *PTPRD* is at 9p23, 13.68 Mbp telomeric to the frequently deleted *CDKN2A* locus. This seems to be a candidate for the "other" melanoma suppressor that has often been speculated to exist on chromosome arm 9p, as another focus for deletions and loss of heterozygosity (43).

3. EMT and cell migration

There is much literature on this topic, and just a few selected points will be made that relate to the pathways already under discussion. An epithelial-mesenchymal transition (EMT) is a term referring to developmental events like gastrulation and neural-crest segregation, when cells emerge from an epithelium and become separated and fibroblast-like. The co-ordinated change in gene expression in these cells also promotes cell migration, through for example protease secretion. EMT-like behavior has also been linked to invasiveness of cancers, including melanoma. In carcinoma cells, it is reported that NFκB can activate another transcription factor, SNAI1 (snail 1) (39), which is implicated in normal EMTs (24). SNAI1 can apparently also effect an EMT in melanoma cells, by modulating expression of specific genes, for example repressing E-cadherin (epithelial cadherin) and inducing N-cadherin and proteases MMP2 and t-PA among others (Fig. 1) (24, 44, 45). It has also been reported that E-cadherin can conversely repress NFκB (4), potentially leading to a bistable circuit or switch effect. For example upregulation of SNAI1 could then amplify NFκB upregulation by repressing E-cadherin. This also connects with WNT5A, a signaling ligand and "non-canonical WNT" that was the gene product most highly correlated with invasive/metastatic behavior in an earlier microarray analysis of melanoma cell lines and tumors (46). WNT5A was recently reported to induce an EMT in melanoma cells in a novel

signaling pathway through PKC and SNAI1 (44) (Fig. 1). This pathway appears to be autocrine, since melanomas can produce WNT5A according to the expression data (46). Incidentally induction of SNAI1 and EMT by PKC provides a neat explanation of the shape change from flat to spindly that is induced in cultured normal melanocytes by phorbol ester TPA (which activates PKC) (47) (Fig. 1), and cautions us to take note of culture additives during gene expression analyses.

On the other hand, some malignant melanomas are epithelial-like and thus have undergone no EMT. Sharpless and colleagues (37) classified a panel of 21 melanoma lines by gene expression profiling and identified a distinct subset expressing epithelial markers, correlating this pattern with absence of NRAS/BRAF mutations and of MAPK pathway activation. HIF1α was also lacking. These tumors presumably have alternative drivers for proliferation (overexpressed cyclin D or CDK4?), and for cell survival, a good candidate being p53 deletion, which they noted in this subset. The epithelioid melanomas also lacked TWIST1, a mesenchymal developmental regulator previously highlighted by Halaban's group in a melanoma expression analysis (48). TWIST1 expression was correlated with EMT-related genes, with low patient survival and with expression of TBX3 (37, 48) (see also section 4 for TBX3). Expression of TWIST1 in the non-epithelioid melanomas may be another outcome of the activation of NFκB, which can induce TWIST1 (49) and EMT (Fig.1).

4. Cellular senescence

It now seems established that a major barrier to the development of cancers, including melanoma, is cell senescence (14, 15, 50, 51). Cell senescence is an irreversible arrest of proliferation, undergone by somatic cells after extensive proliferation, or more rapidly following oncogene activation or some types of cellular stress (51, 52). Benign pigmented nevi have emerged as a prominent example of lesions *in vivo* that proliferate following oncogene activation, then become senescent (14, 15). Cell senescence in melanocytes and in relation to nevi and melanoma progression have been reviewed previously (5, 33, 53) but new data continue to accumulate.

A good many of the recurrent primary events in human melanoma development (Table 1) seem to be those needed to overcome cell senescence. Fig. 2 summarizes a current view of the known pathways of senescence in human cells, with special reference to melanocytes and to how the senescence-related targets of the recurrent events interact with each other. The pathways differ in some respects from those in the mouse, where they have been much studied, but there is also much in common. Triggers for senescence include telomere shortening (replicative senescence), and "oncogenic stress", overactivation of proliferative signaling. A common mechanism between the two has recently emerged; short telomeres were previously found to activate DNA damage signaling (52, 54), and there are now reports that oncogenes and MYC/E2F1 overactivity can also activate DNA damage signaling (55, 56) (Fig. 2). Following these triggers, there are two main known effector pathways of senescence in mammalian cells, the p53 pathway and the p16-RB pathway (Fig. 2). The relative importance of these two seems to vary between cell types, and the p16-RB pathway appears to have special importance in melanoma suppression.

p16/RB: The one locus most commonly altered in sporadic human melanoma, from current data (Table 1), as well as the best-known familial melanoma locus, is *CDKN2A*, encoding senescence mediators p16 and ARF (5, 57). In cancer in general, *CDKN2A* is said to be one of the two most commonly inactivated loci, with p53 (52), but in melanoma p53 mutations are seen in only about 9% of cases (58), compared to about 80% total with defects in p16 (Table 1). Amplification of cyclin D1/*CCND1* (4%) or *CDK4* (rare in cutaneous melanoma other than acral) and mutation of *RB1* (6%) also affect the p16-RB pathway (Fig. 2). This gives a possible 90% of melanomas deficient in this pathway, except that it is not clear whether all these changes are mutually exclusive in uncultured lesions. Exclusion between *CCND1* and *CDK4* amplification was however shown by Curtin et al. (9), while 100% of 22 cultured melanoma cell lines harbored either a *CDKN2A* deletion, an RB1

deletion or a CDK4 mutation (59). It may thus be mandatory to impair this pathway to generate a melanoma.

p16 appears to be regulated normally by changes in its level rather than its specific activity, since no normal post-translational modification or interaction altering its activity is known. It remains unclear how this protein level is raised in senescing human cells, however. p16 is implicated in both replicative senescence (senescence due to cells dividing extensively), and senescence induced by oncogenes including activated *BRAF* (14, 15, 52, 54). Fig 2 shows several speculative pathways whereby p16 might be upregulated by both (dotted lines). First, p16 levels may respond to telomere shortening (like those of p53, the more familiar mediator of telomere-based senescence – Fig.2). This is suggested by p16's role in replicative senescence, by the induction of p16 expression in human cells following forced telomere uncapping (60) and also by a recent report that average nevus size is greater in humans with longer telomeres (61). Since nevus size can also be increased in carriers of germline mutations in *CDKN2A,* and specifically in p16 (5), this suggests that telomere length and p16 act in a common pathway leading to growth-arrest of nevi. Second, there is evidence that the "stress" kinase p38 (MAPK14) can mediate both RAS-MAPK-induced and DNA-damage-induced senescence in other human cells (52, 62), making it a candidate inducer of p16 (Fig 2). There may therefore also be interaction with the MC1R/cAMP pathway, also reported to activate p38 in melanoma cells (63) (Fig. 2). This could help to explain the apparently quicker senescence observed in human melanocytes cultured with cAMP agonists including cholera toxin (53).

p15: Another potential player in the RB1 pathway is the *CDKN2B* locus, very close to *CDKN2A* and encoding p15 (INK4B), a paralogue of p16, and like p16 an inhibitor of the inhibitory phosphorylation of RB1 by CDK4 and CDK6 (Fig. 2). p15 is tentatively included as a senescence effector in Fig. 2, on the basis of Stark and Hayward's confirmation that it is very often included in the deletions of *CDKN2A* (36% of all melanomas) (10), and the recent demonstration that p15 has independent tumor suppressor activity in mice with *Cdkn2a* deleted (64). p15 also has evolutionary credentials as a mediator of senescence, in cells of the chick which has no p16 (65).

ARF: The second product of the *CDKN2A* locus, ARF, must be deleted virtually as frequently as p16, since its coding sequence overlaps with that of p16. It is mutated in familial melanoma less commonly than p16, but a few independent mutations are reported (reviewed: (5)). Arf is established as an important tumor suppressor and mediator of cell senescence in mouse cells, including mouse melanocytes (66). ARF is best known as an activator of p53, through blocking the action of MDM2 (Fig. 2). However, in mouse melanocytes, Arf's mediation of senescence is partly independent of p53, and a novel route for proliferative arrest was recently reported, namely promotion by Arf of the degradation of E2f1 (66). This remains to be tested in human cells.

p53 and p21: The role of p53 in melanoma is unusual. The double coloring in Fig. 2 indicates that p53 is mutated in some melanomas (9%, Table 1), but in many others normal and strongly expressed. p53 does seem to be capable of mediating cell senescence in human melanocytes, as found in two human melanocyte strains lacking normal p16, which both senesced after about 50 doublings with upregulation of p53 and p21. This senescence was greatly delayed compared to other adult melanocytes, evidence that p16 normally limits their proliferation (67). However, in vivo, melanomas often seem to have genetically normal p53, which is expressed at high levels (15) (and work cited). Accordingly it seems that in this cancer type, p53-mediated senescence is usually bypassed not by mutation or loss as in most cancers, but by some other unknown route. It seems potentially significant that growth inhibitor p21 (CDKN1A), a key mediator of p53-induced senescence in other cell types (52, 54), is not expressed in most primary melanomas even where p53 is expressed (15). Why does p53 not activate p21 expression in melanomas? One candidate reason is found in the TBX2 (T-box 2) and related TBX3 transcription factors (Fig. 2). *TBX2* has been reported by Jönsson et al. (11) to show increased copy number in 43% of melanoma cell lines, and has

therefore been entered in Table 1, though this was not noted in other CGH analyses (9, 10); so it may be a broad rather than focal amplification and hence of uncertain significance. At any rate TBX2 is commonly overexpressed in melanoma (68), while TBX3 was detected as part of a "gene expression signature" associated with BRAF mutation (8), and also in a gene set correlated with EMT in melanoma (section 3) (37, 48). Both TBX2 and TBX3 are able to act as "anti-senescence" factors for cancer cells including melanoma cells, by repressing transcription of p21, and also of ARF (68), reviewed in (69).

One other potential route for failure of induction of p21 by p53 in some melanomas is the mutation of the Peutz-Jeghers tumor suppressor, STK11 (serine-threonine kinase 11) or LKB, in 10% of melanomas (Table 1). STK11/LKB is apparently required in some cells for the activation of p21 by p53 (70).

Telomerase: A well-established mechanism of replicative senescence is the shortening of chromosomal telomeres at each cell division, and the eventual activation of a DNA damage signal and p53 by short telomeres (Fig. 2), as reviewed elsewhere, e.g. (52, 54). Even if the p53 and p16 pathways are blocked and the telomeres go on shortening, a limit is reached where chromosomes start fusing and mitosis becomes impossible ("crisis"). Hence cancers including melanomas cannot grow indefinitely (immortalize) without a mechanism to extend telomeres, which is usually through activation of the expression of telomerase (52, 54). Telomerase (in brief) is a heterodimer of the RNA TERC (hTR) and the protein TERT, and it is generally thought that TERC is ubiquitous whereas TERT has very low or no expression in normal cells, so that upregulation of TERT would be sufficient to induce telomerase activity. However, at least in melanoma, there seem to be no reports of primary clonal events affecting TERT. If TERT is indeed not genetically amplified, its upregulation must be a secondary event. Now telomerase activity is indeed upregulated in melanoma progression, as reported in several studies (reviewed in (33)). In fact, TERC is also reported to be upregulated in melanoma progression (71), while the status of TERT has yet to be directly confirmed, though the general observation of low TERT expression in normal cells combined with high telomerase activity in advanced melanoma suggests that TERT would also be upregulated. However we do not know how this happens. One possible route is through MYC, which would be induced by any proliferative stimulus like MAPK activation, and which can activate the TERT promoter (72) (Fig. 2). However, it is unclear whether this would generate enough TERT to maintain telomere length. Clearly not all cell proliferation induces this much TERT, otherwise telomeres would not shorten on cell division.

5. MITF and the differences between melanomas and other cancers

The genetic and epigenetic events in melanoma differ from those of most cancers in various respects, of which two notable differences may be connected: the rarity of p53 mutations and the high frequency of defects in p16. What is specific about regulatory circuits in pigment cells? One potential source of specificity is the protein considered to be the "master regulator" of melanocytic gene expression, namely MITF-M, microphthalmia-associated transcription factor, splicing variant M (melanocytic). MITF is the one entry in table 1 that remains to be discussed. It emerged in a microarray analysis of differences between melanomas and other tumors, and its gene is focally amplified in 10% of melanoma cell lines (73). This indicates that increased abundance of MITF-M can accelerate melanoma growth. However, MITF-M is reported to transactivate both positive and negative growth regulators: p16 (74) and p21 (75) which would inhibit growth, but also TBX2 (68) which can inhibit p21, and HIF1α (38) which can inhibit apoptosis (Fig 1) among others (73). Accordingly, the effects of MITF on proliferation seem unpredictable and may vary according to factors including other genetic changes – for example a *CDKN2A* deletion or a *TBX2* copy-number increase. Among MITF targets, TBX2 seems of special interest. As mentioned, repression of p21 by TBX2 may impair p53-mediated senescence in lesions, providing a possible explanation for the lack of selection against expression of wild-type p53 in melanoma, and the high dependence of melanocyte senescence upon the p16-RB pathway.

Summary and perspectives

An aim of this article was to ask, "what genetic or epigenetic changes are required to convert a melanocyte to a VGP melanoma cell?" Probably no individual genetic change is crucial, but rather any set of changes generating a specific set of biological outcomes. To restate a previous model (53) briefly, it seems logical that the following are needed:

[1] a change that would induce clonal expansion. Without this there is no population available for further clonal selection. Mutational activation of *BRAF* or *NRAS* or amplification of *CCND1* or *CDK4* may provide this initiating step.

[2] the changes needed to overcome melanocyte senescence, which otherwise would halt the lesion as a benign mole. In culture, two changes are required: inactivation of the p16-RB pathway and activation of telomerase (15, 67). As discussed above, at least 80-90% of uncultured melanomas do show primary inactivation of the p16-RB1 pathway. Upregulation of telomerase is also common, possibly universal, in advanced melanoma, but seems to be secondary to some primary change that is currently unidentified.

[3] one or more changes that suppress apoptosis. This proposition has two sources. Firstly many of the known primary changes do suppress apoptosis (Section 2 above). Secondly, p16 deficiency leads to a high level of apoptosis in human melanocytes, but only when they are separated from keratinocytes (in culture) (67). Thus, lesions with only changes [1] and [2] above might be keratinocyte-dependent and grow only in or near the epidermis, like early (radial growth-phase, RGP) melanomas. For progression to the VGP stage, suppression of apoptosis would thus also be needed.

This idea predicts that nodular melanomas (lacking a radial or noninvasive component) would have anti-apoptotic changes from an early stage. Since NRAS seems to activate anti-apoptotic pathways more effectively than BRAF (section 1), this also predicts that melanomas with oncogenic NRAS would tend to be thicker than those with oncogenic BRAF. A recent report supports this (76). Moreover, expression of AKT (antiapoptotic) was reported to convert a human RGP to a VGP-like melanoma in a xenograft model (77).

Finally, do primary changes in melanoma make the best therapeutic targets? In cases where the primary event is also a common one, like *BRAF* mutation, then it would seem a promising target. But in other cases there may be a number of alternative primary events, each relatively uncommon on its own, that result in a common secondary outcome, such as upregulation of NFκB. In this case, the common secondary event may also be an excellent target, so long as there is evidence that it has a causal role in malignancy rather than being a bystander. It does seem likely that our increasing understanding of the nonlinearity and crosstalk in melanoma signalling networks, fed by the hard data from genetic and expression analyses, will be valuable in selection of the best targets.

Note to SMR members: this article will shortly be revised for publication in our new journal PCMR, so comments and suggestions will be welcomed, so that I can get things right next time. Thanks to many colleagues, especially Nick Hayward, for useful discussions, and special thanks to Meenhard H. for his patience as this offering got increasingly overdue.

Table 1: Commonest known genetic/epigenetic changes in human sporadic cutaneous melanoma, ranked by reported frequency

Gene	Locat-ion	Change	% melanomas (no. of samples tested)	Data source (cultured or not)	Also earlier in progression?
CDKN2A **(p16, ARF)**	9p21	Deletion Methylation Mutation (p16) **Total**	50 (119) 19 (59) 9 (760) **78**	**uncultured** (higher in cultured)	Known only for mutations: 0% in nevi, 12% in dysplastic nevi
BRAF	7q34	Activating mutation	47 (2805)	both	51% of benign nevi
TBX2	17q23	Amplification	43 (46)	cultured	?
APAF1	12q23	Methylation	42 (24)	uncultured	No. Advanced only
CDKN2B **(p15)**	9p21	Deletion	36 (74)	cultured	?
PTEN	10q23	Mutation Deletion **Total**	17 (501) 13 (119) **28**	both cultured	?
NRAS	1p13	Activating mutn.	21 (2517)	both	21% of benign nevi.
APC	5q21-22	Methylation (+1 mutation)	16 (94)	both	?
KIT	4q12	Amplification ± activating mutn.	14 (36)	uncultured)	?
MITF	3p14	Amplification	10 (119)	cultured	?
STK11 ***(LKB)***	19p13	Mutation	10 (144)	both	?
TP53 **(p53)**	17p13	Mutation	9 (232)	uncultured	?
CTNNB1 **(β-catenin)**	3p22	Activating mutation	6 (408)	both	?
PTPRD	9p23	Deletion	6 (119)	cultured	?
RB1	13q14	Mutation	6 (67)	both	?
MYC	8q24	Amplification	1-40 (see text)	uncultured	Not nevi. Most in advanced.

Genes are included here if they have a reported total frequency of aberration of over 5%, based on at least 20 independent samples (usually more). Frequencies and rankings given here should be seen as indicative only, as different frequencies are reported in different studies. Data for **mutations** other than p53 are from the COSMIC (Catalogue of Somatic Mutations in Cancer) database (78), selecting cutaneous but not acral nor lentigo melanomas. p53 mutation data are from the IARC TP53 Mutation Database, release R11 (58), with more data than COSMIC. COSMIC data for *CDKN2A* mutations in **uncultured** melanomas were a personal communication from S. Forbes of COSMIC. This figure is used because of the large difference from melanoma cell cultures (50-70% of which have *CDKN2A* mutations). Data for **copy number** changes are from Stark et al. (10), Jönsson et al. (11), (17, 18) for *MYC* and (27) for *KIT*. Deletions, in these reports, are generally homozygous deletions. Methylation data are from (79) for *CDKN2A*, (13) for *APAF1* and (25) for *APC*. The COSMIC database contains some potential duplications where data come from different studies; this has been checked here for smaller sample numbers where they can significantly alter the apparent % aberrant, and duplications removed (cell lines with the same name assumed here to be the same line). Some genes of interest in which a small number of mutations have been reported in melanoma are: *TTN* (titin, 4/6 samples), *PRKDC* (DNA-dependent protein kinase, 3/6), *MAP4K1* (a MAP kinase kinase kinase kinase, and upstream activator of JNK, 2/6 samples), *PIK3CA* (subunit of phosphoinositide-3-kinase, 4/147, 3%), and *HDAC4* (histone deacetylase 4, 2/7 samples) (78). Rarer copy-number changes (10, 11) include *HDAC4* deletion (2q37) (3/119, 3%) and *CCND1* amplification (11q13) (5/119, 4%, but commoner in acral melanoma). *CDK4* amplification (12q14) is also reported in acral and mucosal melanomas (9). A broad chromosomal region (1q) around *AKT3* (1q43-44), see also text, shows copy number increases (40) in around 50% of cases (11), but the significance is unclear.

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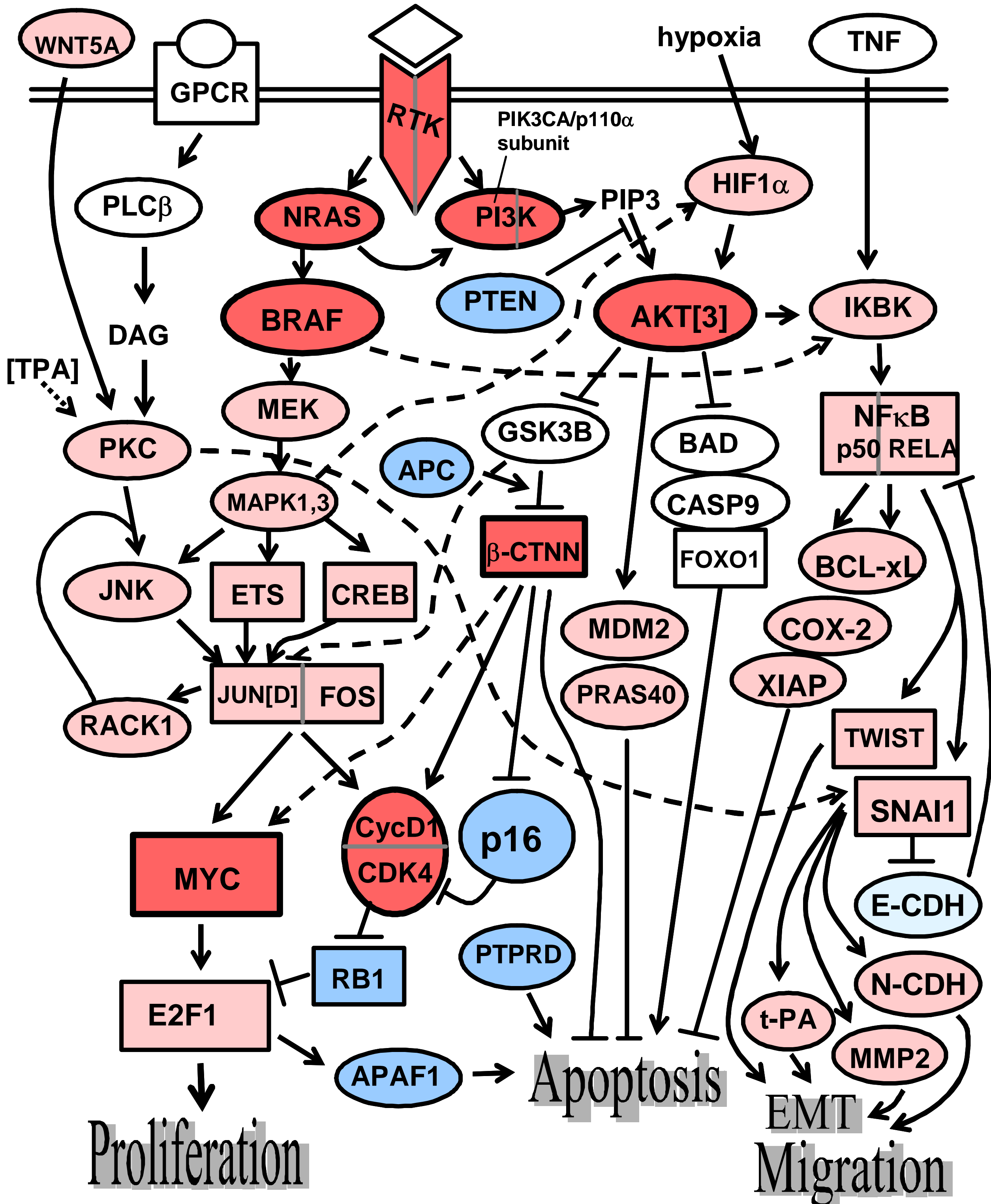
Fig. 1 Signaling pathways affected by the common primary changes in human melanoma, and some inter-relationships



An attempt has been made to focus on molecules and pathways known to be present and active in melanoma cells, although some sections of pathways are derived from the broader literature, and with the help of the wonderful pathway maps of Weinberg (20), though these tend to be centered on fibroblasts and do not always apply to melanoma cells. (contd.)

Molecules shown in **bright red** are those clonally amplified or activated in some melanomas; those in **bright blue** are clonally deleted or inactivated in some melanomas. **Pale red** and **pale blue** indicate proteins that are at least secondarily upregulated or downregulated in melanoma, respectively. **Rectangles** indicate transcription factors. Some components have a **larger** outline; this is just to emphasize apparently important signaling nodes, and does not reflect molecular size.

In general the genetic nomenclature is used here (12), with a few non-genetic terms where they are distinctly better known, e.g. GSK3β. Non-genetic abbreviations used here are: **BCL-xL**: BCL2L1; **β-CTNN**: β-catenin/CTNNB1; **CycD1**: cyclin D1/CCND1; **E-CDH**: E-cadherin/CDH1/ cadherin 1); **HIF1**α: hypoxia-inducible factor-α/HIF1A; **N-CDH**: N-cadherin/CDH2/cadherin 2; **p50,** NFKB1; **t-PA**: tissue plasminogen activator/PLAT. General terms: **GPCR**: G-protein-coupled receptor family; **RTK**, receptor tyrosine kinase family, **EMT**, epithelial-mesenchymal transition. Dashed lines are used for clarity where arrows cross. **MAPK1** and **-3** are also called ERK2 and -1 respectively. The dotted line signifies that TPA (tetradecanoyl phorbol acetate) is of course not a normal cellular component, but its action is included in the diagram for interest, because TPA strongly stimulates normal melanocyte growth, yet typically inhibits advanced melanoma cells or has no effect. PKC (protein kinase C) has at least 9 isoforms, some of which seem to be upregulated in abundance in melanoma and others downregulated; however analysis of phosphorylated (activated) PKC indicates a tendency for upregulation (44).

The assumption that NRAS activates BRAF is based on generic activation of RAFs by RASs in other cells, plus the genetic and cell-physiological evidence that BRAF and NRAS are each the predominant family member for MAPK1/3 signaling in melanoma/melanocytes. AKT3 has been found to be the most commonly overactive AKT family member in melanoma cells (40), but the pathways shown derive partly from AKT1 pathways in other cells.

Fig 2. Pathways of cell senescence in human melanocytes

Colors are used as in Fig. 1; see text concerning the two colors for p53. Key points at which the senescence effector pathways arrest cell division are shown as blue lines. Dotted lines indicate poorly understood pathways. Ub: mediated by ubiquitination. The pathways shown interact at several points with the regulatory pathways in Fig. 1, as can be seen.