# Animal Models of Melanoma

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**Abstract**

Melanoma is a highly aggressive cancer with a significant incidence in western countries and a high mortality rate. Recently developed pathway-targeted or immunotherapies are, at least in part, the fruit of gains of knowledge in chemistry, immunology, genetics, cell signaling, and cell biology. The translation of knowledge from the bench to the bedside was possible because of advanced technologies and techniques but also in vitro and in vivo models that are more refined and relevant for the study of human melanoma. This chapter reviews the different in vivo models that are used to study melanoma, including xenografts of melanoma cell lines, patient-derived xenografts, and genetically engineered animal models.

**Keywords**

Mouse · Zebrafish · NRAS · BRAF · Proliferation · Senescence

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D. E. Fisher, B. C. Bastian (eds.), *Melanoma*,
https://doi.org/10.1007/978-1-4614-7147-9_32

## Introduction

Melanoma is an aggressive cancer and is the leading cause of skin cancer deaths worldwide. Melanoma originates from melanocytes, which are neural crest-derived cells responsible for producing the pigment melanin. Melanocytes are present mainly in the skin, inner ear, meninges, hair follicles, and uveal tract. Epidermal melanocytes make extensive contacts with neighboring keratinocytes, to which they transfer their melanin. During a multi-step process known as melanomagenesis, skin melanocytes are transformed into melanoma. The first steps often involve benign proliferation of melanocytes to form a nevus, or a benign skin lesion, within which the melanocytes are clustered and lose their characteristic contacts with keratinocytes. Eventually, the melanocytes in the nevus cease proliferation and become senescent. As melanomagenesis continues, the melanocytes in the nevus are able to bypass senescence and enter the radial growth phase (RGP), where they typically proliferate superficially toward the epidermal basement membrane. These primary steps constitute melanoma "initiation." Next, during the vertical growth phase (VGP), melanoma cells continue to proliferate actively, and acquire migratory and invasive properties, which enables them to cross the basement membrane and invade the dermis. Eventually the cells progress to acquire metastatic characteristics, as they enter the bloodstream and/or lymphatic vessels and eventually colonize different tissues and organs (Larue and Beermann 2007). These latter steps can be thought of as the "progression" of the disease.

Melanomagenesis is associated with modifications of numerous cellular (proliferation, immortalization, epithelial-mesenchymal transition, and migration) and molecular (signaling pathways, cell cycle, and cell adhesion) processes. At the molecular level, the abovementioned cellular processes are modified primarily in a cell-autonomous manner. For example, the activation of different tyrosine kinase receptors (e.g., KIT, MET, and RET) by the ligands (SCF, HGF, and GDNF, respectively) leads to the induction of a number of signaling pathways (e.g., ERK1/2 MAP kinase, PI3-lipid/PTEN-AKT, and WNT/β-catenin signaling), all of which have been implicated in melanomagenesis both in vivo and in vitro (Easty et al. 2011; Paluncic et al. 2016). The modification of these signaling pathways may act to alter the cell cycle and to promote growth, migration, and invasion of the melanoma cells.

In melanoma, a number of abnormalities of gene activity have been detected, which include genetic and epigenetic lesions and high and low levels of gene expression. In cutaneous melanoma (melanoma that occurs on the skin, which is the most common type of melanoma), abnormalities often involve the activation of oncogenes, the inactivation of tumor suppressor genes, the inhibition of apoptosis, the modification of DNA repair enzyme activities, and the alterations in cell morphology and migration capacity (Larue and Beermann 2007).

Historically, successful therapies to treat melanoma have proven elusive. However in 2011, vemurafenib, an inhibitor of mutationally activated BRAF (V600), was FDA approved for the treatment of advanced melanoma in the USA (Kuzu et al. 2015). While patients initially showed robust responses to this inhibitor, resistance to the drug was almost always observed and patient relapse was frequent. Immunotherapy is another treatment strategy with some clinical success in improving overall melanoma patient survival; however both low patient response rates and relapse have been reported (Kuzu et al. 2015; Zaretsky et al. 2016). Therefore, it is essential to continue to gain a better understanding of the molecular mechanisms regulating melanomagenesis (at both the initiation and progression stages) and to be able to test potential therapeutic agents in the most relevant way possible. The most effective way to accomplish this is to use animal models of melanomagenesis, which are a vital tool in understanding and combating the disease. Since cancer cells exist within a complex tumor microenvironment composed of neighboring cells, blood vessels, host immune cells, and an extracellular matrix, the different animal models must also recapitulate these features and must also allow for the natural proliferation, bypass of

senescence, invasion, and metastasis typically observed during melanomagenesis in humans.

This chapter discusses the various animal models (e.g., mouse, canine, equine, porcine, and zebrafish) that have been used in melanoma research and describes how these different models have contributed to the understanding of melanomagenesis.

## Mouse Melanoma Models

To date, the mouse is the most commonly used organism for studying melanomagenesis in vivo. The majority of the mouse models of melanoma are focused on investigating cutaneous melanoma. Mice are advantageous for melanoma researchers as they are relatively easy to genetically manipulate and are, primarily, readily available for use. In addition, the mouse and human genomes are similar, with many noncoding sequences conserved between the two. In addition, due to the large body of knowledge that exists regarding mouse genetics, extensive analyses can be done using this model. Furthermore, mice and humans have comparable organs and physiology. However, mouse models of melanoma have some acknowledged limitations. First, the localization of melanocytes in human versus mouse skin is different with human melanocytes primarily located in the epidermis, whereas mouse melanocytes are primarily located in hair follicles. Second, mice are not prone to spontaneously develop melanoma in response to ultraviolet light, the most likely carcinogen that promotes melanomagenesis in humans (Zaidi et al. 2011; Noonan et al. 2012).

The mouse has been used to study melanomagenesis through the use of engrafted human melanoma cell lines and melanoma biopsies from patients (patient-derived xenografts, hereafter referred to as PDX) and genetically engineered mice. Xenograft models involve the culture and engraftment of either human melanoma cell lines or patient-derived melanomas into immunocompromised mice. In contrast, genetically engineered mouse models make use of sophisticated genetic manipulations that allow for altered expression of known or suspected melanoma "oncogenes" (e.g., *Nras*, *Braf*, *Rac1*) or "tumor suppressors" (*Cdkn2a*, *Nf1*, *Pten*) with temporal and spatial control of melanocyte-specific genetic alterations.

### Mouse Xenograft Models

#### Cell Line Xenografts

Researchers have established a large number of human melanoma cell lines with genetic alterations that are broadly representative of genes implicated both in the initiation and progression of melanoma. These cell lines are useful for basic manipulations and identification of potential genes important in melanoma progression and maintenance. Melanoma cell line xenografts involve the subcutaneous implantation of these melanoma cell lines into immunocompromised/deficient mice that will not reject the cells (e.g., nude athymic (nu/nu) mice or severe combined immunodeficient (SCID/SCID) mice). In doing so, the implanted cells are able to adhere, to grow, to induce angiogenesis, and to interact directly with the blood and lymphatic vessels, allowing for the in vivo assessment of tumor growth as well as response to various therapeutic interventions (Kuzu et al. 2015). This type of model is simple to use, as numerous melanoma cell lines are readily available for manipulation and implantation. However, many such melanoma cell lines have been cultured for several years under nonphysiological conditions (e.g., growth in 2D, on plastic, presence of calf serum, etc.), and cells that were established and propagated may not accurately reflect the initial tumor from which the cell lines were derived. As a result, these xenograft models have lower frequency of correctly predicting clinical outcomes and drugs that are successful using these models often fail during clinical trials (Kuzu et al. 2015).

#### Patient-Derived Xenografts (PDX)

In this model, tumors from human melanoma patients are surgically removed, cut into small specimens, and subcutaneously implanted into nude mice. PDX have several advantages over

cell line xenografts. For example, tumors that form in these mouse models retain similarities to the original tumors (compared to cell line xenografts) and thus may more accurately reflect the diversity of human melanoma. In several studies, it was observed that PDX have similar histological, transcriptomic, and polymorphic/copy number features to the tumors from which they were derived (Tentler et al. 2012). These PDX are also more useful in accurately predicting therapeutic drug efficiency. This model also allows for the potential of creating a large number of xenografted mice from a single patient tumor, as the tumors can be serially biopsied and injected into different mice. Due to the heterogeneity of the tumors, a variety of different clones, each with different characteristics (including a different potential for therapeutic drug resistance), can be generated and subsequently analyzed (Einarsdottir et al. 2014; Kemper et al. 2015, 2016; Krepler et al. 2016). More recently, genetic screens using PDX have been done (Bossi et al. 2016). In this case, surgically resected melanoma tumors were subcutaneously injected into immunodeficient mice, and the resulting tumors were cultured. Next, the cultured cells were transduced with an shRNA epigenetic library and then re-transplanted into immunodeficient mice. The specific ability of each shRNA-transduced PDX to form tumors would allow for the identification of those genes involved in melanoma tumor formation. PDX also have potential uses in drug screens, since they would provide a more accurate representation of specific responses to specific drug treatments in comparison to cell line xenografts. Treatment of PDX from individual patients with specific drugs may prove useful in identifying specific therapeutic drugs that are useful for individual patients (Kuzu et al. 2015).

Despite these promising features, these models also have limitations. The time for tumors to develop in immunocompromised mice typically ranges from 3 to 9 months, and often, tumors do not develop following implantation. Furthermore, implantation into immunocompromised mice does not accurately reflect the natural, physiological tumor microenvironment. In addition, the PDX are difficult to manipulate genetically in comparison to melanoma cell lines, since traditional gene manipulation protocols are inefficient at inducing a change in gene expression in these tumor xenografts (Kuzu et al. 2015). A major limitation of using PDX models to explore melanoma therapy is that they are largely incompatible with testing various manipulations of the immune system since they are propagated in immunocompromised mice until more cost-effective humanized mice can be produced.

## Genetically Engineered Mouse Models

### Genome-Editing Tools to Generate Melanoma Mouse Models

Despite the fact that spontaneous melanoma formation in mice is rare, melanomagenesis can be readily initiated in mice that have been suitably genetically manipulated leading to the expression of mutant gene products that promote human melanoma or that alter (by either increasing or decreasing) the expression of genes that are implicated in melanomagenesis. In this regard, the mouse genome is exceptionally tractable for sophisticated and extensive genetic manipulation. First, the genome can be modified by random integration of ectopic transgenes into the genome. This is normally achieved by introducing a transgene into fertilized oocytes, after which the DNA will randomly integrate into the genome, and more often than not, the insertion site will not encode for an endogenous gene. Secondly, homologous recombination can be used to alter the genome by replacing or modifying a particular endogenous gene of interest, which is generally achieved using mouse embryonic stem cells (ES). Next, the modified ES cells are injected into mouse blastocysts, eventually generating mice with either the removal (knockout), replacement (knock-in), or particular modification (conditional allele) of a target gene of interest (GOI). As molecular biology techniques have become more refined and sophisticated, the spatiotemporal control of the expression of specific GOIs has become possible and commonly utilized. Finally, CRISPR/CAS9 is likely to provide a fast and

efficient approach to generate novel mouse models for the melanoma field (Singh et al. 2015).

Genetically engineered mouse models of melanoma have been generated employing a basic principle: a specific GOI is placed under the control of a particular promoter, which allows for a specific pattern of expression of that GOI. The expression of the GOI alone may be enough to cause melanomagenesis, or it may need to be expressed simultaneously in the same mouse with other melanoma-associated genes in order to promote melanomagenesis. Alternatively, chemical (e.g., DMBA) or environmental agents/factors (e.g., UVB light) may be applied to mice to promote melanomagenesis (Zaidi et al. 2011; Viros et al. 2014).

To study the function of a particular GOI in the melanocyte lineage, genetically engineered mice have been generated that express the specific gene under the control of a gene promoter expressed solely in the melanocyte lineage. For the most part, genetically engineered mouse (GEM) models with melanocyte-specific transgene expression have been developed using a transgene that is under the control of the tyrosinase (*Tyr*) gene promoter. Tyrosinase is an enzyme involved in melanin synthesis, a process specific to melanocytes. Thus, genes under the control of this promoter will be expressed in the melanocyte lineage. In addition to the promoter, transgenes that contain GOIs under the control of both the tyrosinase enhancer and promoter also exist, adding further specificity to the regulation of gene expression. Other promoters that have been employed to confer melanocyte-specific expression of different GOIs are from the dopachrome tautomerase (*Dct*), melanoma antigen recognized by T cells (*Mart1*), or microphthalmia transcription factor (*Mitf*) genes (MacKenzie et al. 1997; Alizadeh et al. 2008; Aydin and Beermann 2011).

While the majority of genetically engineered melanoma mouse models employ one of the aforementioned promoters, some studies have made use of the metallothionein gene (*Mt*) promoter to drive gene expression. In these mice, since the metallothionein gene is expressed in all tissues, the GOI is also expressed in all tissues (Iwamoto et al. 1991). In this case, melanoma formation usually results from treatment of the mice with a chemical tumor promoter, which seemingly affects the genetically modified melanocytes either alone or in addition to other cell types, which may themselves, become cancerous. For melanoma researchers, two main chemical agents have been used to induce tumor formation in mice. The first of these agents is 7,12-dimethylbenz[a]anthracene (DMBA), which suppresses the immune system and causes organ-specific carcinogenesis after being metabolized in the body and binding to DNA at adenine and guanine nucleotides (Miyata et al. 2001). The second of these carcinogenic agents is 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which binds to and activates protein kinase C (PKC), leading to various outcomes, including tumor formation in mouse skin (Abel et al. 2009).

The reversible induction of specific GOIs from exogenous promoters has also been made possible through the use of the tetracycline/doxycycline inducible system, i.e., the Tet-On and Tet-Off systems (Zaidi et al. 2011; Bockamp et al. 2008). In the Tet-Off system, the tetracycline transactivator protein (tTA), whose expression is under the control of a melanocyte-specific promoter (e.g., Tyr::tTA), is capable of binding to tetO sequences, which are part of a tetracycline response element (TRE) controlling the expression of a target gene of interest (Zaidi et al. 2011). The binding of the tTA to the TRE results in expression of the target gene of interest. When tetracycline is present, it binds to the tTA and does not allow it to bind to the TRE; therefore target gene expression is silenced. In the Tet-On system, the tTA is modified and is actually a reverse tTA (rtTA), which can only bind to the TRE in the presence of tetracycline. This system has been valuable in allowing researchers to look at the inducible and reversible effects of a certain GOI in a particular lineage, including the melanocyte lineage (Chin et al. 1999).

While these technologies have been useful in identifying the role of various genes in melanomagenesis, one important consideration to be made is that an artificially introduced promoter, and not the endogenous promoter, controls

expression of these genes. The consequences are that the temporal and level of expression of the GOI are modified, which may lead to physiological artifacts. To address this concern, genetically engineered mouse models that allow expression of melanoma-relevant genes under the control of their own endogenous promoter have been generated and are now widely used. In this case, the endogenous GOI is modified using homologous recombination in ES cells, which results in a genetically altered ES cell that is then injected into early embryos to generate allophenic (chimeric) mice. Several founder mice containing the altered gene have to be generated and characterized. Gene alteration can be constitutive or conditional. The conditional gene alteration can be performed using the Flp/Frt or Cre/LoxP system (Larue and Beermann 2007). These two systems are naturally present either in yeast or bacteriophage and are based on a recombinase (Flp or Cre) and sequences that are both specifically recognized and recombined (Frt or LoxP). Cre is an enzyme derived from the P1 bacteriophage that is able to homologously recombine internally DNA between two specific DNA sequences (known as LoxP sites) of 34 nucleotides: 5′-ATAACTTCGTATA ATGTATGC TATACGAAGTTAT-3′, with 2 inverted repeats of 13 nucleotides and a spacer of 8 nucleotides (Nagy 2000). The development of this enzyme as a tool for genetic manipulation for genetically engineered mice has been of immense importance and enabled researchers to perform experiments that were not previously possible. In general, Cre is used in the following way to induce the melanocyte-specific expression of a target GOI:

1. The endogenous gene of interest has been modified using homologous recombination in ES cells, and transgenic mice containing the modified allele have been generated. It should be noted that the modified allele (or "floxed" allele), which is present in all cell types, does not affect these mice, since it's mutant/altered form is not present unless Cre-mediated recombination occurs.
2. The Cre enzyme is under the control of a melanocyte-specific promoter such as Mitf or Mart1 (Alizadeh et al. 2008; Aydin and Beermann 2011). However, in most cases the Tyr promoter is used. These Tyr::Cre mice are characterized and, of course, the Tyr::Cre transgene has no effect on melanomagenesis by itself (Delmas et al. 2003).
3. The crosses of Tyr::Cre and floxed mice generate pups that contain the mutant/altered allele of the GOI (also called the defloxed allele) in the melanocyte lineage specifically with intact/floxed germ cells. Importantly, since the *Tyr* gene is expressed at approximately E9.5 during embryonic development, conditional genetic alterations (defloxing) of the gene of interest occur at approximately this time.

This technology has made it relatively simple to study the effects of alterations in a particular GOI in the melanocyte lineage from its endogenous promoter (Aoki et al. 2015; Mort et al. 2014; Wavre-Shapton et al. 2013; Li et al. 2011; Selfridge et al. 2010; Dhomen et al. 2010; Schouwey et al. 2007; Levy et al. 2010; Pshenichnaya et al. 2012). However, as mentioned, since *Tyr* is expressed during embryonic development, the mutant GOI is also expressed at this time, which may not completely reflect melanomagenesis in humans, as mutation primarily occurs after birth. This issue was addressed by the generation of two independent transgenic mouse lines in which a hormone-dependent form of Cre recombinase ($CreER^{T2}$) is expressed under the control of the tyrosinase promoter/enhancer sequences that will be called $Tyr::CreER^{T2(L)}$ and $Tyr::CreER^{T2(B)}$ (Yajima et al. 2006; Bosenberg et al. 2006). $CreER^{T2}$ is a fusion protein comprising Cre recombinase fused to a modified form of the hormone-binding domain of the human estrogen receptor that is activated by 4-hydroxytamoxifen (4-OHT) but not by endogenous estrogens (Feil et al. 1997). Hence, in the absence of 4-OHT, $CreER^{T2}$ is inactive, but in the presence of 4-OHT, the $CreER^{T2}$ protein is activated to perform its enzymatic activity in the nucleus. Consequently, the use of both $Tyr::CreER^{T2}$ mouse models has allowed for the spatial and temporal control of the expression of the altered GOIs in mouse melanocytes.

More recently, as the development of new technologies has advanced, a new technique known as "Replication-competent avian sarcoma-leukosis virus long terminal repeat with splice acceptor/tumor virus A" has emerged. This system, also known as RCAS/TVA, makes use of an RCAS vector to induce efficient and stable delivery of specific genes of interest in a targeted manner (Loftus et al. 2001; von Werder et al. 2012). When the suite of RCAS vectors is used in conjunction with mice carrying a melanocyte-specific *Dct::TVA* transgene (tva800 or tva950), this allows for genetic manipulation of gene expression in mouse melanocytes. In other words, targeted cells of interest are genetically modified to express the proteins tva800 or tva950 (*Dct::TVA* transgene), which are not expressed in mammalian cells. Once these proteins are expressed in the cells of interest, they can be infected with the RCAS vector and will express the gene of interest in the cells of interest.

Finally, recent advances in genome editing have been made possible by the application and refinement of the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system, which has significantly advanced how researchers can edit their desired genome of interest. This system has the potential to generate cell lines with identical genetic backgrounds that only differ in the loss of the CRISPR-targeted GOI. As such, the effects of losing expression of the GOI can be accurately assessed between the parental cell line and the CRISPR cell lines. In this system, the bacterial CAS9 protein, which cleaves DNA to introduce double-stranded breaks, is expressed in conjunction with a specific guide RNA (sgRNA), which is designed to target a specific GOI. This specific RNA sequence is complementary to a genomic region in the GOI that is found adjacent to a protospacer adjacent motif (PAM), which has a particular sequence and is commonly found within the genome. When the sgRNA aligns with the genomic DNA, CAS9 cleaves both strands of the DNA three nucleotides upstream of the PAM resulting in a double-stranded DNA break. When this break is repaired by host DNA repair mechanisms, the end result is often the disruption (knockout) of the targeted gene of interest. In addition, double-stranded break formation may be followed by the insertion of a specific sequence of interest, provided that an exogenous artificial repair template (e.g., a DNA fragment on which the GOI-targeting sequences flank the sequence to be inserted) is provided (Agrotis and Ketteler 2015). Importantly, since PAM sequences are found throughout the genome, CRISPR/CAS9 technology can be useful in manipulating a large number of genes and DNA sequences to assess their effects on cellular processes including melanomagenesis. To date, genetically engineered mouse lines using the CRISPR/CAS9 system for studying melanomagenesis have not been generated, but their potential is enormous. However, several studies have used this system in human melanoma cell lines (Krachulec et al. 2016; Shalem et al. 2014; Benamar et al. 2016; Kim et al. 2016). One of these studies used this system in a human melanoma cell line to identify genes involved in resistance to vemurafenib, one of the few treatments available for melanoma patients, and identified a number of genes that could potentially be involved in this resistance (Shalem et al. 2014). Future studies will be needed to verify the roles of these proteins in resistance associated with vemurafenib.

## Genetically Engineered Mouse Line Tools

In the above section, the different mechanisms that have been used to generate melanocyte-specific expression of particular genes/mutants of interest were described. These mechanisms can also be used to generate transgenic mice that can be useful to help visualize and follow the particular expression or pattern of expression of genes/alleles that have been genetically manipulated (defloxed) following Cre-mediated recombination. This section discusses four such genetically engineered mouse lines that have been very useful in identifying which cells have been genetically altered following recombination events.

### Dct::LacZ Mice

The *LacZ* gene encodes β-galactosidase, an enzyme involved in lactose metabolism in

bacteria. Specifically, this enzyme cleaves the disaccharide sugar lactose into the monosaccharides glucose and galactose. In the case of melanoma research, Dct::LacZ mice were generated, can be molecularly tested for homozygosity, and are still very useful because they can be used in X-gal staining experiments to identify Dct-positive cells including melanoblasts, melanocyte stem cells, melanocytes, and melanomas (MacKenzie et al. 1997; Takemoto et al. 2006; Nishimura et al. 2002). When X-gal, which is an organic molecule that contains galactose linked to an indole group, is added to the tissue section or embryo of interest, LacZ-expressing (i.e., Dct-expressing) cells will cleave the X-gal, liberating the lactose moiety and a 5-bromo-4-chloro-3-hydroxyindole moiety. The latter then forms a homodimer and is oxidized to produce a blue color. Other β-galactosidase substrates can be used for other purposes as well. This tool can be very useful in serving as a control for the identification of melanocyte-lineage cells.

### Z/EG Mice

Another genetically engineered mouse line that is a useful imaging tool is the Z/EG mouse line, which contains the β-galactosidase gene flanked between two LoxP sites. Downstream of the second LoxP site is the gene encoding the green fluorescent protein, EGFP (Novak et al. 2000). Z/EG mice can be genotyped for homozygosity (Colombo et al. 2010) and express β-galactosidase starting from embryonic development. However, when these mice are crossed with Cre mice, Cre-mediated recombination results in the removal of the β-galactosidase gene and allows the expression of EGFP. As such, cells in which Cre has been active appear green under the microscope. The fluorescence can also be viewed in real time on live cells, providing researchers with the unique ability to visualize the kinetics of Cre-mediated recombination as it occurs within the cells. Moreover, as required, EGFP-expressing cells can be isolated using flow cytometry.

### mT/mG Mice

The mT/mG mouse line is another tool for visualization of the spatial and temporal activity of Cre recombinase as well as for lineage tracing and cell morphology studies. In these mice, the gene encoding an N-terminal membrane-tagged tdTomato protein is flanked by LoxP sites, whereas the gene encoding a similarly tagged EGFP is downstream of the second LoxP site. This cassette is encoded within the Rosa26 locus; therefore it is expressed in all cells (Muzumdar et al. 2007). Prior to Cre-mediated recombination, all cells in the mouse express tdTomato and are red fluorescent. Following Cre-mediated recombination, the tdTomato expression is silenced and the cells express EGFP and are green fluorescent. As such, this mouse line is an excellent tool to provide contrast between cells that have or have been subject to Cre-mediated recombination.

### Confetti Mice

The Confetti or Brainbow mouse line consists of a series of genes encoding fluorescent proteins (XFPs) back-to-back and separated by LoxP sites (Muzumdar et al. 2007). The genes encoding the XFPs are downstream of a "road block" cassette, which does not allow the expression of the XFPs prior to Cre-mediated recombination. However, it may contain a mutant XFP (e.g., YFP) that does not fluoresce but that can be detected by immunostaining, which would give an indication of the number of cells containing the transgene. Cre-mediated recombination results in the random removal of XFP genes, resulting in a single characteristic fluorescence for each individual cell. Monitoring of these individual cells is useful for addressing cell of origin, for lineage tracing, and for assessing the clonality of tumor formation.

The abovementioned tools make it possible to study the effects of melanocyte-specific mutations in GOIs. In the following section, the different genetically engineered mouse models of melanoma will be summarized. A complete listing of these mouse models is provided in Table 1.

## Specific Genomic Alterations in Genetically Engineered Mice

Various signaling pathways including the RAS-activated ERK1/2 MAP kinase (MAPK), PI3-kinase, and WNT/β-catenin are involved in melanoma initiation and progression, as are proteins involved in the cell division cycle, such

**Table 1** Summary of genetically engineered melanoma mouse models

| Mouse model | Carcinogen | Melanoma | Met | Reference |
|---|---|---|---|---|
| Cell autonomous: monogenic | | | | |
| Tyr::CreER$^{T2(B)/\circ}$; Braf$^{CA/+}$ | None | No | No | Dankort et al. (2009) |
| Tyr::CreER$^{T2(L)/\circ}$; Braf$^{LoxP\text{-}V600E/+}$ | None | Yes | No | Dhomen et al. (2009) |
| Tyr::CreER$^{T2(B)/\circ}$; Braf$^{LoxP\text{-}V618E/+}$ | None | Yes | No | Perna et al. (2015) |
| Tyr::Braf$^{V600E/\circ}$ | None | No | No | Goel et al. (2009) |
| Tyr::HRAS$^{G12V}$ | None | No | No | Powell et al. (1995) |
| Tyr::HRAS$^{G12V}$ | None | No | No | Chin et al. (1997) |
| Tyr::CreER$^{T2(L)/\circ}$; β-actin::Kras$^{LoxP\text{-}G12V/LoxP\text{-}G12V}$ | None | Yes | No | Milagre et al. (2010) |
| Tyr::NRAS$^{Q61K/\circ}$ | None | Yes | Yes | Ackermann et al. (2005) |
| Tyr::CreER$^{T2(B)/\circ}$; Nras$^{LoxP\text{-}G12D/LoxP\text{-}G12D}$ | None | No | No | Pedersen et al. (2013) |
| Tyr::Cre/°; Nras$^{LoxP\text{-}G12D/LoxP\text{-}G12D}$ | None | Yes | No | Pedersen et al. (2013) |
| Tyr::CreER$^{T2(B)/\circ}$; Nras$^{LoxP\text{-}Q61R/LoxP\text{-}Q61R}$ | None | No | No | Burd et al. (2014) |
| Mt::Hgf | None | Yes | Yes | Takayama et al. (1997) |
| Mt::Ret | None | Yes | Yes | Iwamoto et al. (1991) |
| | | | | Kato et al. (1998) |
| Tyr::CreER$^{T2(B)/\circ}$; Nf1$^{LoxP/LoxP}$ | None | No | No | Maertens et al. (2013) |
| Tyr::CreER$^{T2(B)/\circ}$; Pten$^{LoxP/LoxP}$ | None | No | No | Dankort et al. (2009) |
| Tyr::Cre/°; Pten$^{LoxP/+}$ | None | No | No | Puig et al. (2009) |
| | | | | Conde-Perez et al. (2015) |
| Ink4a$^{-/-}$ | None | No | No | Serrano et al. (1996) |
| p16$^{Ink4a-/-}$; p19$^{Arf+/-}$ | None | Yes | No | Sharpless et al. (2001) |
| Cdk4$^{R24C/R24C}$ | None | No | No | Sotillo et al. (2001) |
| Tyr::bcat*$^{/\circ}$ | None | No | No | Delmas et al. (2007) |
| Tyr::Cre/°; β-catΔex2-6$^{LoxP/LoxP}$ | None | No | No | Luciani et al. (2011) |
| Tyr::Cre/°; β-catΔex3$^{LoxP/+}$ | None | No | No | Yajima et al. (2013) |
| Tyr::Cre/°; Rosa26::Mdm4$^{LoxP/+}$ | None | No | No | Gembarska et al. (2012) |
| Tyr::SV40Tag | None | Yes | Yes | Bradl et al. (1991) |
| | | | | Klein-Szanto et al. (1991) |
| Dct::Grm1 | None | Yes | No | Pollock (2003) |
| Tyr::HRAS$^{G12V}$ | DMBA | Yes | Yes | Gause et al. 1997 |
| p16$^{Ink4a-/-}$ | DMBA | Yes | Yes | Krimpenfort et al. (2001) |
| Cdk4$^{R24C/R24C}$ | DMBA/ TPA | Yes | No | Sotillo et al. (2001) |
| Mt::Hgf | UV | Yes | No | Noonan et al. (2001) |
| Mt::Hgf | UVB | Yes | Yes | De Fabo et al. (2004) |
| Cell autonomous: multigenic | | | | |
| Tyr::CreER$^{T2(L)/\circ}$; Braf$^{LoxP\text{-}V600E/+}$; p16$^{Ink4a-/-}$ | None | Yes | Yes | Dhomen et al. (2009) |
| Tyr::Braf$^{V600E/\circ}$; Cdkn2a$^{+/-}$ | None | Yes | Yes | Goel et al. (2009) |
| Tyr::Braf$^{V600E/\circ}$; p53$^{-/-}$ | None | Yes | Yes | Goel et al. (2009) |
| Tyr::CreER$^{T2(B)/\circ}$; Braf$^{CA/+}$; Pten$^{LoxP/LoxP}$ | None | Yes | Yes | Dankort et al. (2009) |
| Tyr::CreER$^{T2(B)/\circ}$; Braf$^{CA/+}$; Pten$^{LoxP/LoxP}$; β-catΔex2-6$^{LoxP/LoxP}$ | None | No | No | Damsky et al. (2011) |
| Tyr::CreER$^{T2(B)/\circ}$; Braf$^{CA/+}$; Pten$^{LoxP/LoxP}$; β-catΔex3$^{LoxP/LoxP}$ | None | Yes | Yes | Damsky et al. (2011) |

(*continued*)

**Table 1** (continued)

| Mouse model | Carcinogen | Melanoma | Met | Reference |
|---|---|---|---|---|
| Tyr::CreER$^{T2(B)/\circ}$; Braf$^{CA/+}$; Nf1$^{LoxP/LoxP}$ | None | Yes | No | Maertens et al. (2013) |
| Tyr::NRAS$^{Q61K/\circ}$; Cdkn2a$^{-/-}$ | None | Yes | Yes | Ackermann et al. (2005) |
| Tyr::CreER$^{T2(B)/\circ}$; Nras$^{LoxP\text{-}Q61R/LoxP\text{-}Q61R}$; p16$^{LoxP/LoxP}$ | None | Yes | No | Burd et al. (2014) |
| Tyr::NRAS$^{Q61K/\circ}$; Tyr::Cre/$^{\circ}$; Pten$^{LoxP/+}$ | None | Yes | Yes | Conde-Perez et al. (2015) |
| Tyr::NRAS$^{Q61K/\circ}$; Tyr::bcat*$^{/\circ}$ | None | Yes | Yes | Delmas et al. (2007) |
| Tyr::NRAS$^{Q61K/\circ}$; Tyr::Cre/$^{\circ}$; Rosa26::Mdm4$^{LoxP/\circ}$ | None | Yes | No | Gembarska et al. (2012) |
| Tyr::CreER$^{T2(B)/\circ}$; Kras$^{LoxP\text{-}G12D/+}$; p16$^{LoxP/LoxP}$ | None | Yes | No | Monahan et al. (2010) |
| Dct::TVA; Cdkn2a$^{-/-}$; RCAS (NRAS$^{Q61R}$ + Cre) | None | Yes | No | VanBrocklin (2010) |
| Dct::TVA; Cdkn2a$^{-/-}$; RCAS (NRAS$^{G12V}$ + Cre) | None | No | No | VanBrocklin (2010) |
| Tyr::CreER$^{T2(B)/\circ}$; p16$^{LoxP/LoxP}$; p53$^{LoxP/LoxP}$ | None | No | No | Monahan et al. (2010) |
| Tyr::HRAS$^{G12V/\circ}$; Cdkn2a$^{-/-}$ | None | Yes | No | Chin et al. (1997) |
| Tyr::HRAS$^{G12V/\circ}$; Cdkn2a$^{+/-}$ | None | Yes | No | Chin et al. (1997) |
| tetO::HRAS$^{G12V}$; Tyr::rtTA | None | Yes | No | Chin et al. (1999) |
| Tyr::iRasP1A; Cdkn2a$^{LoxP/LoxP}$ | None | Yes | No | Huijbers (2006) |
| Tyr::HRAS$^{G12V}$; Cdk4$^{R24C/R24C}$ | None | Yes | Yes | Hacker et al. (2006) |
| Mt::Ret; EdnrB$^{+/-}$ | None | Yes | Yes | Kumasaka et al. (2010) |
| Mt::Ret; Il6$^{-/-}$ | None | Yes | No | Von Felbert (2005) |
| Cdkn2a$^{-/-}$; Pten$^{-/-}$ | None | Yes | No | You (2002) |
| Dct::rtTA; tetHA-Gnaq$^{Q209L}$; Cdkn2a$^{-/-}$ | None | Yes | No | Feng (2014) |
| Tyr::HRAS$^{G12V}$; Cdk4$^{R24C/R24C}$ | UV | Yes | Yes | Hacker et al. (2006) |
| Tyr::Mip2; Cdkn2a$^{+/-}$ | DMBA | Yes | No | Yang (2001) |
| Non-cell autonomous | | | | |
| K14-CreER$^{T2/\circ}$; RXRα$^{LoxP/LoxP}$ | DMBA/TPA | Yes | No | Indra et al. (2007) |
| K14-CreER$^{T2/\circ}$; Taf4$^{LoxP/LoxP}$ | DMBA/TPA | Yes | No | Fadloun (2007) |

For simplification, the two Tyr::CreERt2 transgenic mouse lines are designated as B and L for the "Bosenberg" and "Larue" lines, respectively. *Met* metastasis

as INK4A-CDK4-RB and ARF-MDM2/4-TP53 signaling. However, it appears that key components of the ERK1/2 MAPK pathway play a crucial role in the early proliferation of initiated melanocytes followed by senescence. By contrast, components of other pathways are more closely associated with the bypass of senescence of initiated melanocytes to melanoma. One current linear model suggests that expression of mutationally activated NRAS or BRAF promotes melanocyte proliferation, resulting in benign melanocytic nevus formation, which ultimately cease proliferation and display features of senescence. Further steps to melanoma therefore require additional genetic/epigenetic events in pathways that promote bypass of senescence leading to melanomagenesis. However, melanoma progression (invasion and metastasis formation) is complex, since it involves multiple cellular mechanisms such as loss of melanocyte-keratinocyte adhesion, loss of melanocyte-basal adhesion, degradation of the basement membrane, migration, invasion, intravasation in blood/lymph vessels, resistance to anoïkis, extravasation, implantation, and angiogenesis. Of course, during the process, melanoma cells must be resistant to apoptosis and the immune system and must also be able to adapt to their environment through their high

molecular and cellular plasticity (phenotypic switch).

The following sections describe different classes of GEM models, which can be thought of as either cell-autonomous or cell non-autonomous (see Table 1). A cell-autonomous melanoma model is defined by the presence of germinal/somatic mutations in the same melanocytes and can be subdivided into three types: (ia) monogenic mouse models associated with proliferation which may or may not form melanoma, (ib) monogenic mouse models associated with immortalization and bypass of senescence, and (ic) multigenic mouse models associated with melanoma formation. A cell non-autonomous melanoma model is defined by at least one modification arising from (iia) the microenvironment (surrounding cells [keratinocytes, fibroblasts, adipocytes] or modification of the amount of nutrients or oxygen) or (iib) the environment (physical irradiation [such as UV] or chemical exposure [such as DMBA and/or TPA]). Here, we will refer to genes/RNA in italics, to human in capital letters, and to mice in lower cases with the first letter capitalized, proteins in non-italicized upper case for human or in lower cases with the first letter capitalized for mouse.

## Cell-Autonomous Models

### Monogenic Mouse Models Associated with Proliferation Which May or May Not Form Melanoma

#### Activated BRAF

The RAF protein family consists of three serine-threonine protein kinases, namely, ARAF, BRAF, and CRAF, that function downstream of GTP-bound RAS, which regulate signaling through the MEK1/2 to ERK1/2 MAP kinase signaling pathway. Mutations in the *ARAF* and *CRAF* genes are rare in human melanoma; hence no melanoma models have been generated for either of these two genes. By contrast, mutational activation of *BRAF* (primarily the $BRAF^{T1799A}$ transversion, resulting in the $BRAF^{V600E}$ oncoprotein) is detected in approximately ~70% of human sun-induced benign nevi and in ~50% of human melanomas (Davies et al. 2002; Pollock et al. 2003a). As such, mutated, oncogenic BRAF induces melanocyte proliferation first and senescence after several cell cycles (Michaloglou et al. 2005).

To study the role of the $BRAF^{V600E}$ oncoprotein kinase in melanomagenesis, four different *Braf* mouse models have been generated (Fig. 1). Due to differences between mouse and human *BRAF*, the human $BRAF^{T1799A}$ mutation is equivalent to $Braf^{T1910A}$ in the mouse. Correspondingly, human $BRAF^{V600E}$ is equivalent to $BRAF^{V637E}$ in the mouse. For convenience, we shall use the human numbering throughout. Two of these models employ a conditionally mutated *Braf* gene (encoding $BRAF^{V600E}$ protein) expressed following Cre-mediated recombination of the endogenous *Braf* gene. Even though Tyr::$CreER^{T2}$-mediated recombination results in the expression of the $BRAF^{V600E}$ oncoprotein in the melanocyte lineage, these two melanoma models display some differences. On one hand, these differences are due to different insertion sites of the transgene (Tyr::$CreER^{T2}$) and to a different sequence of the tyrosinase promoter (Yajima et al. 2006; Bosenberg et al. 2006). On the other hand, the *Braf* knock-in is slightly different as well (Mercer et al. 2005; Dankort et al. 2007). The consequence is that these two crosses, "Marais-Larue" and "McMahon-Bosenberg," led to hyperpigmentation of the skin, tails, ears, and paws and nevi formation. However, "Marais-Larue" mice formed melanoma without additional manipulations, whereas the "McMahon-Bosenberg" model did not (Dhomen et al. 2009; Dankort et al. 2009). Besides the intrinsic molecular differences of the transgenes of these two models, the presence/absence of melanoma may be due to the genetic background of the mice and/or a variety of other factors including the intrinsic quality of the animal colonies. The third Braf allele was slightly different, as the LoxP sequences were located in intron 3, flanking a polyA signal, with a mutated exon 15. These mice also produced melanoma after Cre-mediated recombination displaying hyperpigmentation of the skin, tails, ears, and paws and nevi formation (Perna et al. 2015). In the fourth mouse model

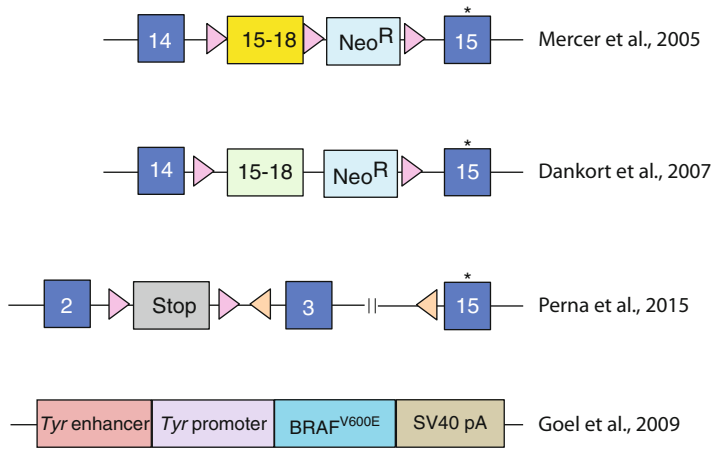


**Fig. 1 Schematic summary of the four different Braf$^{V600E}$ alleles**. Endogenous exons are shown numbered in dark blue boxes. The star indicates that Exon 15 contains the mutation encoding for the V600E mutant protein. The pink triangles denote LoxP sites and the orange triangles denote FRT sites. Note that the mini-gene from Mercer et al. is of mouse origin and from Dankort et al. is of human origin. Note that exon 15–18 are identical at the protein level in human and mouse except at the C-terminal end. Human BRAF has an alanine at the position 762, whereas the mouse equivalent is a glycine. The BRAF V600E cDNA from Goel et al. is of human origin and obtained A375M melanoma cell line

(*Tyr::BRAF$^{V600E}$*), the BRAF$^{V600E}$ oncoprotein is constitutively expressed under the control of the tyrosinase promoter (Goel et al. 2009). These mice displayed hyperpigmentation of the skin, tails, ears, and paws, but did not form melanoma. The efficiency to produce these transgenic mice was low, and the level of BRAF$^{V600E}$ expression was also low. The fact that *Tyr::Cre*-mediated, embryonic expression of BRAF$^{V600E}$ is lethal may explain the properties of the *Tyr::BRAF$^{V600E}$* transgenic mice (Goel et al. 2009; Dhomen et al. 2010).

### Activated RAS

RAS proteins are GTPases that regulate intracellular signaling pathways and thereby serve to propagate extracellular mitogenic signals into appropriate biochemical and biological responses. The most cancer-relevant members of this family are NRAS, HRAS, and KRAS (neuroblastoma, Harvey rat sarcoma, and Kirsten rat sarcoma viral oncogene homologues, respectively). In humans, 20–25% of melanomas express mutationally activated NRAS. While HRAS and KRAS are also mutated in melanoma, the frequency of mutation is significantly lower (1% and 2%, respectively (Fernandez-Medarde and Santos 2011)).

### Activated HRAS

Transgenic mice expressing constitutively active HRAS$^{G12V}$ under the control of the tyrosinase promoter (i.e., Tyr::HRAS$^{G12V}$, also referred to as TPras) displayed melanocytic hyperplasia (characteristic of nevi formation) with intense skin pigmentation, but did not form melanoma (Powell et al. 1995). In another mouse model that contained the HRAS$^{G12V}$ oncogene downstream of both the tyrosinase proximal promoter and upstream enhancer element, HRAS$^{G12V}$ did not promote melanoma (Chin et al. 1997). One should note that in both cases human HRAS was used.

### Activated KRAS

A genetically engineered mouse melanoma model for activated KRAS has also been developed (Milagre et al. 2010), in which a constitutively active KRAS$^{G12V}$ oncoprotein is expressed under the control of the β-actin promoter (i.e., *β-actin::Kras$^{LoxP\text{-}G12V/LoxP\text{-}G12V}$*). Melanocyte-specific expression of KRAS$^{G12V}$, elicited with Tyr::CreER$^{T2}$, led to hyperpigmentation of the back, tail, and ear skin due to the emergence of various melanocytic lesions. The most common of these lesions was similar to human blue nevi.

Furthermore, mice with melanocyte-specific $KRAS^{G12V}$ expression also developed melanoma tumors in all cases, although they appeared not to metastasize throughout the body.

### Activated NRAS

Since *NRAS* mutations are observed at a high frequency in melanoma (20–25%), genetically engineered mice expressing different constitutively active NRAS mutants ($NRAS^{Q61K}$, $NRAS^{G12D}$, and $NRAS^{Q61R}$) have been generated. In human melanomas, 84% and 7% of these mutations localize to codon 61 and 12, respectively. The presence of NRAS mutations in humans induces melanocytic proliferation followed by senescence as shown for giant nevus (Charbel et al. 2014).

The first genetically engineered transgenic *NRAS* mouse line, Tyr::$NRAS^{Q61K/\circ}$, was made using melanocyte-specific expression of human constitutively active $NRAS^{Q61K}$ under the control of both the distal regulatory element (DRE) and the promoter of the mouse tyrosinase gene (Ackermann et al. 2005). These mice displayed hyperpigmentation of the skin, ears, paws, and tails. More significantly, in these mice, melanocytes proliferated at ectopic sites of the skin and in some cases developed cutaneous melanoma with metastases in the lung, liver, and brain.

In the next genetically engineered mouse model, oncogenic $Nras^{G12D}$ was also expressed downstream of the endogenous *Nras* promoter (Pedersen et al. 2013) and was expressed in the melanocyte lineage following the activity of the $CreER^{T2}$ recombinase (*Tyr::CreER*$^{T2/\circ}$*; Nras*$^{LoxP\text{-}G12D/LoxP\text{-}G12D}$). In these mice, melanocyte-specific $Nras^{G12D}$ expression resulted in skin hyperpigmentation and nevi formation, but no tumors developed. When the $Nras^{G12D}$ oncogene was expressed in the melanocyte lineage during development (*Tyr::Cre/*$^{\circ}$*; Nras*$^{LoxP\text{-}G12D/LoxP\text{-}G12D}$), the mice had darker skin, tails, paws, and snouts (compared to controls) and also developed benign lesions similar to human blue nevi, but they did not form cutaneous melanoma. Interestingly, these mice developed neurological symptoms typical of motor dysfunction, which was concurrent with significant darkening of the arachnoid mater and pia mater, the two thinnest membranes surrounding the brain and spinal cord. The melanocytes in these meninges eventually progressed to primary central nervous system (CNS) melanomas, which were both aggressive and invasive.

### Activated RTKs

Many different signaling pathways are implicated in melanocyte development and in melanomagenesis, including receptor tyrosine kinases (RTKs) such as KIT, MET, and RET (Easty et al. 2011; Paluncic et al. 2016). KIT plays an essential role in melanocyte development, proliferation, survival, migration, and differentiation and is overexpressed and/or mutated (V559A) in melanoma (Walker et al. 2011; Stankov et al. 2014). However, no mouse melanoma models with activating mutations in the Kit have been generated. Genetically engineered mouse models studying the Met and Ret RTKs have been generated as described below.

### HGF-MET Signaling

The hepatocyte growth factor (HGF) promotes melanocyte proliferation through its cognate receptor tyrosine kinase MET. HGF-MET-mediated activation of the ERK1/2 MAPK and PI3K pathways most likely promotes melanocyte proliferation leading to melanomagenesis (Hirobe et al. 2004; Li et al. 2001). In a genetically engineered mouse model in which mouse Hgf is expressed under the control of the metallothionein promoter (*Mt::Hgf*), melanomas developed, as did mammary gland tumors and rhabdomyosarcomas (Takayama et al. 1997). Furthermore, it appeared that melanoma formation was driven by an autocrine loop in which the tumors displayed elevated levels of both the Hgf ligand and its receptor Met (Otsuka et al. 1998). Interestingly, while melanocytes in wild-type mice are normally located in the hair follicles, the melanocytes in these transgenic mice were found in the epidermis, in the dermal-epidermal junction, and in the dermis. Thus, since human melanocytes are primarily found in the epidermis, this transgenic mouse model could more accurately reflect the composition of human skin.

### GDNF-RET Signaling

The RTK RET is involved in a wide range of biological processes, including neural crest cell migration, and establishment and maintenance of neurons in the central and peripheral nervous systems (Mulligan 2014). The ligands for RET are the glial cell line-derived neurotrophic factor (GDNF) family of proteins. While mutations in RET have been observed in melanoma, their significance remains uncertain (Mulligan 2014). To better understand the role of RET in tumorigenesis, genetically engineered mice were made that express Ret downstream of the mouse metallothionein 1 promoter-enhancer, which resulted in the ubiquitous expression of oncogenic Ret (Iwamoto et al. 1991; Kato et al. 1998). In these transgenic mice, melanoma tumors spontaneously formed primarily in the dermis of the face around the nose (Iwamoto et al. 1991). These tumors were slow growing and did not metastasize on a mixed strain background (C57BL/6 × BALB/c). However, on a pure C57BL/6 background, these tumors progressed to malignancy and metastasized to multiple sites, including the lymph nodes, lungs, and brain (Kato et al. 1998).

### G-Protein-Coupled-Receptor

G-protein-coupled-receptor has been shown to be involved in melanomagenesis. Metabotropic glutamate receptor 1 (GRM1) is upregulated in some human melanoma and was sufficient to induce melanoma initiation in mice (*Dct::Grm1*) after inducing proliferation and inhibiting apoptosis (Pollock et al. 2003b).

### SV40 Large T-Antigen

The SV40 large T-antigen is an oncoprotein that is derived from the polyoma SV40 virus, which is capable of transforming a wide variety of cell types (for review see An et al. 2012). As the oncogenic activity of the large T-antigen is mediated primarily by its ability to repress the tumor suppressors P53 and RB (An et al. 2012), transgenic mouse models with its expression may display similar phenotypes to those models without expression of both P53 and RB. GEM models with melanocyte-specific expression of this oncoprotein were some of the first mouse models produced. Transgenic mice with expression of the large T-antigen under the control of the *Tyr* promoter (*Tyr::SV40Tag*) spontaneously developed eye and skin melanoma (Bradl et al. 1991; Klein-Szanto et al. 1991; Silvers and Mintz 1998). Moreover, these *Tyr::SV40Tag* melanocytes were prone to form melanoma after UVB irradiation (Larue et al. 1992).

## Monogenic Mouse Models Associated with Immortalization and Bypass of Senescence

### Loss of NF1

Neurofibromin 1 (*NF1*) encodes a GTPase-activating protein (GAP) that has tumor suppressor activity through its activation of the GTPase activity of RAS proteins. Recently *NF1* mutations have emerged as a frequent event in melanomagenesis being mutated in approximately 10–15% of human melanomas, which now constitutes one of the four major subtypes (*NRAS*, *BRAF*, *NF1*, and triple wild-type) used to classify melanomas, based on their mutational profiles (Cancer Genome Atlas Network and Electronic address IMO, Cancer Genome Atlas Network 2015). As a relatively new mediator of melanomagenesis, studies on mice with melanocyte-specific silencing of Nf1 are limited. In a GEM model, the loss of *Nf1* in the melanocyte lineage ($Tyr::CreER^{T2/\circ}$; $Nf1^{LoxP/LoxP}$) resulted in increased ear, tail, and paw pigmentation; however, it did not induce melanoma when induced after birth (Maertens et al. 2013). This finding was surprising, since loss of NF1 should lead to elevated RAS.GTP, which might reasonably be expected to have promoted melanocyte proliferation. This result suggests that NF1 silencing alone is unable to promote sufficient accumulation of RAS.GTP to promote melanocyte proliferation (Maertens et al. 2013; Posch et al. 2016).

### Loss of PTEN

PTEN is a lipid phosphatase that negatively regulates the PI3K signaling pathway in cells and plays an important role in the suppression of melanomagenesis. Indeed, PTEN is mutated or silenced in ~20% of human melanomas (Wu et al. 2003; Whiteman et al. 2002; Zhou et al. 2000; Conde-Perez et al. 2015). However,

GEM models indicate that melanocyte-specific silencing of Pten (*Tyr::CreER*$^{T2/o}$*; Pten*$^{LoxP/LoxP}$ or *Tyr::Cre/*$^{o}$*; Pten*$^{LoxP/+}$) has little or no phenotypic effect on melanocytes and is not sufficient to promote melanomagenesis (Dankort et al. 2009; Conde-Perez et al. 2015; Puig et al. 2009).

### Loss of CDKN2A, Encompassing INK4A and ARF

The *CDKN2A* gene, which is mutated in at least half of all human melanomas, comprises two genes (*INK4A* and *ARF*) that encode two melanoma suppressor proteins, P16$^{INK4A}$ and P14$^{ARF}$ (p19$^{Arf}$ for the mouse) (Bennett 2016). P16$^{INK4A}$ is a stoichiometric inhibitor of D-type cyclin-dependent kinases (CDK) 4 and 6. Expression of P16$^{INK4A}$ inhibits the CDK4/6-mediated phosphorylation of RB and its close homologues p107 and p130, leading to arrest of the cell division cycle prior in G1. By contrast, P14$^{ARF}$ (p19$^{Arf}$ in mice) inhibits MDM2/MDM4 thereby leading to stabilization and activation of TP53 (Bennett 2016). The initial studies looking at the role of the *Cdkn2a* gene (also referred to as Ink4a) in tumorigenesis showed that while Ink4a$^{-/-}$ transgenic mice developed various malignancies, including fibrosarcomas and lymphomas, they did not form melanomas (Serrano et al. 1996). DMBA and/or UV treatment decreased tumor latency, demonstrating that the loss of the *Cdkn2a* gene resulted in increased sensitivity to carcinogenic agents. However, transgenic mice lacking p16$^{Ink4a}$ but containing one copy of p19$^{Arf}$ developed melanoma, although they developed soft tissue sarcoma and lymphoma more frequently (Sharpless et al. 2001). Mice lacking p16$^{Ink4a}$ (p16$^{Ink4a-/-}$) were also more prone to form tumors (including melanoma) following DMBA treatment than those with one functional copy of p16$^{Ink4a}$ (p16$^{Ink4a+/-}$).

### Gain of CDK4

CDK4 is implicated in melanomagenesis since a mutationally altered form of the gene, encoding CDK4$^{R24C}$, was identified as a FAMM family gene (Wolfel et al. 1995). CDK4 regulates early events in the cell division cycle through the phosphorylation of the RB family tumor suppressors. Furthermore, activating mutations in CDK4 (i.e., CDK4$^{R24C/H}$) have been observed in melanoma (Zuo et al. 1996; Puntervoll et al. 2013). However, studies using genetically modified mice expressing the mutant CDK4$^{R24C}$ in the place of the normal protein showed that the mice displayed a wide range of tumors, but not melanomas unless the mice were treated with DMBA/TPA (Sotillo et al. 2001).

### Altered β-Catenin Levels

β-Catenin (*CTNNB1*) is an armadillo repeat-containing protein that is both a mediator of cell-cell adhesion through cadherins and a transcriptional co-regulator that regulates gene expression (Aktary et al. 2016). Following WNT-mediated inhibition of the APC destruction complex (among other pathways), the abundance of β-catenin is increased allowing it to translocate into the nucleus where it interacts with transcription factors (including LEF/TCF) to regulate mRNA production. While mutations in the *CTNNB1* gene itself are rare in melanoma, β-catenin cytoplasmic or nuclear localization has been observed in approximately 30% of human melanoma patients, suggesting that its transcriptional activity may be increased (Rimm et al. 1999). To date, three different genetically engineered mouse models have been developed that can be used to look at the role of β-catenin in melanomagenesis. In the first model (*Ctnnb1*$^{\Delta ex2\text{-}6LoxP}$), Cre-mediated recombination results in a truncated and inactive β-catenin (Brault et al. 2001). Two other mouse models allow for expression of a stabilized and activated form of β-catenin. *Tyr::*βcat* mice express a stabilized form of β-catenin (S33A/S37A/T41A/S45A) under the control of the mouse tyrosinase promoter. Importantly, serines/threonine, which are encoded in exon 3 of the *CTNNB1* gene, are essential for regulating the stability (degradation) of β-catenin (Delmas et al. 2007). In *Ctnnb1*$^{\Delta ex3LoxP}$ mice, exon 3 has been flanked by loxP sites such that Cre-mediated recombination results in expression of a truncated but stabilized and constitutively active form of β-catenin (Harada et al. 1999).

Different studies have shown that melanocyte-specific overexpression or loss of β-catenin by

itself is insufficient to elicit melanoma in genetically manipulated mice (Delmas et al. 2007; Luciani et al. 2011; Yajima et al. 2013). However, these studies have shown that any alterations in β-catenin levels appear to have deleterious effects on melanocyte proliferation and pigmentation in mice. *Tyr::*βcat* mice display a gray coat color and a white belly spot similar to mice with hypomorphic allele of $Mitf^{Wh/+}$ (Delmas et al. 2007; Gallagher et al. 2013). By contrast, mice with melanocyte-specific silencing of β-catenin (*Tyr::Cre/°; Ctnnb1*$^{\Delta ex2\text{-}6LoxP/\Delta ex2\text{-}6LoxP}$) displayed a white coat color with a dramatic reduction of the number of melanocytes due a reduction of melanoblast proliferation (Luciani et al. 2011).

### Gain of MDM4

The transcription factor TP53 is activated in response to various forms of cellular stress (including DNA damage) and plays an important role in maintaining genome integrity by regulating the expression of genes involved in DNA repair, cell cycle arrest, and apoptosis (Eischen 2016). The stability of TP53 and its transcriptional activity are regulated by a number of its interacting partners, including MDM4 (MDMX), a negative regulator of TP53 function (Eischen 2016). Consistent with TP53 being commonly mutated in many types of cancer, ~15% of human melanomas display alterations in *TP53.* Moreover, recent mouse studies have indicated that TP53 serves as a potent suppressor of melanoma progression in mice with melanocyte-specific expression of oncogenic NRAS or BRAF. Moreover, to study the role of the MDM4 in melanomagenesis, a GEM model was generated in which the Mdm4 was conditionally expressed from the Rosa26 locus (Gembarska et al. 2012). However, no tumors were observed in mice with melanocyte-specific overexpression of Mdm4 (*Tyr::Cre/°; Mdm4*$^{LoxP/°}$).

### Multigenetic Mouse Melanoma Associated with Melanoma Formation

While the genetically engineered mouse models described above have been useful in identifying the role of individual genes in melanoma initiation, the majority of GEM models have demonstrated that a single mutated gene is insufficient for the formation of advanced melanomas with the ability to metastasize. However, when a number of these genetically modified alleles are combined, the rate of melanoma initiation and progression (invasion with metastatic dissemination) is very frequently increased.

### Multigenic GEM Models of BRAF-Mutated Melanoma

Melanocyte-specific expression of oncogenic $BRAF^{V600E}$ leads to formation of benign nevus-like lesions (Dhomen et al. 2009; Dankort et al. 2009). However, $BRAF^{V600E}$ expression in combination with $p16^{Ink4a}$ silencing (*Tyr::CreER*$^{T2/°}$; *Braf*$^{LoxP\text{-}V600E/+}$; *p16*$^{Ink4a-/-}$) decreased the latency of melanoma initiation and increased the penetrance, number, and metastatic dissemination of melanomas in the mice (Dhomen et al. 2009). Consistent with these results, silencing of Ink4a/Arf or Tp53 promoted melanoma progression in two other $Braf^{V600E}$-driven melanoma models (*Tyr::Braf*$^{V600E/°}$; *Cdkn2a*$^{-/-}$ & *Tyr::Braf*$^{V600E/°}$; *Trp53*$^{-/-}$) (Goel et al. 2009).

Mutational silencing of Pten also strongly potentiates progression of Braf-mutated melanoma. In this case, mice with melanocyte-specific expression of $Braf^{V600E}$ combined with Pten silencing (*Tyr::CreER*$^{T2/°}$; *Braf*$^{CA/+}$; *Pten*$^{LoxP/LoxP}$) displayed rapid onset, fully penetrant primary melanomagenesis with evidence of micrometastases in numerous organs including the lungs and lymph nodes (Dankort et al. 2009). Consistent with its ability to regulate PI3′-lipid signaling in melanocytes, $Braf^{V600E}$ also cooperated with mutationally activated Pik3CA, encoding the catalytic subunit of PI3′-kinase-a (Vredeveld et al. 2012; Marsh Durban et al. 2013; Deuker et al. 2015).

An important role for β-catenin has been discerned in the $Braf^{V600E}$/$Pten^{null}$ GEM model of melanoma. Silencing of β-catenin (*Tyr::CreER*$^{T2/°}$; *Braf*$^{CA/+}$; *Pten*$^{LoxP/LoxP}$; *Ctnnb1*$^{\Delta ex2\text{-}6LoxP/LoxP}$) delayed melanoma formation and inhibited the appearance of lymph node metastases. This result may be explained by previous results showing that melanocyte-specific loss of β-catenin in mice (*Tyr::Cre/; Ctnnb1*$^{\Delta ex2\text{-}6LoxP/\Delta ex2\text{-}6LoxP}$) resulted in a white coat color that is due to an inhibition of proliferation of melanocytes and

in consequence a decreased number of melanocytes in the skin of these mice (Luciani et al. 2011). Furthermore, activation of β-catenin in the $Braf^{V600E}$/$Pten^{null}$ melanocytes (*Tyr::CreER*$^{T2/+}$; *Braf*$^{CA/+}$; *Pten*$^{LoxP/LoxP}$; *Ctnnb1*$^{\Delta ex3LoxP/\Delta ex3LoxP}$) resulted in a significant increase in melanoma growth and metastasis (Damsky et al. 2011).

Melanocyte-specific silencing of Nf1 combined with $Braf^{V600E}$ expression (*Tyr::CreER*$^{T2/+}$; *Braf*$^{CA/+}$; *Nf1*$^{LoxP/LoxP}$) resulted in increased melanoma compared with $Braf^{V600E}$ alone (*Tyr::CreER*$^{T2/+}$; *Braf*$^{CA/+}$) alone (Maertens et al. 2013). Taken together, these results suggest that oncoproteins such as $Braf^{V600E}$ that promote initial melanocyte proliferation can cooperate with genetic alterations pathways that promote melanoma initiation and which may be involved in the bypass of the senescence-like arrest that restrains the continuous proliferation of $Braf^{V600E}$-driven benign nevus cells.

Finally, the sleeping beauty transposon-mediated mutagenesis has also been used in a $Braf^{V600E}$ context (Mann et al. 2015). This study made use of the sleeping beauty transposase, an enzyme that is capable of excising a transposon from DNA (either plasmid or genomic) and then inserting it into another DNA site with a specific sequence (Dupuy et al. 2009). Transposon insertion would then result in the loss or altered expression of a number of different genes, which could potentially affect tumor formation. Analysis of the different tumors formed in each mouse would potentially identify a different gene implicated in tumor formation. In this study, Cre-mediated activation of the sleeping beauty transposase resulted in the melanocyte-specific disruption in the expression of different genes and allowed for the identification of 1,232 candidate melanoma genes. Specifically, it was shown that CEP350, a protein thought to be involved in the organization, binding and anchoring of microtubules at the centrosome, acts as a tumor suppressor (Mann et al. 2015).

### Multigenic Mouse Models on a Mutant NRAS Background

Mice with melanocyte-specific expression of $NRAS^{Q61K}$ develop melanomas with evidence of metastases, although the time to tumor formation was approximately 1 year (Ackermann et al. 2005). However, expression of $NRAS^{Q61K}$ in combination with Ink4a-Arf silencing (*Tyr::NRAS*$^{Q61K/+}$; *Cdkn2a*$^{-/-}$) resulted in reduced latency and increased melanoma formation and metastases. Similarly, melanocyte-specific silencing of $p16^{Ink4a}$ combined with $Nras^{Q61R}$ expression (*Tyr::CreER*$^{T2/+}$; *Nras*$^{LoxP\text{-}Q61R/LoxP\text{-}Q61R}$; *Ink4a*$^{LoxP/LoxP}$) also resulted in melanoma, but these tumors did not metastasize (Burd et al. 2014). In the most rigorous analysis of the effects of oncogenic NRAS on melanomagenesis to date, mice with melanocyte-specific expression of either $Nras^{G12V}$ (*Nras*$^{LSL\text{-}G12V}$) or $Nras^{Q61R}$ (*Nras*$^{LSL\text{-}Q61R}$) were compared with an $Ink4a^{Null}$ background. Remarkably, whereas $Nras^{Q61R}$/$Ink4a^{Null}$ melanocytes progressed to melanoma, $Nras^{G12V}$/$Ink4a^{Null}$ melanocytes did not. This is perhaps the clearest example of mutation-specific effects of RAS genes on tumorigenesis to date and may also explain the preponderance of $NRAS^{Q61X}$ versus $NRAS^{G12X}$ alterations in human melanoma (Burd et al. 2014).

In addition to Ink4a and/or Arf silencing, mutational inactivation of Pten expression also contributed to melanomagenesis on an NRAS mutant background (Conde-Perez et al. 2015). Mice with both melanocyte-specific expression of $NRAS^{Q61K}$ combined with reduced Pten expression (*Tyr::NRAS*$^{Q61K}$; *Tyr::Cre/+*; *Pten*$^{LoxP/+}$) showed that diminished Pten expression accelerated melanomagenesis in conjunction with oncogenic NRAS. Furthermore, when melanocytes with one functional copy of Pten (i.e., Tyr::Cre/+; $Pten^{LoxP/+}$) were isolated, they displayed low efficiency (~25%) of establishment of immortalized cell lines. However, Pten-deficient melanocytes were completely established in culture as immortalized cell lines very efficiently, thereby suggesting that Pten silencing was a contributing factor in the bypass of senescence required for the immortalization of melanocytes in cell culture.

β-catenin has also been shown to promote melanomagenesis in the context of oncogenic NRAS. While *Tyr::βcat** mice alone did not form tumors, mice with combined expression of $NRAS^{Q61K}$ and activated β-catenin (*Tyr::NRAS*$^{Q61K/+}$; *Tyr::βcat**/+) developed melanomas with shorter latency and higher penetrance than mice with $NRAS^{Q61K}$ expression alone.

Mechanistically, β-catenin repressed *Ink4a* transcription in these tumors, which resulted in the bypass of senescence. The presence of the oncogenic form of β-catenin also promoted the formation of lung metastases. These results revealed the association of induction of proliferation ($NRAS^{Q61K}$) with the bypass of senescence in these mice (Delmas et al. 2007).

Finally, melanocyte-specific overexpression of Mdm4 in mice with melanocyte-specific expression of oncogenic $NRAS^{Q61K}$ (Tyr::$NRAS^{Q61K/\circ}$; Tyr::Cre/°; Mdm4 $^{LoxP/\circ}$) resulted in increased melanoma tumor formation in comparison to mice without the overexpressed Mdm4 (Tyr::$NRAS^{Q61K/\circ}$; Tyr::Cre/°). This study also showed that MDM4 protein levels were increased in human melanomas compared to normal melanocytes and benign nevi, which would suggest that while the *TP53* gene may be mutated in melanoma at low frequency, the TP53 pathway may still be inactivated by other means during melanomagenesis.

Constitutive expression of an oncogenic form of Nras in mouse melanocytes represents only partially the situation in humans. Several mouse models were generated including the iNras melanoma model based on the Tet-On system in which Nras activated form is produced in melanocytes after doxycycline induction on a Cdkn2a-null background. iNras mice produce melanoma in 17 weeks with a 50% penetrance (Kwong et al. 2012).

### Other Multigenic Mouse Models

While most multigenic mouse models of melanoma are built on a platform of mutationally activated Nras or Braf, a number of studies have been performed using other oncoprotein drivers of melanocyte proliferation. For example, melanocyte-specific silencing of $p16^{Ink4a}$ cooperated with expression of $Kras^{G12D}$ (*Tyr::CreER*$^{T2/\circ}$*; Kras*$^{LoxP\text{-}G12D/+}$*; Ink4a*$^{LoxP/LoxP}$) to promote melanomagenesis, although no metastases were observed in this model (Monahan et al. 2010). In this study, the importance of the oncogenic Kras in promoting melanomagenesis was demonstrated by the fact that mice with melanocyte-specific silencing of $p16^{Ink4a}$ and TP53 (*Tyr::CreER*$^{T2/\circ}$*; p16*$^{LoxP/LoxP}$*; p53*$^{LoxP/LoxP}$) did not form melanoma. When both alleles of the *Cdkn2a* gene were deleted in *Tyr::HRAS*$^{G12V}$ mice (*Tyr::HRAS*$^{G12V/\circ}$*; Cdkn2a*$^{-/-}$), melanomagenesis, but not metastasis, was significantly increased compared to mice with an intact *Cdkn2a* locus (Tyr::$HRAS^{G12V/\circ}$; $Cdkn2a^{+/-}$ Chin et al. 1997). The importance of oncogenic HRAS in the context of *Cdkn2a* null mice was further demonstrated using a doxycycline-inducible $HRAS^{G12V}$ mouse model (i.e., Tyr/Tet-RAS; Chin et al. 1999). In these mice, induced expression of $HRAS^{G12V}$ combined with *Cdkn2a* deletion resulted in melanomagenesis. Furthermore, doxycycline withdrawal from $HRAS^{G12V}$/Ink4a-$Arf^{null}$ melanoma-bearing mice resulted in dramatic regression of pre-existing melanomas. Furthermore, re-administration of doxycycline resulted in prompt melanoma recurrence at the original primary sites.

In another study, mice with melanocyte-specific $HRAS^{G12V}$ expression combined with ubiquitous expression of $Cdk4^{R24C}$ (*Tyr::HRAS*$^{G12V}$*; Cdk4*$^{R24C/R24C}$) developed melanoma more frequently than $Cdk4^{R24C/R24C}$ mice alone (Hacker et al. 2006). At this point, the cooperation was not studied at the cellular level.

Finally, the Ret melanoma model has been used to show that the endothelin receptor B (EdnrB), which plays an important role in the development of neural crest cells (including melanocytes), can also contribute to melanomagenesis (Kumasaka et al. 2010). Specifically, when melanomas emerged in the *Mt::Ret* mice, it was observed that the expression of EdnrB was decreased in malignant compared to benign tumors. To examine the role of EdnrB in tumor progression in the *Mt::Ret* model more carefully, mice were engineered to express RET but with reduced EdnrB expression (*Mt::Ret; EdnrB*$^{+/-}$). These mice directly developed metastatic melanoma without displaying an evolution from a nevus-like phenotype. Moreover, these mice displayed evidence of lung metastases. While this model might be useful for studying de novo melanomagenesis, the lack of increased RET

signaling in human melanoma may reduce the clinical relevance of this model.

### Cell Non-autonomous Models

#### Cell Non-autonomous Models Associated with the Microenvironment

While studying melanocyte-specific gene mutations and their effect on melanomagenesis is of paramount importance, it must be remembered that, in vivo, melanocytes in the epidermis make contacts with neighboring keratinocytes. In this regard, it may be possible that alterations (e.g., mutations and/or alterations in expression of various genes) within keratinocytes may, in certain contexts, promote melanomagenesis. The first indication of this was from work in mice with keratinocyte-specific silencing of the retinoic acid receptor Rxrα as well as the Taf4 subunit of TFIID, a general transcription factor. In these mice, DMBA and TPA treatment led not only to papilloma formation but also to the formation of nevi and invasive dermal melanoma tumors (Indra et al. 2007). This cell non-autonomous melanoma formation, which occurred in response to genetic changes in the neighboring keratinocytes, reinforces the fact that environmental cues from keratinocytes (e.g., α-MSH) can trigger melanocyte hyperproliferation and/or oncogenic transformation.

#### Cell Non-autonomous Models Associated with the Environment

Different treatments have been used with different genetically engineered mouse models to induce melanomagenesis: including treatment with DMBA with or without TPA and UV irradiation.

Administration of DMBA to mice with melanocyte-specific oncogenic $HRAS^{G12V}$ resulted in malignant melanoma development (Powell et al. 1995; Gause et al. 1997), which then metastasized to the lungs and the lymph nodes (Gause et al. 1997). Mice lacking $p16^{Ink4a}$ expression are not melanoma-prone; however treatment of these mice with DMBA induced melanoma with evidence of metastases (Krimpenfort et al. 2001). Furthermore, combination treatment of mice with ubiquitous expression of $Cdk4^{R24C}$ with DMBA and TPA induced nevus formation, which eventually progressed to melanoma (Sotillo et al. 2001).

The *Mt::Hgf m*odel has been particularly useful for determining the role of UV irradiation in melanoma. In one study, a single dose of UV radiation of neonates was both necessary and sufficient to induce melanoma (Noonan et al. 2001; Wolnicka-Glubisz and Noonan 2006). This model was also used to demonstrate that it is UVB irradiation, and not UVA, that promotes melanoma initiation in this model (De Fabo et al. 2004). UV irradiation of $Tyr::HRAS^{G12V}$; $Cdk4^{R24C/R24C}$ mice increased tumor development (compared to non-irradiated mice) and resulted in lymph node metastases (Hacker et al. 2006).

The Braf mouse melanoma model was used to evaluate that the single dose of UVR that mimicked mild sunburn in humans induced clonal expansion of the melanocytes, and repeated doses of UVR increased melanoma burden. A large proportion of UVR tumors exhibited Trp53 mutations, and mutant Trp53 accelerated melanoma initiation on a $Braf^{V600E}$ background (Viros et al. 2014).

## Other Mammalian Melanoma Models

### Canine Melanoma Models

Dogs are now viewed as potentially useful models of human melanoma and can be used in treatment studies. Unlike in mice, malignant melanoma occurs spontaneously in domestic dogs and is relatively common, compared to other animals. In this regard, canine models of melanoma are useful for studying the human disease, since they are heterogeneous and since both tumor formation and metastasis occur spontaneously in immunocompetent animals. In dogs, the most frequent type of melanoma is of mucosal origin, which typically originates in the oral cavity. This type of canine melanoma is highly aggressive and metastasizes rapidly to numerous sites including the lungs and the lymph nodes (for review see van

der Weyden et al. 2016). Other types of melanomas, including cutaneous (occurring in the hairy skin), acral (occurring in the footpad and nails), and uveal (occurring in the eye), also occur in dogs but are less frequent. Importantly, it must be noted that canine cutaneous melanomas are usually benign, which is in contrast to human cutaneous melanomas, which are invariably malignant.

Following the completion of the canine genome project, it was noted that human and dog nucleic acid and protein sequences are more similar to one another than are mouse and human sequences, further supporting the utility of the dog melanoma model in studying the human disease (Lindblad-Toh et al. 2005). However, some differences exist between the human and dog diseases. As mentioned above, the primary disease subtype in humans is cutaneous melanoma, whereas in dogs, it is mucosal melanoma. As such, exposure to UV is not a risk factor in mucosal melanoma in dogs, whereas it is a factor in cutaneous melanoma in humans. This observation appears intuitive since dogs are protected against UV by their fur. As with human melanomas, mutational activation of genes encoding BRAF, NRAS, PTEN, and KIT have all been observed in canine melanomas, albeit to differing extents than the human disease (van der Weyden et al. 2016). Nonetheless, as with human melanoma, multiple signaling pathways (e.g., MAPK, PI3K, WNT) are involved in and responsible for canine mucosal and cutaneous melanomas.

One study looking at over 2,000 dogs with melanoma showed that certain dog breeds, including Labradors, Rottweilers, and Dobermans, developed melanoma more frequently than other breeds. Furthermore, melanomas were more frequent among dogs with black coats, compared to those with white coats (Gillard et al. 2014). This finding is somewhat in disagreement with the occurrence of melanoma in humans, where light-skinned individuals are more likely to develop melanoma than those who are dark-skinned, although this is a comparison made between mucosal and cutaneous melanomas. This may suggest that the genetic predisposition for these two types of melanoma is different.

Typical treatments for mucosal melanoma in dogs include surgical resection and ionizing radiation of the primary tumor. In addition, other therapies such as chemotherapy (e.g., carboplatin or cisplatin) and immunotherapy (e.g., allogeneic cancer vaccines expressing interleukin-2) have also been employed (van der Weyden et al. 2016). Similar to humans, melanoma metastases in dogs are difficult to treat. While immunotherapy has been tested as a potential therapeutic avenue, these trials have shown limited success (van der Weyden et al. 2016). Clinical trials in canines provide the ability for researchers and veterinarians to assess the effects of a particular therapy in a shorter time frame (due to the shorter lifespan of dogs) compared to humans, while assessing the effects on a complex and heterogeneous animal population, which spontaneously forms melanoma and metastases.

## Equine Melanoma Models

As in dogs, spontaneous melanomas also occur in horses. There are five types of melanocytic lesions that have been characterized in horses, which can be considered as cutaneous melanomas:

(i) Melanocytoma, melanocytic nevi sometimes resembling human nevi that occur primarily on the legs, body, and neck of horses of any coat color.
(ii) Dermal melanoma, which typically occur in gray horses, are characterized as discrete tumors/nodules with a low propensity to metastasize that typically develop in the anal, perianal, and genital regions as well as in the perineum, lips, and eyelids and under the tail root.
(iii) Dermal melanomatoses are usually characterized as multifocal dermal lesions, which are typically found in the genital or perianal regions. These tumors arise in white and gray horses, and they can eventually become malignant.
(iv) Anaplastic malignant melanoma occurs in all horses, but the risk is higher for non-gray horses.

(v) Besides cutaneous and mucosal melanomas, ocular melanomas may occur in horses with a very low frequency (Valentine 1995).

Unlike humans, exposure to UV irradiation is not considered to be a risk factor for melanoma in horses. The disease is however associated with the age-related development of a gray hair coat color, which is caused by a germline intronic duplication in the *STX17* gene, which encodes syntaxin 17. This mutation leads to the constitutive activation of the ERK1/2 MAPK pathway in the melanocytes of the gray horses. As such, this observation further emphasizes the importance of the ERK1/2 MAPK pathway in melanoma, regardless of the species. The bypass of senescence would be favored with age with an unknown mechanism that could be associated with sFRP2 and β-catenin (Delmas et al. 2007; Kaur et al. 2016).

Horses with mutation in the agouti signaling protein gene (*ASIP*) have increased propensity to develop melanoma, thereby pointing to a role for the melanocortin-1 receptor pathway in the development of equine melanoma (Rosengren Pielberg et al. 2008). Horse melanoma cell lines were established from primary and metastatic tumors, and all of them lacked TP53 expression. However equine melanoma cell lines established from metastases lacked both $P16^{INK4A}$ and PTEN expression (Seltenhammer et al. 2014). Horse melanomas present molecular characteristics similar to humans; therefore a better understanding of their genetics and epigenetics may be useful to discover novel genes and pathways involved in horse melanomagenesis with potential implications for the treatment of the human disease.

## Swine Melanoma Models

In pigs, cutaneous melanoma occurs spontaneously around birth but frequently regresses (Baco et al. 2014). Such regression occurs in humans with melanoma and was thought to reflect the patient's immune system gaining the upper hand over the melanoma and therefore spurred research in melanoma immunotherapy. Comparative genomic analyses have demonstrated that the pig and human genomes are very similar. In addition, the skin of humans and pigs is similar with their melanocytes mainly located in the basal layer of the epidermis. The postnatal onset, the lack of contribution of an obvious mutagen, and the practicalities of research in porcine models aside, such similarities could be exploited to discover novel molecular players and therapies (Rambow et al. 2008).

Three breeds of pigs develop melanoma spontaneously and have been used to study melanoma: Sinclair, Munich Troll, and MeLiM (melanoblastoma-bearing Libechov Minipig). The phylogenetics of these three breeds remains unknown, but it does not mean that these breeds are unrelated. More than any other animal model, porcine melanomas have been essential for gaining a better understanding of the natural history of the spontaneous melanoma regression with a complete tumor regression rate of 90%. Regression of the primary melanoma is characterized by the flattening, drying, and loss of pigmentation of the tumors (Vincent-Naulleau et al. 2004). Large-scale analyses of these various cases may be informative to decipher the mechanism(s) responsible for these phenomena.

## Nonmammalian Melanoma Models

While all of the abovementioned melanoma animal models involve mammals, nonmammalian models also exist and have played important roles in the understanding of the disease. More specifically, various species of fish have been used to study melanomagenesis and have been successful in identifying important factors that regulate disease initiation and progression.

Work using the swordtail fish *Xiphophorus* has shown that these fish can develop melanoma. More specifically, when different *Xiphophorus* species (*Xiphophorus maculatus*, which is a pigmented platyfish, and *Xiphophorus hellerii*, which is a nonpigmented swordtail fish) were mated, the resulting hybrid offspring developed melanoma. These melanomas were shown to result from the aberrant expression of Xmrk, which is the *Xiphophorus* ortholog of EGF

receptor (Wittbrodt et al. 1989). This tyrosine kinase receptor was able to promote melanophore proliferation, protect against apoptosis, and induce migration (Wellbrock et al. 2002). Reintroduction of the Xmrk gene into medaka (*Oryzias latipes*), another fish species, resulted in the formation of melanoma, confirming the role of Xmrk in the initiation of melanomagenesis in fish (Winnemoeller et al. 2005).

Genetically engineered zebrafish have provided an excellent tool for researchers to perform in vivo imaging experiments as well as large-scale chemical screens and genetic analyses to identify important molecular players and potential therapeutic targets for treatment of melanoma. Overall, the zebrafish and human genomes show approximately 70% similarity, and orthologs of an estimated 80% of human disease-associated genes have been identified in zebrafish (Howe et al. 2013).

Despite the emphasis on GEM models of melanoma, the first model of $BRAF^{V600E}$-driven melanoma was developed in zebrafish (Patton et al. 2005). In this study, zebrafish expressing oncogenic $BRAF^{V600E}$ under the control of the MITF promoter formed nevi. In addition, $BRAF^{V600E}$ expression was combined with TP53 silencing (*Mitf::BRAF$^{V600E}$; Trp53$^{-/-}$*); melanoma formation was increased compared to $BRAF^{V600E}$ expressing fish alone. The involvement of somatic gain-of-function mutations in *BRAF* has since been confirmed in mice and observed in dogs, thus demonstrating the utility and relevance of the zebrafish model for the better understanding of human melanoma.

Genetically engineered zebrafish have been useful in identifying other genes that are potentially involved in promoting melanomagenesis. More specifically, in one study, a list of several genes that were overexpressed in a set of human melanoma cell lines and tumor cultures was compiled, and each gene was co-expressed in the *Mitf::BRAF$^{V600E}$; Trp53$^{-/-}$* fish. In doing so, a number of genes that accelerate melanoma progression were identified (Ceol et al. 2011), many of which are also implicated or upregulated in human melanoma.

In addition to mutant $BRAF^{V600E}$, zebrafish models expressing mutant oncogenic $NRAS^{Q61K}$ have also been generated, which by itself resulted in hyperpigmentation of the fish (Dovey et al. 2009). When $NRAS^{Q61K}$ was expressed in $TP53^{null}$ zebrafish, melanomagenesis was again increased. These melanomas were invasive and could be transplanted into other zebrafish that were previously irradiated. Importantly, these tumors overexpressed a number of genes that are typically upregulated in human melanoma (Subramanian et al. 2005).

Genetically modified zebrafish have also been useful in demonstrating the role of the transcription factor MITF in melanomagenesis. MITF is a master regulator transcription factor in the melanocyte lineage and is responsible for the regulated expression of many genes essential for appropriate melanocyte development, migration, and function, including those genes that are involved in the production of melanin. In one study using a transgenic zebrafish model containing a temperature-sensitive MITF allele ($mitfa^{vc7}$), it was shown that while *Mitf::BRAF$^{V600E}$; mitfa$^{vc7}$* zebrafish did not form melanoma at the non-permissive temperature (due to a loss of MITF activity resulting from a splicing defect and lack of melanocytes), the same fish formed melanomas at the permissive temperature. These tumors appeared to be less differentiated than tumors from *Mitf::BRAF$^{V600E}$; Trp53$^{-/-}$* fish, as they had lower levels of the melanocyte markers DCT and TYR but higher levels of the oncogenic signaling protein c-MET. This result showed that mutated MITF, together with oncogenic $BRAF^{V600E}$, were sufficient for melanomagenesis in zebrafish. More impressively, when the fish at the permissive temperature were shifted to the non-permissive temperature, there was a regression of the melanoma tumors. Finally, the melanoma tumors recurred when the fish were shifted back to the permissive temperature (Lister et al. 2014). Thus, this study clearly demonstrated the necessity of MITF in maintaining melanoma tumors, at least in the context of $BRAF^{V600E}$ mutations.

Zebrafish can also be used for transplantation experiments, where human melanoma cells can be transplanted into either the early embryos, the larvae, or the adult animals. Melanoma cell lines

transplanted into the early embryos prior to gastrulation have been useful in identifying important signaling pathways, since the transplanted cells may alter the development of the embryos. Transplantation into the larvae can result in melanoma lesions within several days. Since the larvae are transparent, these types of experiments would allow for the visualization, under the microscope, of tumor-induced vascularization and metastatic spread. Coupling these types of experiments with zebrafish that contain fluorescently tagged vasculature would allow for the live visualization of angiogenesis and/or lymphoangiogenesis. Melanoma cells themselves that are fluorescently labeled can also be visualized in the embryos and larvae. This type of live visualization may be useful in discovering how different tumor cells behave and interact with one another in vivo during angiogenesis or invasion. Transplantation of melanoma cells in adult zebrafish is also useful; however, the issue of immune suppression must be addressed (potentially by gamma irradiation prior to transplantation). These transplantation experiments are useful for examining the tumorigenic and metastatic potential of various cells of interest (van der Weyden et al. 2016).

## Conclusion

While experiments done in cell culture and in vitro provide valuable information regarding the processes that regulate melanomagenesis, they are limited in their applicability to human melanoma due to their artificial nature. Therefore, animal models are essential in helping to gain a more relevant biological understanding of the molecular alterations that affect the different biological processes that lead to melanomagenesis. All of the animal models listed in this chapter have their advantages and disadvantages regarding the ease and availability of use and their relevance to human melanoma. Collectively, these models have helped in identifying and confirming a number of genes and proteins that are implicated in the initiation and progression of melanoma (e.g. BRAF, NRAS, $P16^{INK4A}$, β-catenin, PTEN, and TP53). It is this collectivity that is essential for the work on understanding and treating this disease. Each model makes important contributions and the findings from each help to advance the field as a whole.

As molecular biological techniques continue to improve and more options become available for use in animal models, our understanding of the molecular and physiological events that contribute to melanomagenesis will only increase. This will also allow for a more comprehensive strategy for the design and melanoma-specific targeting of various therapeutic compounds/agents, with the eventual goal of more effective treatments for melanoma patients.

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