A preclinical model of malignant peripheral nerve sheath tumor-like is characterized by infiltrating mast cells

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Abstract
Human melanomas exhibit considerable genetic, pathological, and microenvironmental heterogeneity. Genetically engineered mice have successfully been used to model the genomic aberrations contributing to melanoma pathogenesis, but their ability to recapitulate the phenotypic variability of human disease and the complex interactions with the immune system have not been addressed. Here we report the unexpected finding that immune-cell poor pigmented and immune-cell rich amelanotic melanomas developed simultaneously in Cdk4R24C mutant mice upon melanocyte-specific conditional activation of oncogenic BrafV600E and a single application of the carcinogen DMBA. Interestingly, amelanotic melanomas showed morphological and molecular features of malignant peripheral nerve sheath tumors (MPNST). A bioinformatic cross-species comparison using a gene expression signature of MPNST-like mouse melanomas identified a subset of human melanomas with a similar histomorphology. Furthermore, this subset of human melanomas was found to be highly associated with a mast cell gene signature, and accordingly, mouse MPNST-like melanomas were also extensively infiltrated by mast cells and expressed mast cell chemoattractants similar to human counterparts. A transplantable mouse MPNST-like melanoma cell line recapitulated mast cell recruitment in syngeneic mice demonstrating that this cell state can directly reconstitute the histomorphological and microenvironmental features of primary MPNST-like melanomas. Our study emphasizes the importance of reciprocal, phenotype-dependent melanoma-immune cell interactions and highlights a critical role for mast cells in a subset of melanomas. Moreover, our BrafV600E-Cdk4R24C model represents an attractive system for the development of therapeutic approaches that can target the heterogeneous tumor microenvironment characteristic of human melanomas.

Precis:
Findings highlight the ability to study human melanoma heterogeneity in a mouse model, revealing how melanocyte-immune cell interactions contribute to the development of distinct subsets of melanomas within a single individual.
**Introduction**

Malignant melanoma, the most aggressive type of skin cancer, is well known for its heterogeneity in clinical behavior and histomorphological appearance (1). This heterogeneity is in part due to the variety of oncogenic driver mutations in genes such as BRAF, NRAS or NF1 that initiate the malignant phenotype. These driver mutations cooperate with genetic deletions or loss of function mutations in critical tumor suppressor genes such as CDKN2A (p16), a negative regulator of the cell cycle kinase CDK4, or PTEN, a negative regulator of PI3K/AKT signaling. In addition to the landscape of genomic alterations, there is now an emerging interest in the phenotypic and microenvironmental heterogeneity of melanomas. This includes dynamic changes from proliferative to more invasive and migratory cell states during metastatic disease progression which are controlled in part by the melanocyte master transcription factor MITF (2,3). Reciprocal interactions with surrounding immune and endothelial cells facilitate the adaptation of melanoma cells to changing environments. Little is known how these principally reversible functional and epigenetic changes in melanoma cells are regulated on the background of persistent oncogenic genomic alterations.

Genetically engineered mouse models are powerful tools to scrutinize the reciprocal communication between melanoma cells and their microenvironment in vivo. Using the Hgf-Cdk4\textsuperscript{R24C} melanoma mouse model, we observed that pro-inflammatory mediators can shift the phenotype of melanoma cells towards a dedifferentiated state as part of a resistance mechanism to an adoptive T cell therapy targeting a melanocytic antigen (4,5). In a different experimental setting we found that UV-induced neutrophilic skin inflammatory responses promoted the emergence of migratory, angiotropic melanoma cell phenotypes and increased melanoma metastasis (6). Similar phenotypic shifts of melanoma cells towards more invasive, dedifferentiated and angiogenic phenotypes have been observed in a hypoxic microenvironment (7,8). Altogether, melanoma cell phenotypes and inflammatory as well as hypoxic responses in their microenvironment have an intricate relationship that needs to be further elucidated.

Here we investigated the phenotype of mouse melanomas which were initiated by melanocyte-specific activation of the oncogenic Braf\textsuperscript{V600E} mutation in the context of an oncogenic Cdk4\textsuperscript{R24C} germline mutation and further accelerated by a single application of the carcinogen DMBA. Unexpectedly, we observed melanomas with two distinct morphologies arising on the same genetic background and even in the same animal. A bioinformatic cross-species comparison of gene expression profiles obtained for mouse and human melanomas together with syngeneic mouse melanoma re-transplantation assays revealed how distinct cell states actively shape the histological appearance and the immune cell infiltrates of melanomas.
Materials and methods

Mice. Tyr::Cre<sup>ERT2</sup> mice (kindly provided by Lionel Larue, Orsay, France), LSL-Braf<sup>600E</sup> mice (kindly provided by Richard Marais, Cancer Research Institute, Manchester, UK) and Cdk4<sup>R24C</sup> mice (kindly provided by Mariano Barbacid, CNIO, Madrid, Spain) were crossed and genotyped as previously described (9-11). All animal experiments were conducted on the C57BL/6 background according to the institutional and national guidelines for the care and use of laboratory animals with approval by the local government authorities (LANUV, NRW, Germany).

Induction of primary melanomas. Braf<sup>600E</sup> expression was induced in melanocytes at an age of 6-8 weeks in Tyr::Cre<sup>ERT2</sup>-LSL-Braf<sup>600E</sup>-Cdk4<sup>R24C</sup> mice (called “Braf V600E-Cdk4R24C mice”) with topical application of 1mg tamoxifen (≥99%, Z-Isomer, Sigma T5648) dissolved in 100% acetone on their shaved backs for four consecutive days. Synchronous development of primary melanomas was initiated by a single epicutaneous application of 100nmol 7,12-dimethylbenz(a)anthracene (DMBA) on the shaved back one day after the last tamoxifen application. Tumor growth was monitored by inspection, palpation and digital photography. The size of the largest tumor was measured weekly using a vernier caliper and recorded as mean diameter. Mice were sacrificed when progressively growing melanomas exceeded 10 mm in size and tissues were harvested for further analyses.

Tumor transplantation assays. The BCmel4 melanoma cell line was derived from a primary amelanotic Braf V600E-CDK4R24C melanoma. In brief, fresh melanoma tissue was collected, dissociated mechanically, incubated with 1mg/ml of collagenase D (Roche) for 30 min at 37°C, and filtered through a 70µm cell strainer (BD Bioscience). Cells were seeded into collagen-coated 6-well plates and cultured in complete RPMI 1640 (Gibco) medium supplemented with 10% FCS (Biochrom), 2 mM L-glutamine (Gibco), 10 mM non-essential amino acids (Gibco), 1 mM HEPES (Gibco), 100 µg/ml streptomycin (Invitrogen). Groups of syngeneic C57BL/6 (purchased from Charles River) were injected intracutaneously with 4x10<sup>5</sup> BCmel4 melanoma cells into the flanks. Mice with tumors >10mm were sacrificed and tissue was harvested. The HCmel3 cell line was previously established from a primary pigmented Hgf-Cdk4<sup>R24C</sup> melanoma (5).

Histomorphological analyses of melanoma tissues. Mouse tissue samples were immersed in a zinc-based fixative (BD Pharmingen) and human melanomas in buffered paraformaldehyde (DAKO). Informed consent to use melanoma biopsy material for scientific purposes was obtained from all patients. Tissues were embedded in paraffin and sections stained with H&E and toluidine blue according to standard protocols. Heavily pigmented melanomas were bleached before staining (20 minutes at 37°C in 30% H<sub>2</sub>O<sub>2</sub> and 0.5% KOH, 20 seconds in 1% acetic acid and 5 minutes in TRIS buffer). Immunohistochemistry was performed with rabbit anti-mouse gp100 polyclonal antibody (Novus Biologicals, NBP1-69571) and goat anti-mouse NGFR polyclonal antibody (R&D Systems, BAF1157), followed by enzyme-conjugated secondary antibodies and the LSAB-2 color development system (DAKO). Intratumoral mast cells of mouse melanomas were counted in 3 sequential high-power fields (20x magnification) and mean number of intratumoral mast cells was...
expressed as mast cells/3 high-power fields. All stained sections were examined with a Leica DMLB microscope.

**Analyses of human melanoma histopathologies in the TCGA cohort.** Digital images of H&E-stained tissue sections for the 44 human melanoma samples in the TCGA data base with a gene expression profile similar to MPNST-like mouse Brαf\(^{V600E}\)-CDK4\(^{R24C}\) melanomas were retrieved from the Cancer Digital Slides Archive (http://cancer.digitalslidearchive.net/). The images were carefully analysed for the presence of amelanotic melanoma cell subpopulations with scanty pale cytoplasm and wavy nuclei in a collagen-rich stroma or with mesenchymal-like morphology and fascicular growth patterns were scored as histomorphologically similar to MPNST-like mouse Brαf\(^{V600E}\)-CDK4\(^{R24C}\) melanomas.

**Microarray analysis of gene expression.** RNA from mouse tumor tissues and cultured mouse melanoma cell lines was isolated as described (5), quantified fluorimetrically and assayed for integrity with the Agilent Bioanalyser (Agilent Technologies). 100ng RNA was converted to biotinylated cRNA using one round of amplification with the Illuma Labelling Kit (Illumina) and one round of T7 polymerase amplification and hybridized to Illumina Murine Beadchips v. 2.0. After hybridization and staining, the arrays were scanned in an Illumina Bead Station, and the images processed using Illumina Bead Studio software. Gene expression data have been deposited in the GEO repository with the following accession number: GSE71879.

**Gene expression analysis of Brαf\(^{V600E}\)-CdK\(^{R24C}\) mouse melanomas.** Raw microarray data was extracted from the Illumina BeadStudio software and imported into the R statistical programming environment and the Bioconductor platform using the beadarray package. Variance stabilization and normalization were performed followed by quality assessment of the fit. Differential gene expression between amelanotic MPNST-like and pigmented Brαf\(^{V600E}\)-CdK\(^{R24C}\) melanomas (n=5 each) was calculated by a linear model and empirical Bayes-moderated t-statistics (eBayes function, Bioconductor limma package) adjusted for multiple testing using the procedure by Benjamini and Hochberg (BH) to determine the false discovery rates (FDR = BH adjusted p-values). The NF/MPNST-like signature was generated by stringent selection criteria (BH-corrected p-value <0.01 and log2 fold-change >2 between amelanotic MPNST-like and pigmented Brαf\(^{V600E}\)-CdK\(^{R24C}\) melanomas). The stringency of selection criteria was arbitrarily chosen to generate gene sets that fulfill the default recommendation for gene set sizes (25-500) for GSEA (see http://www.broadinstitute.org/gsea/doc/GSEAUserGuideFrame.html).

**Gene set enrichment analysis.** Pathway analysis of pigmented versus amelanotic MPNST-like Brαf\(^{V600E}\)-CdK\(^{R24C}\) mouse melanomas was performed using gene set enrichment analysis (GSEA) and the curated C2 and C3 BROAD molecular signature gene set collection (MSigDb v4.0, http://www.broadinstitute.org/gsea/msigdb/index.jsp). The t-test statistics results of the empirical Bayes-moderated t-test method were used as metric for running the pre-ranked gene list algorithm (GSEAPreranked) of the BROAD GSEA tool (http://www.broadinstitute.org/gsea/index.jsp) with 10K
permutations (permutation type = gene set as the only available option in GSEAPreranked). The VERFAILLIE_INVASIVE and VERFAILLIE_PROLIFERATIVE gene sets were retrieved from the original publication (12). The HOEK_INVASIVE and HOEK_PROLIFERATIVE gene sets were retrieved from http://www.jurmo.ch/work_97.php. These gene sets were manually included in the C2 gene set collection for GSEA.

Cross-species comparison with TCGA human cutaneous melanoma cohort. Human homologs (n=241) of the mouse MPNST-like signature genes (n=265) were identified using the NCBI homologene database (ftp://ftp.ncbi.nih.gov/pub/HomoloGene/). Gene expression data (RNA-seq) were accessed through the cBioportal for Cancer Genomics (http://www.cbioportal.org) and the R-based package CGDS-R (13,14). We retrieved individual gene expression values as median z-scores as one of the default gene expression formats provided by TCGA and the cBioportal project. We reasoned that z-scores emphasize variation in gene expression across the cohort rather than global mRNA abundance with a favorable performance for building integrated signature scores. To calculate an MPNST-like signature z-score (=mean z-score) for each sample we took the mean of the z-scores of the signature genes. To identify (define) BCMPNST-like positive samples we applied an unbiased statistical cutoff (>2xMAD, median absolute deviation). TCGA samples were then sorted and plotted by increasing gene signature z-scores. Pigmentation and immune cell subtype signature z-scores were generated as described for the BCMPNST-like signature above. Bioinformatic analysis of TCGA melanoma samples was finalized by end of February 2015 and all melanoma samples provided through cBioportal at this time were included.

BCMPNST-like signature expression in different human tumor entities. The following raw affymetrix CEL files (all samples Hgu133plus2 platform) were downloaded from the GEO repository: Basal cell carcinoma (GSE7553), breast cancer (GSE12276), neurofibroma/MPNST (GSE14038), Ewing sarcoma (GSE34620), GIST (GSE20708, GSE8167, GSE17743), malignant melanoma (GSE7553, GSE19234, GSE23376, GSE10282, GSE19293), medulloblastoma (GSE10327, GSE12992), neuroblastoma (GSE13136, GSE16237), soft tissue sarcoma (GSE21050), skin squamous cell carcinoma (GSE7553). All samples were normalized using RMA (Robust Multi-array Average) and expression values were log2-transformed followed by gene-wise z-score transformation. Corresponding affymetrix probes were identified for each of the genes of the human homologues of the BCMPNST-like signature and one probe per gene with the maximum expression was selected. Then averaged z-score across all genes were calculated for each sample and the sample-wise signature z-scores were plotted in a boxplot grouped by the different tumor entities. Significance of group-wise comparisons was determined by an unpaired two-sided and pairwise t-test with Benjamini&Hochberg (=false discovery rate) correction for multiple comparisons.

Characterization of genomic aberrations of BCMPNST-like TCGA melanomas. We retrieved data of genomic aberrations either through the webpage or the R-interface of the cBioportal project: total count of non-synonymous mutations and non-synonymous mutations (BRAF, NRAS and NF1).
Refinement of immune cell subtype signature (ICSS) for the use in melanoma samples. From the study by Bindea et al. (15) we used the affymetrix gene probe identifiers for each ICSS of interest (n=8) in our study: T cells, CD8 T cells, B cells, NK cells, neutrophils, macrophages, mast cells, eosinophils. As the work by Bindea et al. addressed a different tumor context (colon cancer) we performed a melanoma-specific adjustment to exclude genes with overlap expression in melanoma that had no overlap expression in colon cancer as determined by Bindea et al. The expression of each ICSS gene probe was determined across a panel of human melanoma cell lines (n=88, BROAD melanoma cell line panel) and then represented by a boxplot to visualize variation in gene expression. The BROAD melanoma cell line panel and the ICSS were analyzed by the same microarray platform (Affymetrix) which facilitated the melanoma-specific adjustment of the ICSS. Affymetrix raw CEL files from the BROAD melanoma cell line panel of 88 melanoma cell lines were downloaded from the BROAD melanoma portal (https://www.broadinstitute.org/software/cprg/?q=node/46) and normalized by Robust Multichip Average (RMA) using the affy package and expression values were log2 transformed.

Statistical tests. All statistical tests were performed with the R-computing platform. We specify within the manuscript or legends the type of test, parametric or non-parametric and direction (e.g. two-sided).
Results
Simultaneous development of immune-cell poor pigmented and immune-cell rich amelanotic melanomas in TAM+DMBA-treated Braf\(^{V600E}\)-Cdk4\(^{R24C}\) mice

In previous studies we showed that Hgf-Cdk4\(^{R24C}\) mice exposed to a single epicutaneous dose of the carcinogen DMBA rapidly developed pigmented immune-cell poor melanomas which imitate the histomorphology observed in a subgroup of patients with primary cutaneous melanomas (5,6,16). Transgenic Hgf (hepatocyte growth factor) expression provides continuous mitogenic stimulation through the receptor tyrosine kinase Met. The oncogenic Cdk4\(^{R24C}\) mutation impairs p16-dependent cell cycle control as an intrinsic barrier to malignant transformation. Given that BRAF is mutated in about half of human melanomas and a key therapeutic target, we decided to generate a Braf\(^{V600E}\)-driven model by crossing Tyr::Cre\(^{ERT2}\), LSL-Braf\(^{V600E}\) and Cdk4\(^{R24C}\) mice. Epicutaneous application of tamoxifen (TAM) on the shaved back skin of Tyr::Cre\(^{ERT2}\)-LSL-Braf\(^{V600E}\)-Cdk4\(^{R24C}\) mice (henceforth called Braf\(^{V600E}\)-Cdk4\(^{R24C}\) mice) for four consecutive days allows for selective and conditional activation of oncogenic BRAF\(^{V600E}\) exclusively in the melanocytic lineage (17). In addition, these mice received a single topical dose of the carcinogen DMBA in order to allow for a comparative evaluation of melanoma development with DMBA-treated Hgf-Cdk4\(^{R24C}\) mice (Figure 1A). TAM+DMBA-treated Braf\(^{V600E}\)-Cdk4\(^{R24C}\) mice developed progressively growing skin melanomas after 60 days (+/- 13 days SEM, Figure 1B). They had to be sacrificed because melanomas exceeded a size of 10mm in diameter after 131 days (+/- 34 days SEM, Figure 1B). The kinetics of tumor onset and growth were highly similar to that observed previously in DMBA-treated Hgf-Cdk4\(^{R24C}\) mice (5,6). Of note, a single topical dose of DMBA fails to induce melanomas in Cdk4\(^{R24C}\) mice as previously shown (18).

Unexpectedly, TAM+DMBA-treated Braf\(^{V600E}\)-Cdk4\(^{R24C}\) mice developed not only pigmented but also amelanotic tumors at an almost equal ratio in the same animal (Figure 1C,D). Microscopic analysis of hematoxylin and eosin (H&E) stained tissue sections of the pigmented tumors showed an indistinguishable morphology from pigmented DMBA-induced Hgf-Cdk4\(^{R24C}\) melanomas with a highly melanocytic, immune cell-poor phenotype (Figure 1E, left panel). In contrast, the amelanotic tumors lacked expression of the melanocytic marker gene gp100 but instead stained strongly positive for Ngfr, an inflammation-responsive TNF-receptor family member that is found on dedifferentiated melanoma cells and neural crest progenitors (6) (Figure 1E, right panel). Intriguingly, the amelanotic tumors were also infiltrated by increased numbers of CD45+ immune cells when compared to the melanotic tumors (Figure 1E, bottom).

Morphological features of malignant peripheral nerve sheath tumors in amelanotic TAM+DMBA-induced Braf\(^{V600E}\)-Cdk4\(^{R24C}\) melanomas

A closer histopathologic scrutiny revealed considerable morphologic heterogeneity within amelanotic tumors, which consisted of alternating hypocellular areas with myxoid stroma and hypercellular areas with spindle-shaped melanomas cells. Many tumor cells had a fusiform appearance with wavy or comma-shaped hyperchromatic nuclei often embedded in a fibrillar collagen-rich stroma. Nests of pigmented more epitheloid melanoma cells were sparsely found in
amelanotic tumors. Mitotic figures were predominantly found in cell-rich regions with fascicular growth patterns and only rarely in cell-poor regions with myxoid components. Amelanotic melanomas also showed some areas with fibrosarcoma-like herringbone growth patterns as well as hemangiopericytoma-like branching vascular structures (Figure 2). These histological features are reminiscent of malignant peripheral nerve sheath tumors (MPNST) suggesting that the amelanotic melanomas of Braf<sup>V600E</sup>-Cdk4<sup>R24C</sup> mice have a MPNST-like phenotype.

Comparative transcriptomic characterization of amelanotic, MPNST-like and pigmented Braf<sup>V600E</sup>-Cdk4<sup>R24C</sup> mouse melanomas

To better understand the phenotype of the amelanotic, MPNST-like Braf<sup>V600E</sup>-Cdk4<sup>R24C</sup> melanomas we performed genome-wide transcriptomic analyses and determined genes that were differentially expressed when compared with the pigmented melanomas using a moderated t-test statistic (Figure 3A). We obtained a ranked gene list (t-test values as ranking parameter) and performed gene set enrichment analysis (GSEA) using the C2 curated gene set collection of the BROAD molecular signature database (MSigDB) that was extended by gene sets representing the so-called "proliferative" and "invasive" melanoma cell states (Supplementary Tables 1 and 2). "Invasive" human melanoma cell lines are characterized by an epithelial-mesenchymal transition (EMT)-like phenotype in contrast to the melanocytic "proliferative" cell state, as described by Hoek et al. and Verfaillie et al. (12,19). We found that MPNST-like mouse melanomas showed significant enrichments of the "invasive" (Hoek/Verfaillie), EMT, collagen and (neural crest) stem cell gene signatures suggesting reactivated neural crest pathways (Figure 3B and Supplementary Figure 1A). These developmental programs normally allow melanocytes to emerge from pluripotent neural crest cells. On the contrary, the pigmented melanomas were enriched for the "proliferative" (Hoek/Verfaillie) gene signatures in addition to metabolic pathways associated with oxidative phosphorylation, glucose metabolism and lysosomal biogenesis (Figure 3B and Supplementary Figure 1B). This is consistent with recent studies that identified MITF, the key inducer of the melanocytic differentiation program, as regulator of mitochondrial respiration and driver of lysosomal biogenesis (20,21). The "proliferative" melanoma gene signatures are largely defined by the MITF-driven transcriptional program and contain numerous <i>bona fide</i> MITF targets like <i>tyrosinase</i> that are involved in pigment production.

We also assessed the transcription factor framework defining the pigmented versus MPNST-like melanomas in our mouse model (Supplementary Figure 2a). Besides MITF, pigmented melanomas showed high expression of Sox10, Ppargc1a (PGC-1alpha) and Tfap2a (AP-2alpha) that are key components of the "proliferative" signature and cooperate with MITF (Supplementary Figure 2b) (20,22). MPNST-like melanomas strongly expressed EMT-driving transcription factors like Zeb1, Twist1, Wwtr1 (TAZ), Gli2 and Tead1 (Supplementary Figure 2c). GSEA using the C3 transcription factor target gene set collection revealed a potential role for Sox9, Engrailed-1, CCAAT/enhancer binding protein (C/EBP) and POU transcription factors in the orchestration of the MPNST-like phenotype (Supplementary Table 1). We found significantly higher expression of Engrailed-1 (En1), a key factor for midbrain development and survival of mesencephalic dopaminergic neurons, as...
well as Pou3f1 (Oct-6) and Egr2 (Krox-20) that are both master regulators of myelination in Schwann cells (23,24) (Supplementary Figure 2d,e). Thus, MPNST-like melanomas express neuronal and Schwann cell lineage transcription factors, in addition to the EMT-related transcriptions factors described by the "invasive" melanoma phenotype. High levels of neuronal and Schwann cell markers like Gap43, Gldn (Gliomedin), Nefm (Neurofilament), Ngrf or Map2 (Microtubule-associated protein 2) further argue for the neuronal traits of MPNST-like mouse melanomas in addition to their mesenchymal phenotype with expression of collagens and other extracellular matrix components (Figure 3C). Together, this molecular characterization supports our morphological designation as MPNST-like. In contrast, pigmented Braf<sup>V600E</sup>-Cdk4<sup>R24C</sup> melanomas showed a gene expression profile that was highly similar to the pigmented Hgf-Cdk4<sup>R24C</sup> melanomas with strong expression of typical melanocytic differentiation markers (Figure 3C).

For further analyses and cross-species comparisons we decided to build a core signature characterizing the amelanotic MPNST-like mouse melanomas (see Methods). Using stringent cutoff criteria we obtained 265 genes strongly expressed in MPNST-like melanomas (Braf<sup>V600E</sup>-Cdk4<sup>R24C</sup> MPNST-like signature; abbreviated as BC<sup>MPNST-like</sup>) versus 99 genes (Pigmented signature) highly associated with the pigmented melanoma phenotype (Figure 3D and Supplementary Table 3). The BC<sup>MPNST-like</sup> signature (converted to human homologues) showed a partial overlap with the Hoek and Verfaillie "invasive" gene signature derived from human melanoma cell cultures (Supplementary Figure 3 and Supplemental Table 2). Then we investigated the expression of the BC<sup>MPNST-like</sup> melanoma signature in different human tumors including neuroectodermal tumors like neurofibroma/MPNST, neuroblastoma, medulloblastoma and Ewing sarcoma. Indeed, neurofibromas/MPNSTs and soft tissue sarcomas showed the highest expression of the BC<sup>MPNST-like</sup> signature supporting our morphological and transcriptomic characterization (Figure 3E). This multi-tumor analysis also indicated that subsets of human melanomas express high levels of the BC<sup>MPNST-like</sup> signature and may therefore exhibit MPNST-like traits.

**Identification of human melanomas with a MPNST-like histomorphology in the TCGA data base using a bioinformatic cross-species comparison of gene expression signatures**

Human melanomas indeed show a broad range of histological phenotypes because they can reactivates the phenotypic plasticity of their embryonic precursors from the neural crest. This also includes MPNST-like phenotypes which have occasionally been described in metastatic lesions (25). We hypothesized that amelanotic Braf<sup>V600E</sup>-Cdk4<sup>R24C</sup> melanomas resemble MPNST-like human melanomas and therefore we analyzed the TCGA melanoma cohort by calculating BC<sup>MPNST-like</sup> signature z-scores (see Methods) to identify and characterize human MPNST-like melanomas. Indeed a small subset (44/384, 11%) of the human TCGA melanomas highly expressed the BC<sup>MPNST-like</sup> gene signature using an unbiased statistical cutoff (Figure 4A and Supplementary Figure 4). To assess the melanocytic differentiation status of this subgroup we manually curated a pigmentation-related gene set (n=17) and calculated signature z-scores. As shown in Figure 4B most of the human TCGA melanomas with a BC<sup>MPNST-like</sup> signature lacked expression of pigmentation genes similar to their mouse counterparts.
Next we asked whether human melanomas with high expression of the BC\textsuperscript{MPNST-like} gene signature have a histological appearance that is similar to the mouse MPNST-like melanomas. For this purpose we made use of the Cancer Digital Slide Archive project (http://cancer.digitalslidearchive.net/) that provides high-resolution images of H&E stained tissue sections (diagnostic slides) from TCGA cohorts. Indeed, a careful histopathological evaluation revealed a remarkable morphological similarity of human and mouse melanomas correlating with the expression of the BC\textsuperscript{MPNST-like} gene signature (Figure 4C). As the TCGA project also characterizes the tumor samples by whole-exome sequencing, we utilized this data to explore the mutational landscape of human melanomas with high BC\textsuperscript{MPNST-like} gene signature expression. An analysis of oncogenic driver mutations revealed BRAF, NRAS and NF1 mutations rates of 67%, 22% and 22% in melanomas with the BC\textsuperscript{MPNST-like} gene signature versus 56%, 32% and 14% in the other samples (Figure 4C). The total number of non-synonymous mutations was also high. Hence, BC\textsuperscript{MPNST-like} melanomas of the TCGA cohort have typical melanoma driver mutations and a high mutation load, ruling out that these samples have been erroneously misclassified as melanomas and thus represent "true" melanomas. Altogether, our bioinformatic cross-species comparison identified a subgroup of human melanomas with a transcriptional profile and histomorphology that closely resembles amelanotic, MPNST-like Braf\textsuperscript{V600E}-Cdk4\textsuperscript{R24C} mouse melanomas.

**High expression levels for mast cell-related genes in human melanomas with a transcriptional profile of MPNST-like Braf\textsuperscript{V600E}-Cdk4\textsuperscript{R24C} mouse melanomas**

Previously we showed that inflammatory signals promote phenotype switching of melanoma cells in vivo. Therefore, we investigated the immune landscape of the subgroup of human melanomas that showed the MPNST-like transcriptional profile of Braf\textsuperscript{V600E}-Cdk4\textsuperscript{R24C} mouse melanomas using immune cell subtype specific gene expression signatures (ICSS) as recently described by Bindea et al. in the context of colon cancer (15). In this study the classifiers were built by differential gene expression analysis of various immune cell subtypes including B cells, T cells and macrophages. A colon cancer cell line served as reference to exclude genes from the signatures with overlapping expression in the tumor cells. We reasoned that the immune cell signatures needed to be adjusted, as melanoma and colon cancer cells likely differ in the set of genes with potential overlap expression in immune cells (Figure 5A). To determine the expression level of individual ICSS-genes (e.g. CD3D, CD19, CD163, FCER1A) in melanoma cells we employed data from a published melanoma cell line panel (n=88) that was profiled with the same microarray platform as used for the ICSS identification by Bindea et al. (Supplementary figure 5). This uniformity in technical platforms allowed us to use the identical and validated gene probes for comparison. As expected we found that several ICSS-genes showed moderate to high levels in melanoma cells and c-KIT is an obvious example, as it is expressed both in melanoma and mast cells. Hence, all overlapping ICSS-genes like c-KIT were excluded as a melanoma-specific adjustment prior to further analysis (Supplemental Table 2).
Next, we retrieved mRNA expression values of the ICSS-genes from the TCGA melanoma cohort and determined ICSS z-scores (Supplementary Figure 6). To identify significant associations of human melanomas with the BC\textsuperscript{MPNST-like} transcriptional profile of \textit{Braf\\textsuperscript{V600E}-Cdk4\\textsuperscript{R24C}} mouse melanomas we performed non-parametric testing with Bonferroni correction for multiple comparisons. We found enrichments of ICSS for myeloid cell lineages and NK cells (Figure 5B). Notably, the mast cell gene signature was the top ranking ICSS that was significantly linked to the MPNST-like transcriptional profile in human melanomas. This is an intriguing finding as mast cell infiltration is a well-known phenomenon in human and mouse neurofibromas and MPNSTs. Mast cell recruitment and activation is governed by a set of characteristic chemokines including KIT ligand (KITLG), CCL11 (eotaxin-1) and IL33. We performed a literature search and compiled a gene set of chemokines that have been linked to mast cell recruitment (26). Consistently, this mast cell chemotaxis gene set was significantly higher expressed in human melanomas with the BC\textsuperscript{MPNST-like} transcriptional profile, as there was also an overlap between the two signatures (Figure 5C-E and Supplementary Figure 7A). To corroborate our findings we analyzed an independent cohort of human metastatic melanomas and confirmed that the expression of the BC\textsuperscript{MPNST-like} gene signature positively correlated with the mast cell gene sets (Figure 5F-G) (27). Furthermore, we screened a human melanoma cohort with available tissue specimens for MPNST-like histomorphological appearance and indeed we found mast cells in melanomas with MPNST-like features by toluidine blue staining, whereas mast cell infiltration is extremely rare in regular melanomas (Supplementary Figure 7B-D).

**Recruitment of mast cells in amelanotic, MPNST-like Braf\\textsuperscript{V600E}-Cdk4\\textsuperscript{R24C} mouse melanomas**

Next we investigated whether MPNST-like Braf\\textsuperscript{V600E}-Cdk4\\textsuperscript{R24C} mouse melanomas also expressed mast cell-related gene sets. This was indeed the case as amelanotic Braf\\textsuperscript{V600E}-Cdk4\\textsuperscript{R24C} melanomas showed high expression of mast cell markers and mast cell chemoattractants whereas pigmented Braf\\textsuperscript{V600E}-Cdk4\\textsuperscript{R24C} and Hgf-Cdk4\\textsuperscript{R24C} melanomas did not (Figure 6A and Supplementary Figure 8). Furthermore, toluidine blue staining of amelanotic, MPNST-like Braf\\textsuperscript{V600E}-Cdk4\\textsuperscript{R24C} melanomas revealed a substantial mast cell infiltration, whereas the pigmented Braf\\textsuperscript{V600E}-Cdk4\\textsuperscript{R24C} as well as the pigmented Hgf-Cdk4\\textsuperscript{R24C} melanomas lacked mast cells (Figure 6B). Next, we speculated that mast cell recruitment is directly dictated by the MPNST-like melanoma cell state. We established the BCmel4 cell line from a MPNST-like Braf\\textsuperscript{V600E}-Cdk4\\textsuperscript{R24C} melanoma which displayed an amelanotic and mesenchymal-like phenotype \textit{in vitro}. BCmel4 expressed MPNST-like marker genes and mast cell chemoattractants like Kitlg and Il33, but not the melanocytic differentiation genes when compared to the pigmented mouse melanoma cell line HCmel3 (Figure 6C). Intracutaneous transplantation of BCmel4 cells into syngeneic C57BL/6 mice led to amelanotic tumors that macroscopically and microscopically resembled the primary MPNST-like Braf\\textsuperscript{V600E}-Cdk4\\textsuperscript{R24C} melanomas. Importantly, toluidine blue staining of BCmel4 tumors also showed a dense infiltration of mast cells (Figure 6D). In contrast, the pigmented HCmel3 cell line generated syngeneic melanomas that entirely lacked mast cells. Altogether, these results suggest that mast cell recruitment is directly and intrinsically linked to the MPNST-like melanoma cell state and the
expression of mast cell chemoattractants is a species-conserved characteristic of this melanoma subtype.

Discussion
In the present study we show that \( \text{Braf}^{\text{V600E}} - \text{CdK4}^{\text{R24C}} \) mice simultaneously develop both pigmented, highly melanocytic as well as amelanotic melanomas with morphology features reminiscent of malignant peripheral nerve sheath tumors even in the same animal. The appearance of amelanotic melanomas has previously been described in other genetically engineered mouse melanoma models. Histomorphological features of neural crest and Schwann cell differentiation were first reported for melanomas from a different genetically engineered \( \text{Braf}^{\text{V600E}} \) mouse melanoma model (28). Other mouse models where primary skin melanomas are driven by mutant \( \text{Braf} \) in combination with loss of \( \text{Pten} \) or \( \text{NF1} \) also appear to develop predominantly amelanotic melanomas with smaller areas of pigmented tumor cells (29,30). In line with our results, it was shown that amelanotic melanomas driven by mutant \( \text{Nras} \) in combination with loss of \( \text{p53} \) also express genes related to neural differentiation, including genes of the Schwann cell lineage (31).

The simultaneous development of highly pigmented and amelanotic melanomas on the same genetic background similar to our model has been observed in the TiRP mouse model where melanocytes are transformed by Cre-mediated conditional expression of the mutant \( \text{H-ras}^{\text{G12V}} \) oncogene in combination with conditional deletion of the \( \text{Ink}4\text{a/Arf} \) tumor suppressor gene specifically in melanocytes. In agreement with our findings in \( \text{Braf}^{\text{V600E}} - \text{CdK4}^{\text{R24C}} \) mice, the pigmented melanomas in TiRP mice were immune cell poor whereas the amelanotic melanomas were immune cell-rich. Accordingly, genome-wide transcriptomic analyses revealed an increased expression of proinflammatory genes as well as genes related to the \( \text{Tgf-β} \) pathway and to epithelial-mesenchymal transition in amelanotic tumors when compared with melanotic tumors, suggesting a link between a dedifferentiated phenotype and an inflammatory microenvironment (32).

We performed a bioinformatic cross-species comparison using a gene expression signature of amelanotic, MPNST-like mouse melanomas and identified a subset of human melanomas with a similar histomorphology in the TCGA database. Human melanomas with features of Schwann cell differentiation have been first described as a new histological variant of desmoplastic melanoma, termed “neurotropic” melanoma, by Reed and Leonard in 1979 (33). Such desmoplastic melanomas with features of neural crest differentiation are often amelanotic and tend to occur in elderly patients with a predilection for the head and neck area (34-36). Conversely, malignant peripheral nerve sheath tumors can express melanocytic markers and display a pigmented melanoma phenotype. These tumors have previously been described as pigmented neurofibromas, melanotic or melanocytic schwannomas or cutaneous melanocytoneuromas (35). Our observations in both mice and humans further support the notion that melanomas can present with a wide range of neural crest-related phenotypes and provide evidence that genetically engineered mouse models can recapitulate this phenotypic heterogeneity.
It has been thought that amelanotic, desmoplastic melanomas arise from transformed melanocytes as a consequence of dedifferentiation. An inflammatory microenvironment would favor this dedifferentiation process (6). Alternatively, it has been postulated that amelanotic melanomas may originate directly from neural crest precursor cells. This may have happened in the Braf<sup>V600E</sup>-Cdk4<sup>R24C</sup> mice used in our experiments, since the tyrosinase promoter used to drive the CRE<sup>ERT2</sup> gene and conditionally activate the Braf<sup>V600E</sup> oncogene may be expressed in other neural crest-derived cells (37). Pigmented tumor cells could arise through melanocytic differentiation of these precursors, a process that has been shown to occur even under physiologic conditions during development using genetic lineage tracing (38). The concept of principally reversible phenotype switching towards a dedifferentiated cell type in melanoma progression has recently been supported by the observation that Braf-induced transformation of melanocytes (Melan-A cells) leads to a reversible suppression of the MITF-driven melanocyte differentiation program through activation of the EMT-inducing transcription factors ZEB1 and TWIST1. Expression of ZEB1/TWIST1 at the invasion front of primary human melanomas was associated with poor prognosis indicating a role of dedifferentiated phenotypes during melanoma progression in humans (39).

One of the most intriguing findings in our work is the identification of a species-conserved mast cell infiltration in MPNST-like melanomas that appears to be directly orchestrated by the respective melanoma cell state. Mast cell recruitment is a known phenomenon of neurofibromas and MPNSTs, that predominantly develop in patients with neurofibromatosis type I (40). This hereditary disease is caused by germline mutations in NF1, a GTPase-activating protein that negatively regulates RAS. NF1 is a well-documented tumor suppressor in melanoma, neuroblastoma and several other cancers. Interestingly, Braf<sup>V600E</sup> activation and NF1 loss in the melanocytic lineage of mice causes melanomas that were described as hypopigmented/amelanotic with a fascicular growth pattern and rarely interspersed melanophages (30). As the immune cell composition of Braf<sup>V600E</sup>-NF1<sup>−/−</sup> mouse melanomas was not assessed in this study, we can only speculate whether mast cell recruitment is a conserved feature in this model. Nevertheless, this histological description is indeed reminiscent of MPNST-like melanomas found in our study and suggests a potential mechanistic link between NF1-loss and the MPNST-like melanoma phenotype. However, NF1 loss is most likely a cooperating rather than a sufficient genomic event driving the MPNST-like phenotype of human melanomas, as NF1 mutations also occurred in many non-MPNST-like melanomas of the TCGA cohort. Several elegant studies using Nf1 knockout mice as a model for neurofibromatosis type I revealed a critical contribution of mast cells to the development of neurofibromas and MPNSTs (40-42). Thus, our study indicates that mast cells play an important role in the MPNST-like melanoma subtype and it requires further investigation how they precisely modulate the tumor microenvironment.

The role of mast cells for melanoma pathogenesis is still unclear. Several histomorphological studies showed increased mast cells in primary melanomas compared to benign melanocytic...
lesions (43). Intratumoral mast cell infiltration positively correlates with microvessel density, suggesting that the tumor promoting capacity of mast cells is attributed to their release of proangiogenic and tissue degrading mediators (44). Experimental mouse studies have shown that mast cells promote B16 melanoma growth and/or metastatic spread through mast cell derived HIF-1a (45). Histomorphological analyses of nine primary melanomas with a distinct MelanA negative clone next to a MelanA positive clone, discovered a significant correlation of the loss of MelanA expression with an increased infiltration of CD163+ macrophages and a spindle-shaped tumor cell morphology in comparison to MelanA expression tumor areas (46). These observations as well as our results suggest that reciprocal tumor-immune cell interactions shape the microenvironment and the melanoma cell phenotype in mouse and man.

By comparison with the TGCA melanoma cohort we identified human B0MPNST-like melanoma counterparts with species-conserved immune cell compositions and histological appearance. Our bioinformatic approach demonstrates the usefulness of consortium-driven cancer genomics projects like TCGA with free data access to researchers around the globe. Most studies employing TCGA data focus on gene signatures, genomic aberrations and prognosis. Our work is an example how TCGA data can be used beyond these approaches to match histomorphological with molecular phenotypes and to correctly position genetically engineered mouse tumor models against the background of the respective human malignancy.

Our study also highlights that genetically engineered mouse melanoma models can recapitulate cross-species conserved histological appearance and immune cell infiltrates of the human disease. Our data further support the idea that a proinflammatory microenvironment favors melanoma cell plasticity and facilitates the reactivation of their embryonic developmental program. Recent studies suggest that phenotypic plasticity contributes to therapy resistance in melanoma (47). Low MITF expression of melanoma cells has been identified as common feature of most BRAF-inhibitor resistant cell lines and patient biopsies indicating that distinct cell states influence resistance to MAPK pathway inhibitors in BRAF mutant melanomas (48). The proinflammatory cytokine TNF which is known to be abundant in the tumor microenvironment has been shown to dynamically regulate Twist1, an epithelial-mesenchymal transition regulator, and induce an EMT-like melanoma cell phenotype that is resistant to BRAF inhibition (49). TNF has also been identified to induce a dedifferentiated melanoma cell phenotype that contributes to resistance to an adoptive T cell therapy (6). Taken together, emerging evidence indicates that melanoma cells can switch between different phenotypes as adaptation to environmental factors.

In conclusion, BrafV600E-Cdk4R24C mice develop distinct melanoma phenotypes that are relevant to the human diseases, as they recapitulate cross-species conserved histological appearance and immune cell infiltrates. We propose that our mouse melanoma model is useful for the preclinical development of phenotype-directed therapies which exploit synergisms with current treatments and counteract resistance-driving phenotypic heterogeneity (50).
References


correlated with dense infiltration of CD163 macrophages and loss of E-cadherin. Melanoma Res. 2015;
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Figure Legends

Figure 1. Simultaneous development of immune-cell poor pigmented and immune-cell rich amelanotic melanomas in TAM+DMBA-treated Bra\textsubscript{V600E}-Cdk\textsubscript{4R24C} mice.
A. Experimental protocol for melanoma induction in Bra\textsubscript{V600E}-Cdk\textsubscript{4R24C} mice using sequential topical application of tamoxifen (TAM) to activate oncogenic Bra\textsubscript{V600E} expression in melanocytes and a single application of the carcinogen DMBA to accelerate and synchronize tumor growth. B. Mean diameter of the largest primary tumor in individual TAM+DMBA-treated Bra\textsubscript{V600E}-Cdk\textsubscript{4R24C} mice (n=10). C. Representative macroscopic pictures of TAM+DMBA-induced pigmented and amelanotic melanomas in a Bra\textsubscript{V600E}-Cdk\textsubscript{4R24C} mouse. Upper panel: Outside view of tumor-bearing skin flap. Lower panel: View from dermal side of the same skin flap. D. Number of pigmented and amelanotic tumors in individual mice at the time of death. E. Representative tissue sections of a pigmented (left panels) and an amelanotic melanoma (right panels) stained with H&E (left) and immunohistochemically for gp100, Ngfr and CD45. Size bars indicate magnifications of the tissue sections.

Figure 2. Morphological features of malignant peripheral nerve sheath tumors in amelanotic TAM+DMBA-induced Bra\textsubscript{V600E}-Cdk\textsubscript{4R24C} melanomas.
Representative H&E stained tissue sections demonstrating the spectrum of histomorphological appearances of an amelanotic Bra\textsubscript{V600E}-Cdk\textsubscript{4R24C} tumor. The larger panel in the middle shows an overview of the tumor at lower magnification (2.5x). The smaller panels exemplify the heterogeneity of the histological appearance found in a single tumor (magnification 40x). Top left: Nest of melanocytic tumor cells. Top right: Hemangiopericytoma-like vascular growth pattern. Bottom left: Cell-poor myxoid growth pattern. Bottom right: Tumor cell-rich fascicular growth pattern. Size bars indicate magnifications.

Figure 3. Comparative transcriptomic characterization of amelanotic MPNST-like and pigmented Bra\textsubscript{V600E}-Cdk\textsubscript{4R24C} melanomas.
A. Outline of experimental approach for transcriptomic characterization of MPNST-like (n=5) versus pigmented (n=5) Bra\textsubscript{V600E}-Cdk\textsubscript{4R24C} melanomas using microarrays and GSEA. B. Vulcano plot summarizing GSEA results by significance (-log10 of FDR-adjusted raw p-values) and normalized enrichment scores. Top-ranking gene sets are highlighted. C. Heatmap of top differentially expressed genes (selected categories from GSEA) between Hgf-Cdk\textsubscript{4R24C} (n=6), amelanotic MPNST-like (n=5) and pigmented (n=5) Bra\textsubscript{V600E}-Cdk\textsubscript{4R24C} tumors. Gene expression data (log2) was transformed to row z-scores. D. Vulcano plot visualizing the strategy to identify top differentially expressed genes between amelanotic MPNST-like (n=5) and pigmented (n=5) Bra\textsubscript{V600E}-Cdk\textsubscript{4R24C} tumors using moderated empirical Bayes t-test statistics for building the MPNST-like (=BC\textsuperscript{MPNST-like}) signature. FDR-corrected p-values are represented as -\log_{10} values on the y-axis. The x-axis shows differences (log2) in gene expression between the groups. Magenta dots represent genes (n=265) that were used to build the BC\textsuperscript{MPNST-like} signature. E. Expression of the BC\textsuperscript{MPNST-like} signature (human homologues converted) in different human tumor entities. Significance
was determined by an unpaired two-sided pairwise t-test with FDR-adjustment for multiple comparisons. **, p<0.01; ***, p>0.001.

**Figure 4. Identification of human melanomas with a BC<sup>MPNST-like</sup> histomorphology in the TCGA data base using a bioinformatic cross-species comparison of gene expression signatures.**

A. TCGA human melanoma samples (n=384) ranked by increasing BC<sup>MPNST-like</sup> signature z-scores from left to right. BC<sup>MPNST-like</sup> samples (n=44) were identified by a z-score larger than twice the median absolute deviation (MAD) and highlighted in magenta. B. Expression of the pigmentation signature in the human TCGA melanoma cohort. Samples ranked by increasing BC<sup>MPNST-like</sup> signature z-scores. Significance determined by a non-parametric two-sided Wilcoxon test. C. Comparison of the histological appearance of human BC<sup>MPNST-like</sup> melanomas (TCGA) and murine amelanotic melanomas (Braf<sup>V600E-Cdk4R24C</sup>). Digital images of H&E-stained tissue sections from the human TCGA melanomas were retrieved through the webpage of the Cancer Digital Slides Archive (http://cancer.digitalslidearchive.net). The matrix indicates BRAF, NRAS and NF1 somatic mutation status and number of non-synonymous mutations in BC<sup>MPNST-like</sup> melanomas. Grey bars indicate that high quality H&E slides or genomic data was not available. Brown bars indicate non-V600 BRAF mutations.

**Figure 5. Expression of mast cell genes and mast cell chemoattractants in association with BC<sup>MPNST-like</sup> human melanomas.**

A. Bioinformatic approach using immune cell subtype signatures (ICSS). B. Significant association of the mast cell ICSS with BC<sup>MPNST-like</sup> samples in the human TCGA melanoma cohort, as determined by a two-side non-parametric Wilcoxon test. Eos: eosinophils; Mϕ: macrophages, NK: Natural killer cells. C. Plot of mast cell and D. mast cell chemotaxis signature z-scores in TCGA samples ranked by increasing NF/MPNST-like signature z-scores. BC<sup>MPNST-like</sup> samples are highlighted in magenta. E. Heatmap of mast cell and mast cell chemotaxis signature gene expression in TCGA samples ranked as described in C-D. F. Heatmap of mast cell, mast cell chemotaxis, pigmentation and BC<sup>MPNST-like</sup> signature gene expression in the Lund melanoma metastasis cohort (n=214). G. Plot of mast cell and mast cell chemotaxis signature z-scores in the cohort of melanoma metastasis from Lund (n=214). Samples in F-G are ranked by increasing BC<sup>MPNST-like</sup> signature z-scores from left to right.

**Figure 6. BC<sup>MPNST-like</sup> melanoma cell phenotype determines mast cell recruitment upon intracutaneous transplantation into syngeneic wildtype mice.**

A. Heatmap of mast cell/mast cell chemotaxis, pigmentation and MPNST-like signature gene expression in pigmented Hgf-Cdk4<sup>R24C</sup> (n=6) or Braf<sup>V600E-Cdk4R24C</sup> versus amelanotic MPNST-like Braf<sup>V600E-Cdk4R24C</sup> melanomas (n=5 each). A subset of representative MPNST-like signature genes was used for the heatmap generation. B. Comparison of mast cell infiltration in pigmented Hgf-Cdk4<sup>R24C</sup> or Braf<sup>V600E-Cdk4R24C</sup> versus amelanotic MPNST-like Braf<sup>V600E-Cdk4R24C</sup> melanomas. Mast cells were stained by toluidine blue and representative images are shown (20x and 40x magnification as indicated by the size bars in each panel). C. Heatmap of signature gene
expression in HCmel3 and BCmel4 in vitro cultures as shown for the primary melanomas in A. D. Experimental protocol of intracutaneous injection of the pigmented HCmel3 (Hgf-Cdk4R24C-derived) and the amelanotic MPNST-like BCmel4 mouse melanoma cell line (BrafV600E-Cdk4R24C-derived) in syngeneic C57BL/6 wildtype mice. Representative toluidine blue stains (mast cells) of syngeneic HCmel3 and BCmel4 melanomas (20x and 40x magnification as indicated by the size bars in each panel).
Figure 1

A. Diagram showing the genetic alterations (Tyr::Cre<sup>ERT2</sup>, Braf<sup>SVL60E</sup>, Cdk4<sup>R24C</sup>) and treatments (Tamoxifen [1 mg], DMBA [100 nmol]) in mice over a timeline of 4 days.

B. Graph showing the tumor diameter (mm) over time after tamoxifen administration.

C. Images showing pigmented and amelanotic tumors.

D. Table showing the number of melanomas in different mouse groups:

<table>
<thead>
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<th>Mouse</th>
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Mean: Amelanotic 1.6 ± 0.7; Pigmented 2.2 ± 2.2

E. Staining images for pigmented and amelanotic tumors with markers gp100, NGFR, and CD45.
Figure 5

A. Immune cell subtype signatures: ICSS (T-cells, neutrophils, …) and melanoma-specific refinement of ICSS. Bindea et al. Immunity 2013

B. Expression of ICSS in BCMPNST-like vs. non-BCMPNST-like TCGA melanomas.

C. Mast cell signature

D. Mast cell chemotaxis

E. TCGA samples (ranked by BCMPNST-like signature z-score)

F. Lund metastatic melanomas (ranked by BCMPNST-like signature z-score)

G. Mast cell & Mast cell chemotaxis signature

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Figure 6

A. Primary murine melanomas

B. Pigmented  MPNST-like

C. Murine melanoma cell lines (in vitro)

D. Tx of pigmented HCme13 (Hgf-Cdk4R24C) or MPNST-like & amelanotic BCme14 (Braf^600E-Cdk4R24C)

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A preclinical model of malignant peripheral nerve sheath tumor-like is characterized by infiltrating mast cells

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