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FBXW7 inactivation in a *Braf*^{V600E}-driven mouse model leads to melanoma development

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Abstract

Human melanocytic nevi driven by *BRAF*^{V600E} are in a growth-arrested state referred as oncogene-induced senescence. *FBXW7* tumor suppressor is mutated in melanomas, but not in benign precursor melanocytic nevi. In order to characterize whether inactivation of FBXW7 in cooperation with *BRAF*^{V600E} (*Braf*^{V637E} in the mouse) is sufficient to bypass from the growth-arrested state, we generated a murine model by conditionally silencing *Fbxw7* in an established system, *Tyr::CreER; Braf*^{CA} (or *Tyr::CreER; Braf*^{V600E}). We show that loss of *Fbxw7* in the presence of *Braf*^{V600E} mutation is consequential and sufficient to drive tumorigenesis. This model provides further evidence that *Fbxw7* is a tumor suppressor in the melanocytic lineage, and can serve as a tool for future pre-clinical studies.

Keywords

FBXW7; BRAF; nevus; melanoma; transformation; mouse model

Dear Editor

Oncogene-induced senescence (OIS) is a tumor suppressive mechanism that has been described in several cell types, including the melanocytic lineage (Michaloglou et al., 2005). In this phenomenon, cells initially undergo a proliferative phase due to an oncogenic insult followed by activation of a cell cycle arrest program (Peeper, 2011). Bypass from the senescent-like state can occur upon loss of key tumor suppressors such as *CDKN2A*, *PTEN*,

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TP53, or *NF1* leading to malignant transformation (Vredeveld et al., 2012, Dankort et al., 2009, Viros et al., 2014, Maertens et al., 2013). It is critical to discern the combination of oncogene activation and tumor suppressor loss that is required for tumor initiation.

The best *in vivo* example of OIS or OIS-like state in humans is the melanocytic nevus (mole on the skin) (Michaloglou et al., 2005). Melanoma can arise from a pre-existing nevus, thus representing bypass from this condition (Shain et al., 2015). Approximately 85% of benign or precursor lesions (common acquired nevi) harbor the oncogenic mutation $BRAF^{V600E}$, whereas 60% of melanomas harbor the same mutation, as well as other genetic and epigenetic events necessary for malignant transformation (Shain et al., 2015, Cancer Genome Atlas, 2015). $BRAF^{V600E}$-induced senescence has been recapitulated in the murine system by the conditional expression of $Braf^{V600E}$ ($Braf^{V637E}$ in the mouse) in the melanocytic lineage, resulting in melanocytic hyperplasia resembling nevus formation (Dankort et al., 2009). By employing this model, inactivation of *Pten*, *Nf1*, and *Tp53* has been demonstrated to bypass from an OIS-like state and result in tumor initiation (Dankort et al., 2009, Maertens et al., 2013, Viros et al., 2014).

FBXW7 is a tumor suppressor frequently altered in cancer (Aydin et al., 2014). The gene is deregulated by point mutations, mono- or bi-allelic deletion, promoter hypermethylation, microRNA-modulation, and transcriptional inhibition (Kourtis et al., 2015). *FBXW7* encodes an E3 ubiquitin ligase responsible for regulating the stability of substrates containing a CPD domain (CDC4 phosphodegron motif). Several oncoproteins are substrates of FBXW7 including mTOR, CYCLIN E, NOTCH1, MYC, JUN, and PCG1α (Kourtis et al., 2015, Davis et al., 2014). We have previously reported *FBXW7* mutations in melanoma identified through exome sequencing, its inactivation in over 40% of melanomas, and FBXW7-NOTCH1-mediated tumor promotion (Aydin et al., 2014). Here, in this study, we aimed to determine if conditional inactivation of *Fbxw7* in cooperation with $Braf^{V600E}$ in melanocytes is sufficient to bypass from an OIS-like condition in an established murine system, *Tyr::CreER;* $Braf^{CA}$ (or *Tyr::CreER;* $Braf^{V600E}$) (Dankort et al., 2009) and drive tumorigenesis.

Mice carrying $Braf^{CA}$ alleles were crossed with *Fbxw7* floxed mice, followed by crossing to $Tyr::CreER^{T2}$ to generate mice with genotypes $Tyr::CreER^{T2}$; $Braf^{CA/CA}$; $Fbxw7^{flox/flox}$ (*Braf/Fbxw7*) and $Tyr::CreER^{T2}$; $Braf^{CA/CA}$ (*Braf*). Activation of CreER with topical 4-hydroxytamoxifen treatment at birth resulted in melanocytic-specific conversion of $Braf^{CA}$ to $Braf^{V600E}$ and the conversion of $Fbxw7^{flox}$ alleles to null alleles (Figure S1A). Approximately 3–4 weeks post-treatment, *Tyr::CreER;* $Braf^{CA/CA}$ mice showed hyperpigmentation of the skin and melanocytic hyperplasia within the dermis as determined by histology, resembling blue nevus-like clones as previously described (Figure S1B, C). Similarly, we observed hyperpigmentation of the skin in the *Tyr::CreER;* $Braf^{CA/CA}$; $Fbxw7^{flox/flox}$ mice, but with significantly pronounced pigmentation and enhanced thickening of the skin as compared to *Tyr::CreER;* $Braf^{CA/CA}$ mice (Figure S1B). The thickening of the skin was mostly due to spindle cell tumor component beneath the epidermis and the nevus based on histologic examination (Figure S1C, upper panels).

Tyr::CreER; Braf$^{CA/CA}$*; Fbxw7*$^{flox/flox}$ *mice* developed tumors that were characterized as melanomas. In these mice, the tumor penetrance was 81% and the melanoma-free survival (latency) was 6.1 months (6.17 ± 2.25 months), whereas mice harboring the *Tyr::CreER; Braf*$^{CA/CA}$ genotype alone developed tumors with 22.7% penetrance and 7.1 month latency (7.1 ± 2.46 months) (Figure 1A, B). Our findings on the control group (*Tyr::CreER; Braf*$^{CA/CA}$) were in line with previous studies that have described ~10% of mice harboring *Braf*^{V600E} mutations developing tumors beyond melanocytic hyperplasia within the first 6 months of life, increasing up to 50% within the first year (Dankort et al., 2009, Maertens et al., 2013). Importantly, melanomas of *Tyr::CreER; Braf*$^{CA/CA}$*; Fbxw7*$^{flox/flox}$ *mice* were unique and compellingly different than tumors observed in *Tyr::CreER; Braf*$^{CA/CA}$ mice. Histologically, they were characterized by increased cellularity, spindle cell morphology, fascicular growth pattern, and atypical cells extending deep into the subcutis (Figure 1C). While the tumor cells were non-pigmented (amelanotic), many tumors had clusters of coarse melanin granules in the upper and deeper parts of the dermis. Tumor cells were positive for S100, Ki67, and MITF (Figure 1C, D). Of note, tumor cells retained high levels of p16INKA expression (Figure S1C, lower panels).

To evaluate the signaling pathways involved in promoting tumorigenesis upon loss of FBXW7, we investigated the activity of mTOR, an established oncogene and target of FBXW7 (Kourtis et al., 2015). We examined phosphorylated ribosomal protein S6 (p-S6), a direct mTOR target (mTORC1 complex) and phosphorylated AKT (p-AKT) that is activated by the mTORC2 complex, in tumors of *Tyr::CreER; Braf*$^{CA/CA}$*; Fbxw7*$^{flox/flox}$ mice. Growth-arrested nevus clones of the *Tyr::CreER; Braf*$^{CA/CA}$ mice were used as controls (Figure 2A). We noted significant up-regulation of p-S6 (S235/S236) as well as p-AKT (S473) in S100-expressing melanoma tumors throughout the dermis extending deep into the subcutis of the *Tyr::CreER; Braf*$^{CA/CA}$*; Fbxw7*$^{flox/flox}$ mice (Figure 2B, C). These data suggest that both mTORC1 and mTORC2 signaling are activated in *Fbxw7* inactivated and *Braf*^{V600E}-mutant murine melanocytes. These results propose a potential therapeutic avenue for melanomas with FBXW7 inactivation.

While we noted micro-metastases of melanoma to the lymph nodes in *Tyr::CreER; Braf*$^{CA/CA}$*; Fbxw7*$^{flox/flox}$ mice, overt systemic metastasis could not be evaluated as the mice needed to be euthanized due to the large and fast growing primary tumors. The lymph node metastasis showed similar histologic features of the primary tumor and displayed spindle-shaped melanoma cells with coarse melanin granules growing in a fascicular pattern in the sub-capsular region invading into the lymph node parenchyma (Figure 2C). Tumor cells in the lymph nodes were positive for S100 and Ki67 (Figure 2C).

In summary, we report a mouse model that provides further evidence that Fbxw7 is a *bona fide* tumor suppressor in the melanocyte lineage. We show that loss of Fbxw7 in the presence of *Braf*^{V600E} mutation (mitogen-activated protein kinase pathway activation) is consequential and sufficient to drive tumorigenesis, but does not lead to an aggressive phenotype with short latency period and overt systemic metastasis. As expected, the FBXW7 substrate, mTOR, and mTOR-mediated signaling were activated (mTORC1 and mTORC2). It remains to be established whether FBXW7-driven tumorigenesis is due to bypass from OIS. To this end, high levels of p16INK4A expression in these tumors suggest

mechanisms other than those previously described where p16INK4A inactivation in the context of other mutations leads to bypass from OIS. It is possible that the non-aggressive phenotype observed may be due to retention of p16INK4A in this model. The model described here can serve as a tool for future pre-clinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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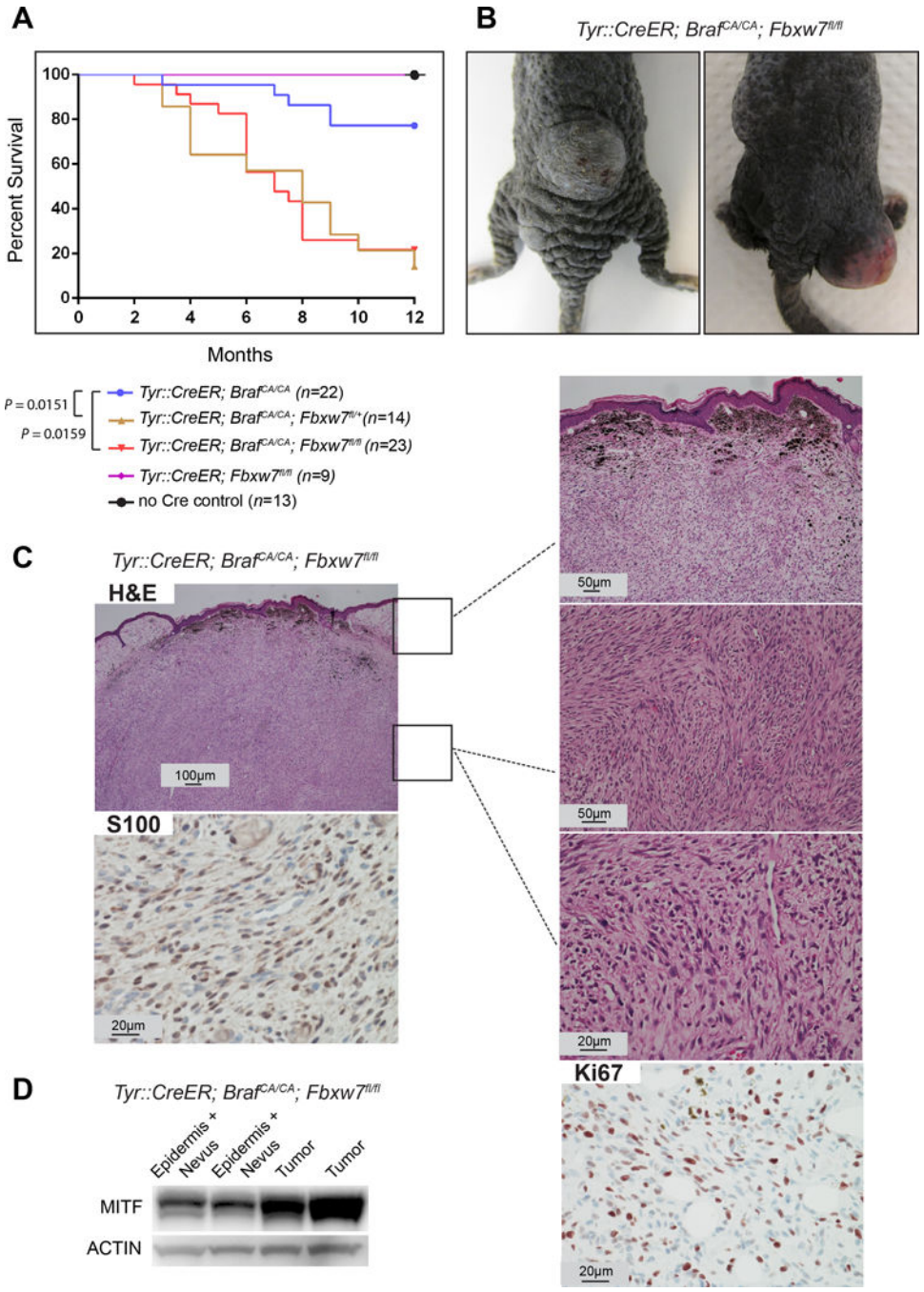

Figure 1.

FBXW7 inactivation together with *Braf*^{V600E} mutation cooperates *in vivo* to drive melanoma formation. (A) Survival curves of the mouse cohorts. Animals with tumor size greater than 1 cm^3 or at 12 months after birth were euthanized. Log rank test was used for statistical analysis. (B) Representative examples of melanomas in the *Tyr::CreER*^{T2}*; Braf*$^{CA/CA}$*; Fbxw7*$^{flox/flox}$ mice are indicated. (C) Histology sections of the melanomas stained with hematoxylin and eosin (H&E) and immunohistochemistry for S100 and Ki67 are shown in the micrographs. (D) Tumors from *Tyr::CreER*^{T2}*; Braf*$^{CA/CA}$*; Fbxw7*$^{flox/flox}$

mice (without the overlying nevus cells and epidermis) were lysed and analyzed for MITF by western blotting. Actin was used as loading control.

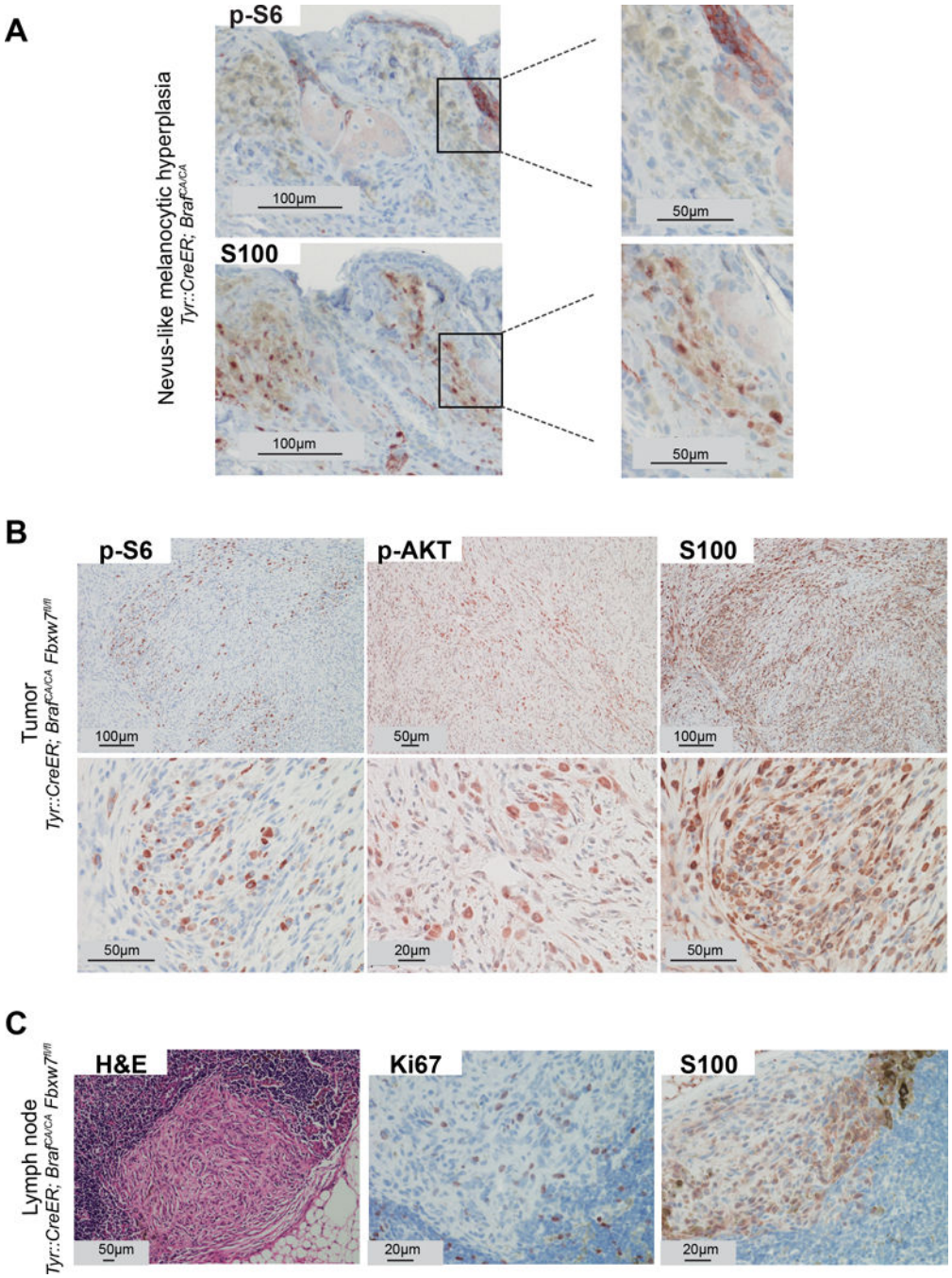

Figure 2.

mTOR signaling is activated during loss of FBXW7-mediated tumorigenesis. (A) Melanocytic hyperplasia (blue nevus-like clones) from *Tyr::CreER^{T2}; Braf$^{CA/CA}$* mice stained with p-S6 (upper panel) and S100 (lower panel) using immunohistochemistry. Positive staining for p-S6 within the follicular epithelium is indicated as an internal control. (B) Melanomas (tumors within the deep portion of the dermis) from *Tyr::CreER^{T2}; Braf$^{CA/CA}$; Fbxw7$^{flox/flox}$* mice stained with p-S6, p-AKT (S473), and S100. Low (upper panel) and high power (lower panel) magnifications are shown..(C) Histologic and

immunohistochemistry analysis of lymph nodes from *Tyr::CreER^{T2}; Braf$^{CA/CA}$; Fbxw7$^{flox/flox}$* mice stained with H&E, S100, and Ki67. Representative micrographs are indicated.