A Transcriptionally Inactive ATF2 Variant Drives Melanomagenesis

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Abstract: Melanoma is one of the most lethal cutaneous malignancies, characterized by chemoresistance and a striking propensity to metastasize. The transcription factor ATF2 elicits oncogenic activities in melanoma, and its inhibition attenuates melanoma development. Here, we show that expression of a transcriptionally inactive form of Atf2 (Atf2(Δ8,9)) promotes development of melanoma in mouse models. Atf2(Δ8,9)-driven tumors show enhanced pigmentation, immune infiltration, and metastatic propensity. Similar to mouse Atf2(Δ8,9), we have identified a transcriptionally inactive human ATF2 splice variant 5 (ATF2(SV5)) that enhances the growth and migration capacity of cultured melanoma cells and immortalized melanocytes. ATF2(SV5) expression is elevated in human melanoma specimens and is associated with poor prognosis. These findings point to an oncogenic function for ATF2 in melanoma development that appears to be independent of its transcriptional activity.

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**Highlights**
- Transcriptionally inactive ATF2 (Atf2\(^{\Delta 8,9}\)) induces pigmentation
- Atf2\(^{\Delta 8,9}\) induces melanoma in Braf\(^{V600E}\) mice
- Genes implicated in immune cell recruitment and metastasis are induced by Atf2\(^{\Delta 8,9}\)
- Human ATF2 splice variant 5 phenocopies Atf2\(^{\Delta 8,9}\) and coincides with poor prognosis

**Graphical Abstract**

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**In Brief**
Claps et al. demonstrate that a transcriptionally inactive isoform of ATF2 lacking exons 8 and 9 is sufficient to promote melanoma in mouse models. They also identify a transcriptionally inactive human splice variant of ATF2, which resembles the mouse isoform, that is expressed in melanoma specimens and is associated with poor prognosis.

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A Transcriptionally Inactive ATF2 Variant Drives Melanomagenesis

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INTRODUCTION

Melanoma is one of the most lethal cutaneous malignancies, characterized by chemoresistance and a striking propensity to metastasize. The transcription factor ATF2 elicits oncogenic activities in melanoma, and its inhibition attenuates melanoma development. Here, we show that expression of a transcriptionally inactive form of ATF2 (Atf2\textsuperscript{SV5}) promotes development of melanoma in mouse models. Atf2\textsuperscript{SV5}-driven tumors show enhanced pigmentation, immune infiltration, and metastatic propensity. Similar to mouse Atf2\textsuperscript{SV5}, we have identified a transcriptionally inactive human ATF2 splice variant 5 (ATF2\textsuperscript{SV5}) that enhances the growth and migration capacity of cultured melanoma cells and immortalized melanocytes. ATF2\textsuperscript{SV5} expression is elevated in human melanoma specimens and is associated with poor prognosis. These findings point to an oncogenic function for ATF2 in melanoma development that appears to be independent of its transcriptional activity.

INTRODUCTION

Melanoma is one of the most lethal cutaneous malignancies due to its metastatic propensity and resistance to therapy (Lo and Fisher, 2014). Rewired signal transduction pathways, which underlie melanoma lethality, are driven by networks of protein kinases and related transcription factors. Activating Transcription Factor 2 (ATF2) is a member of the ATF/CREB bZIP family of transcription factors, which heterodimerizes with members of the JUN and FOS transcription factor families (Lau and Ronai, 2012; Lopez-Bergami et al., 2010).

In melanoma, nuclear ATF2 expression is associated with poor prognosis and metastatic burden, whereas cytoplasmic localization correlates with sensitization of melanoma to genotoxic stress (Lau et al., 2012; Lau and Ronai, 2012) and better clinical outcome (Berger et al., 2003). Consistent with this, inhibition of ATF2 reduces melanoma growth in BRAF and NRAS mutant melanoma cell lines in culture and in xenografts (Bhounik et al., 2002, 2004a, 2004b). Also in agreement, Nras\textsuperscript{Q61K,Ink4a/−/−} mice selectively expressing the transcriptionally inactive form of Atf2 (Atf2\textsuperscript{SV5}), which lacks the DNA-binding domain and part of the leucine zipper domain (Breitwieser et al., 2007), show attenuated melanoma development (Shah et al., 2010).

In contrast, mice without melanoma susceptibility genes, but harboring Atf2\textsuperscript{SV5} selectively expressed in keratinocytes, display increased papilloma development when subjected to a two-stage skin carcinogenesis protocol (Breitwieser et al., 2008). Similarly, mouse embryonic fibroblasts expressing Atf2\textsuperscript{SV5}, but lacking its homolog ATF7, show increased proliferation and formation of tumors exhibiting a JNK-ATF2-dependent gene signature in orthotopic liver cancer models (Gozdecka et al., 2014). Likewise, mutant p53 mice with deletions in the Atf2 DNA-binding domain (amino acids 327–395) develop mammary tumors (Maekawa et al., 2008).

These observations suggest that ATF2 oncogenic or tumor suppressor function varies among tumor and tissue types depending on the landscape of genetic factors. Here, we assessed the role of ATF2 in a well-characterized genetic mouse model of Braf/Pten mutant melanoma (Dankort et al., 2009). Surprisingly, we found that transcriptionally inactive Atf2\textsuperscript{SV5} has a tumor-promoting effect in Braf mutant melanocytes, which is enhanced in Braf/Pten mutant animals.

RESULTS

ATF2\textsuperscript{SV5} Accelerates Melanoma Development and Metastasis Formation in Braf\textsuperscript{V600E/V600E;Pten\textsuperscript{+/−}} Mice

To assess Atf2 function in BRAF mutant melanoma, we crossed mice expressing a transcriptionally inactive form of Atf2 (Atf2\textsuperscript{SV5}) (Shah et al., 2010) with Braf\textsuperscript{V600E/V600E;Pten\textsuperscript{+/−}} mice. The Tyr:\textsuperscript{CreER,Atf2\textsuperscript{lox/lox,Braf\textsuperscript{WT/WT;Pten\textsuperscript{lox/lox}}} mice show melanocyte-specific expression of Atf2\textsuperscript{SV5}, BRAF\textsuperscript{V600E/V600E;Pten\textsuperscript{+/−}} following administration of 4-hydroxytamoxifen (4-HT). Cre-dependent deletion of Atf2 exons 8 and 9 results in a protein lacking the DNA-binding domain and a portion of the leucine zipper (bZip) (Figures 1A and 1B) (Breitwieser et al., 2007). We
detected low levels of full-length ATF2, which may originate from non-tumor cells or degree of ATF2 deletion (<100% of cells; Figure 1C) in this model. Of note, the presence of full-length ATF2 in the *Atf2*Δ8,9/Δ8,9 mice accurately reflects the co-expression of full-length and splice variant forms of ATF2 in human melanoma (see below). Further, co-expression of full-length ATF2 did not compromise the dominant activity of the ATF2Δ8,9 mutant.

To determine the role of *Atf2*Δ8,9 in melanoma development, 4-HT was administered systemically to *Atf2*WT/WT; *Braf*V600E/V600E; *Pten*−/− (n = 30) and *Atf2*Δ8,9/Δ8,9; *Braf*V600E/V600E; *Pten*−/− (n = 28) or *Atf2*WT/Δ8,9; *Braf*V600E/V600E; *Pten*−/− (n = 18) mice (Figure S1A, upper). 4-HT-treated *Atf2*Δ8,9/Δ8,9 mice were more heavily pigmented than *Atf2*WT/WT animals (Figure 2A). Increased pigmentation was seen throughout the dermis and subcutis of *Atf2*Δ8,9/Δ8,9 mice (n = 6) compared with *Atf2*WT/WT controls (n = 6), with spreading into the epidermis (Figure 2B). Despite these changes, the median survival time of the *Atf2*WT/WT, *Atf2*Δ8,9/Δ8,9, and *Atf2*WT/Δ8,9 mice did not differ (18–19 days), possibly due to the rapid melanoma development in this model (Figure S1B).

To monitor tumor growth over a prolonged period, we administered 4-HT locally on the dorsal skin of 3-week-old *Atf2*WT/WT; *Braf*V600E/V600E; *Pten*−/− (n = 19) and *Atf2*Δ8,9/Δ8,9; *Braf*V600E/V600E; *Pten*−/− (n = 12) mice (Figure S1A, lower). *Atf2*Δ8,9/Δ8,9 animals
developed larger tumors (Figure 2C) that appeared earlier in control mice (Figure 2D). The median survival time of BravV600E/V600E, Pten−/− mice was decreased from 64 to 47 days by Atf2Δ8,9Δ8,9 expression (Figure 2E). The Atf2Δ8,9Δ8,9 mice had a larger number of highly pigmented cells, which were confirmed to be of melanocytic origin based on S100 immunostaining (Figure 2F). These data suggest that expression of Atf2Δ8,9 in BravV600E/V600E; Pten−/− mice promotes melanoma development.

S100 staining revealed an increased number of metastatic melanoma cells in the lymph nodes of ATF2Δ8,9 mice subjected to local 4-HT treatment compared with similarly treated ATF2WT mice (Figures 2G and 2J, left). To determine whether these lesions represent metastatic melanoma, we collected lymph nodes from Atf2Δ8,9Δ8,9 and control Atf2WT mice bearing tumors, which were (either the whole lymph nodes or following partial digestion; Figure S1C) transplanted into nude mice. Under both approaches, the recipient mice developed tumors, confirming that the clusters of subcapsular S100-positive cells in the lymph nodes of Atf2Δ8,9Δ8,9;BravV600E/V600E; Pten−/− mice are metastatic melanoma cells. S100 staining of tumors emerging after transplantation confirmed the presence of non-pigmented melanoma cells, as seen in the non-pigmented lymph node source (Figures 2H and S1D). In addition, the number of lesions in the lungs was higher in BravV600E/V600E; Pten−/− mice harboring Atf2Δ8,9Δ8,9 versus Atf2WT (Figures 2I and 2J, right). These observations point to the higher metastatic propensity of ATF2Δ8,9 expressing melanocytes.

**Atf2Δ8,9 Exerts Oncogenic Activity in BravV600E/V600E, Pten−/− Mice**

As the finding that a transcriptionally inactive form accelerates melanoma development and promotes metastasis in the BravV600E/V600E; Pten−/− murine melanoma model was unanticipated, we set to further characterize the activities of ATF2Δ8,9 in BRAF mutant melanoma.

Using the congenic YUMM1.3 melanoma cell line, which was derived from a C57BL/6 BravV600E/V600E; Pten−/−;Cdkn2a−/− melanoma, we compared the effects of Atf2Δ8,9 expression versus small hairpin (sh)RNA-mediated knockdown (KD) of endogenous ATF2. ATF2 shRNA-expressing cells formed fewer colonies than control YUMM1.3 cells (Figures 3A and S2A–S2C), consistent with earlier studies. In contrast, ATF2Δ8,9 overexpression (OE) conferred a growth advantage on YUMM1.3 cells compared with ATF2WT OE (Figures 3A and S2C). Similarly, ATF2 KD attenuated and ATF2Δ8,9 OE increased migration of YUMM1.3 compared with their respective controls (Figures 3B and S2C). These observations are consistent with observations made in the ATF2Δ8,9 mouse model.

Gene expression profiles of tumor samples from Atf2Δ8,9Δ8,9, BravV600E/V600E, Pten−/− and Atf2WT/WT, BravV600E/V600E, Pten−/− mice identified 579 genes (655 probes) that were significantly differentially expressed (Table S1). Of these, 305 genes (337 probes) were upregulated and 274 gene (318 probes) were downregulated in Atf2Δ8,9Δ8,9 mice compared with Atf2WT/WT tumors. Genes with significant FDR-adjusted p values were subjected to Ingenuity Pathway Analysis (IPA), which revealed immune receptor and response signaling, angiogenesis, and ROS/NOS signaling components to be among those most significantly altered in the ATF2Δ8,9 tumors (Figure 3C; Table S2A). The respective functional networks predicted to be activated in Atf2Δ8,9Δ8,9-expressing tumors included pigmentation, inflammation, cell motility, and invasion (Table S2B). Representative genes, including S100A8, implicated in macrophage recruitment; Mmp3, Mmp9, and Ccr7, implicated in tumor cell invasion and metastasis; Cxcl9 and Cita4; and Mif and related pigmentation genes were confirmed for their increased expression in Atf2Δ8,9Δ8,9 tumors (Figure S2D). Atf2Δ8,9-driven melanoma indeed exhibits greater pigmentation (Figure 2F), markedly increased immune cell infiltration (CD45+ and F4/80+; Figures S2E and S2F), and an enhanced propensity to metastasize (Figures 2G–2I), compared to Atf2WT melanoma.

**Mouse Atf2Δ8,9 Structurally Resembles an ATF2 Splice Variant Expressed in Human Melanoma**

To determine whether human tumors express an analogous form of ATF2, we interrogated RNA-sequencing (seq) expression data obtained from >70 melanoma lines and three melanocyte lines (Dutton-Regester et al., 2012). Of the four reported human ATF2 isoforms (http://www.ncbi.nlm.nih.gov/gene/1386), we confirmed the expression of three in the human melanoma set.

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Figure 2. Systemic or Local Induction of Atf2Δ8,9 Expression in Melanocytes Accelerates Melanoma Development and Metastasis in BravV600E/V600E; Pten−/− Mice

(A) Representative images of Atf2WT/WT and Atf2Δ8,9Δ8,9 animals 18 days following perinatal 4-HT administration.

(B) H&E staining analysis of skin from Atf2WT/WT and Atf2Δ8,9Δ8,9 animals 18 days following perinatal 4-HT administration (left) and quantification of the percentage of migrating melanoma cells in the skin (right). The data are the mean ± SEM of skin samples from n = 6 mice per genotype. The scale bars represent 500 μm.

(C) Representative picture of Atf2WT/WT, Atf2Δ8,9Δ8,9, and Atf2WT/Δ8,9Δ8,9 animals 40 days following local 4-HT administration.

(D) Growth curves for tumors from Atf2WT/WT (n = 19) and Atf2Δ8,9Δ8,9 (n = 12) mice from six different litters following local administration of 4-HT. The data are the mean ± SEM (p value was calculated by two-tailed unpaired t test).

(E) Kaplan-Meier survival curves for Atf2WT/WT (n = 19) and Atf2Δ8,9Δ8,9 (n = 12) mice following local administration of 4-HT. The mice from six different litters were analyzed (p < 0.0001 by log rank [ Mantel-Cox] test).

(F) H&E analysis (left) and S100 immunostaining (right) of tumors from Atf2WT/WT and Atf2Δ8,9Δ8,9 mice following local 4-HT administration. The scale bars represent 400 μm (H&E) and 100 μm (immunofluorescence [IF]).

(G) H&E analysis (left) and S100 immunostaining (right) of lymph nodes from Atf2WT/WT and Atf2Δ8,9Δ8,9 mice following local 4-HT administration. The scale bars represent 500 μm (H&E) and 600 μm (IF).

(H) (Upper) Representative images of nude mice transplanted with whole lymph nodes from Atf2Δ8,9Δ8,9 or Atf2WT/WT (control) animals. S100 immunostaining of Atf2Δ8,9Δ8,9 tumors from lymph node-transplanted nude mice is shown (lower). The scale bar represents 100 μm.

(I) H&E analysis of lungs from Atf2Δ8,9Δ8,9 and Atf2WT/WT mice following local 4-HT administration. The scale bars represent 3 mm.

(J) Quantification of metastasis in lymph nodes (left) and lungs (right) in Atf2WT/WT, BravV600E/V600E, Atf2WT/WT, BravV600E/V600E, Pten−/−, and Atf2Δ8,9Δ8,9, BravV600E/V600E, Pten−/− mice. The data are the mean ± SEM of n = 4 mice (p < 0.0001 and p = 0.002 by two-tailed unpaired t test). See also Figure S1.
using a mixture of isoforms (MISO) analysis (Figure S3A) (Katz et al., 2010; Wang et al., 2008) and in a smaller panel of 23 melanoma cell lines by quantitative (q)PCR (Figure S3B). These analyses verified the expression of full-length ATF2 (isoform 1; ATF2WT); isoform 3, a splice variant (SV3) that lacks the N-terminal region (ATF238,9); and isoform 5, a splice variant (SV5) that lacks the DNA-binding and the leucine zipper domains (ATF258,9) and thus partially resembles mouse Atf28,9 (Figure 3D). ATF2WT and ATF2SV5 are expressed at varying levels in colon, breast, and prostate tumor cells, whereas ATF258,9 exhibited a more selective expression (breast cancer and melanoma cells; Figure S3C).

**Human ATF2SV5 Phenocopies Mouse Atf28,9 Function**

To determine whether ATF2SV5 elicits oncogenic activities similar to mouse Atf28,9, we examined human UACC1113 cells, which express high levels of ATF2SV5 in addition to ATF2WT and ATF2SV3 (Figure S3B). Silencing of ATF2SV5 in UACC1113 cells reduced their migration compared to cells in which both ATF2SV3 and ATF2WT were silenced (Figures 3E and S4A). Furthermore, although colony formation by the UACC91 melanoma cell line (which does not express ATF2SV5, Figure S3B) was reduced by silencing of endogenous ATF2 (Figures S4B and S4C), it was rescued to a greater extent by reconstitution with ATF2SV5 than by reconstitution with either ATF2WT or ATF2SV3 (Figures 3F and S4D). Likewise, ectopic expression of ATF2SV5 in human Mel501 cells (which do not express ATF2SV5, Figures S3B and S4E), without silencing endogenous ATF2, also increased colony formation compared with ATF2WT and ATF2SV3 (Figures 3F, S4E, and S4F). In these cells, the expression level of ectopic ATF2SV5 was comparable to that of ectopic ATF2WT, and both proteins were expressed at ~3-fold higher levels than the endogenous proteins (data not shown). These observations suggest that human ATF258,9 phenocopies mouse Atf28,9.

**Atf28,9 Induces Nevus Formation and Promotes Melanoma in BrafWT/V600E;PtenWT/+ Mice**

The finding that ATF28,9 augments melanoma development when combined with Pten deletion and Braf mutation prompted us to assess its possible role in melanomagenesis in the absence of Pten inactivation. Strikingly, Atf28,9/+;BrafWT/WT;PtenWT/WT mice developed more and larger nevi than Atf2WT/WT;BrafWT/WT;PtenWT/WT mice (Figures 4A and S4G). Similarly, Atf28,9,8,9 mice had an increased number of pigmented hair follicles than Atf2WT/WT mice (Figures S4H and S4I).

When crossed with BrafWT/V600E animals, Atf28,9,8,9 mice developed melanoma within 250 days (Figures 4B–4D and S4J). Tumor penetrance was 50% for the Atf28,9,8,9 genotype, compared with 28% and 0% for the Atf2WT/8,9 and Atf2WT/WT genotype, respectively (Figure 4B). On their own, Atf28,9 mice did not develop tumors, even when maintained for 250 days. Within the first 50 days, the Atf28,9 mutation did not affect the pigmentation level in BrafWT/WT;Pten+/− animals (data not shown). These findings identify Atf28,9 as a driver of melanocyte biogenesis, highlighted by the degree of pigmentation, and demonstrate that when combined with mutant BrafV600E, it is sufficient to induce melanoma development. The slow rate of melanoma development and progression seen in the Atf28,9 animals recapitulates the time course of human melanoma (Balch et al., 2009).

To compare the activities elicited by ATF28,9 and ATF2SV5, we silenced endogenous ATF2WT and ATF2SV5 in non-transformed human melanocytes (Hermes 3A [H3A]) and ectopically expressed either ATF28,9 or ATF2SV5 (Figure S4K). While silencing of ATF2 in these cells reduced colony formation (Figure S4L), consistent with earlier studies, expression of either ATF2SV5 or ATF28,9 increased H3A proliferation and migration compared with ATF2WT expression (Figures 4E and 4F). Moreover, H3A cells expressing ATF2SV5 or ATF28,9, and either constitutively active BrafV600E or NRASQ61K (Figures S4M and S4N) showed enhanced colony formation compared with cells reconstituted with ATF2WT (Figures 4G and 4H). Finally, migration of H3A cells was decreased to a greater degree by silencing of ATF2SV5 compared with silencing of ATF2WT/SV3 (Figures 4I and S4O).

RNA-seq analysis of H3A cultures identified 434 genes that were upregulated and 580 genes that were downregulated after treatment with siATF2SV5 compared with control small interfering (si)RNA, but that were not significantly altered following siATF2WT treatment (Table S3). IPA analysis identified a number of pathways deregulated by ATF2SV5 KD (Figure 4J). To validate genes that may be commonly deregulated by ATF2SV5 and ATF28,9 expression, siRNA-mediated KD of ATF2SV5 and ATF28,9 in the human melanoma cell lines U11205 and CO54 (Figure S4P) allowed us to assess the changes in the expression of genes identified in our expression studies (Table S3) and IPA analysis (Figure 4J). Among the genes confirmed to be altered by both ATF2 variants were CCL4, CCR7, S100A8 (implicated in metastasis), TIM3 (immune checkpoint), and MITF (Figure 4J).

IPA analyses to identify potential common mechanisms of oncogenic activities for both mouse Atf28,9 and human ATF2SV5, identified signaling and networks associated with immune cell infiltration and cell motility in both melanocytes that were subjected to ATF2SV5 KD in H3A cells and in tumors from Atf28,9,8,9; BrafV600E/V600E;Pten−/− mice (Tables S2B, S4A, and S4B).

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**Figure 3. Human ATF2SV5 Phenocopies Mouse Atf28,9**

(A and B) Colony forming assay (A) and Transwell migration assay (B) of YUMM1.3 cells stably transduced with control vector (Scr) or vectors expressing ATF2 shRNA (shATF2), ATF2WT, or ATF28,9. The data are the mean ± SEM (p value was calculated by two-tailed unpaired t test).

(C) Heatmap showing gene expression in ATF2WT/WT or ATF28,9 tumors. The pathways indicated on the right are deregulated by ATF28,9.

(D) Schematic of three ATF2 isoforms (WT, SV3, and SV5) identified in human melanoma cell lines by MISO analysis. The mouse Atf28,9 isoform is shown at the bottom.

(E) Migration assay of human melanoma UACC1113 cells transduced with control (Scr) or with a vector expressing shATF2 (targeting ATF2WT and ATF2SV5, but not ATF28,9) and transfected with control siRNA or shRNA targeting ATF2SV5. The data are the mean ± SD (p value was calculated by two-tailed unpaired t test).

(F) (Upper row) Colony forming YUMM1.3 human melanoma cells transduced with control (Scr) or with vectors expressing shATF2 plus ATF2WT, ATF2SV5, or Atf28,9. (Lower row) Mel501 human melanoma cells stably transduced with pLXSN4 (empty vector, EV) or vectors expressing ATF2WT, ATF2SV5, or ATF28,9 (lower row). The data are the mean ± SEM (p value was calculated by two-tailed unpaired t test). See also Figures S2 and S3 and Tables S1 and S2.
Figure 4. Atf2Δ8,9 Expression in Melanocytes Accelerates Nevus Formation in BrafbWT/WT Mice and Melanoma Development in BrafbWT/V600E Mice

(A) Quantification of nevi per mouse (left) and nevi size (right) in Atf2WT/WT (n = 9) and Atf2Δ8,9/Δ8,9 mice (n = 8) on a BrafbWT/WT background, 250 days following perinatal 4-HT administration. The data are the mean ± SEM (p value was calculated by two-tailed unpaired t test).

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Importantly, we also assessed the possible importance of ATF2SV expression in human melanoma tumors. The relative expression of ATF2SV and ATF2WT / ATF2SV3 were measured in 33 melanoma tumor specimens that were obtained from 21 consenting metastatic melanoma patients at the time of tumor resection or upon autopsy. A total of 18 (54.5%) of the biopsies came from autopsy samples. Notably, a higher level of ATF2SV (>-3-fold) was found to coincide with poorer prognosis (Figure 4K). Of the top 11 highest ATF2SV expressing tumors (quantification cycle (cq) < 33.5), seven (63.6%) were from tumor biopsies obtained shortly after death and four (36.4%) were from patients who were double BRAF/NRAS wild-type. These observations substantiate the findings made in the genetic mouse model as in the cultured melanoma and melanocytes, where transcriptionally inactive ATF2 variants elicit a gain of function oncogenic activity.

**DISCUSSION**

We identified an unexpected role for transcriptionally inactive ATF2 in melanocyte homeostasis and melanoma development. Atf28,9 alone is able to drive the formation of nevi within 2–3 months and induction of the melanomagenesis. When combined with the Braf mutant genotype, Atf28,9 was able to drive the development of melanoma over 250 days. These animals also exhibit upregulation of select gene networks, including pigmentation-related genes, chemokines/cytokines implicated in the recruitment of immune cells to the tumor sites, and genes related to the enhanced propensity for metastasis. Further, Atf28,9 accelerated the formation of melanomas and increased their propensity to metastasize in Braf/Pten mutant animals. These data establish the ability of a transcriptionally inactive form of ATF2 to promote BrafV600E melanoma development and progression. Although Atf28,9 enhanced the pigmentation in both Nras and Braf mutant melanocytes, it attenuated melanoma development in Nras mutant mice and enhanced it in Braf mutant mice. These differences may be attributed to an effect of Atf28,9 on specific signaling pathways that cooperate with Braf, but not Nras, and/or to its effect on tumor microenvironment (i.e., immune editing; tumor stroma).

While full-length transcriptionally active ATF2 is largely oncogenic in melanoma, its transcriptionally inactive splice variants, represented by human ATF2SV and modeled by mouse ATF28,9, exhibit a super-oncogenic function. Genes that were commonly deregulated by expression of both human and mouse variants (ATF2SV and ATF28,9, respectively) were primarily implicated in metastasis (CCL4, CCR7, S100A8, and MIF), immune cell infiltration (CCL4, CCR7, and TIM3), and melanoma progression / drug resistance (MIF).

Expression of ATF2SV in a series of 33 melanoma biopsies coincides with poor prognosis, consistent with our findings in ATF28,9 mouse melanoma models. Notably, although the activity of ATF2SV is expected to supersede that of other ATF2 forms, it is co-expressed with other ATF2 forms (full-length and other splice variants).

Collectively, our studies provide a genetic support for the involvement of a gain-of-function ATF2 isoform in melanoma. Our data demonstrate that the transcriptionally inactive variant is able to drive melanomagenesis and, in cooperation with mutant Braf, induce melanoma development at the slow rate often seen in human melanoma. Our findings highlight the importance of ATF2 function also as a transcriptionally inactive form.

**EXPERIMENTAL PROCEDURES**

For additional experimental procedures, see Supplemental Experimental Procedures.

**Animal Studies and In Vivo Experiments**

All animal studies were approved by the Institutional Animal Care and Use Committee of the Sanford Burnham Prebys Medical Discovery Institute. Atf28,9;BrafV600E, mice, described in earlier studies (Shah et al., 2010), were crossed with BrafV600E/Pten−/− mice (Dankort et al., 2009), Atf28,9;Nras8,9 and Atf28,9;Nras8,9;BrafV600E/Pten−/− are C57BL/6 and Atf28,9;Nras8,9;BrafV600E/Pten−/− are C57BL/6 x 129.

**Activation of the Tyr::CreERT2 Transgene**

Topical administration of 4-HT (Sigma-Aldrich) was performed by application of 10 μl of a 50 mg/ml solution in DMSO with a paintbrush onto the dorsal skin of pups on days 1, 3, and 5 after birth. Local administration of 4-HT was performed by application of 1.5 μl of a 7.8 mg/ml solution in ethanol onto the shaved dorsal skin of 3-week-old mice (Scortegagna et al., 2014).

**Cell Lines**

The YUMM1.3 cell line was generated from a BrafV600E/Pten−/−;Cdkn2a−/− melanoma in C57BL/6 mice (Scortegagna et al., 2015). The human UACC91,
UACC2427, and UACC1113 cell lines were kindly provided by Drs. Brown and Lin. H3A immortalized melanocyte cells were provided by Dr. Bennet (Gray-Schopfer et al., 2006). All cell lines except H3A were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. H3A cell lines were propagated in 254 media (Gibco) and transferred to DMEM medium prior to initiating the experiments.

**Statistical Analysis**

Data are presented as means ± SEM or SD and the statistical significance (p value) was determined by two-tailed unpaired t test. Kaplan-Meyer survival curves were compiled using Prism statistical analysis software and significance was assessed using the log rank (Mantel-Cox) test. A p value of < 0.05 was considered significant.

**ACCESSION NUMBERS**

Microarray datasets were deposited in the NCBI GEO database under GEO: GSE79917 and GSE81014.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.072.

**AUTHOR CONTRIBUTIONS**


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