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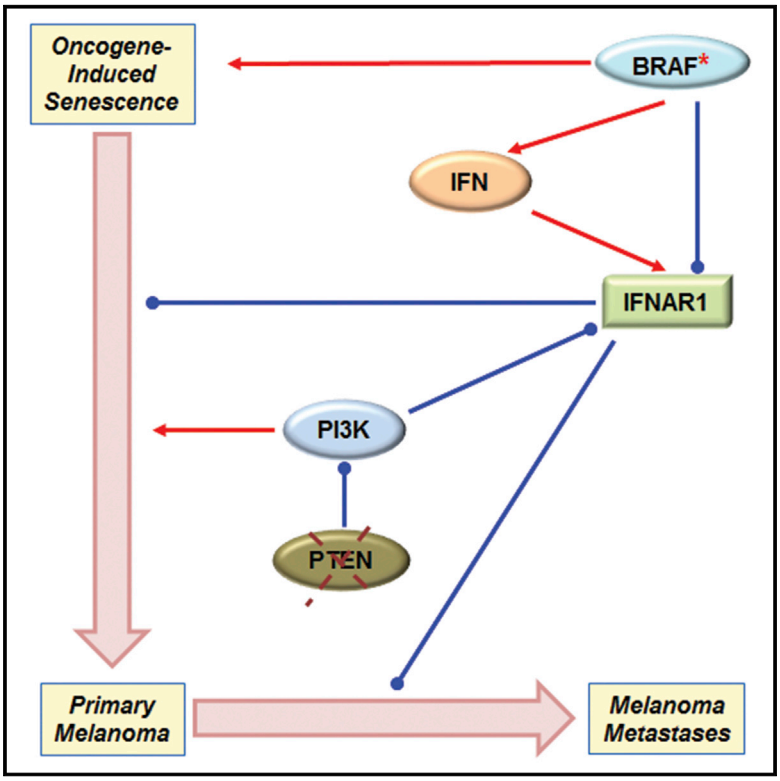
Cell Reports

Suppression of Type I Interferon Signaling Overcomes Oncogene-Induced Senescence and Mediates Melanoma Development and Progression

Graphical Abstract



Highlights

- Type I IFNs contribute to development of the oncogene-induced senescence
- Suppression of IFN signaling suffices to induce melanoma by activated Braf
- IFN signaling in melanocytes and other cell types counteracts melanoma development
- Downregulation of IFN receptor IFNAR1 promotes melanoma development and progression

Authors

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In Brief

Katlinskaya et al. demonstrate that oncogene-induced senescence is promoted by type I interferons (IFNs). Both cell-autonomous and non-cell-autonomous effects of IFN contribute to its role as a tumor-suppressive pathway counteracting development of melanoma. Downregulation of IFN receptor IFNAR1 promotes melanoma development and limits its responses to molecular and immunotherapy.

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Suppression of Type I Interferon Signaling Overcomes Oncogene-Induced Senescence and Mediates Melanoma Development and Progression

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SUMMARY

Oncogene activation induces DNA damage responses and cell senescence. We report a key role of type I interferons (IFNs) in oncogene-induced senescence. IFN signaling-deficient melanocytes expressing activated *Braf* do not exhibit senescence and develop aggressive melanomas. Restoration of IFN signaling in IFN-deficient melanoma cells induces senescence and suppresses melanoma progression. Additional data from human melanoma patients and mouse transplanted tumor models suggest the importance of non-cell-autonomous IFN signaling. Inactivation of the IFN pathway is mediated by the IFN receptor IFNAR1 downregulation that invariably occurs during melanoma development. Mice harboring an IFNAR1 mutant, which is partially resistant to downregulation, delay melanoma development, suppress metastatic disease, and better respond to BRAF or PD-1 inhibitors. These results suggest that IFN signaling is an important tumor-suppressive pathway that inhibits melanoma development and progression and argue for targeting IFNAR1 downregulation to prevent metastatic disease and improve the efficacy of molecularly target and immune-targeted melanoma therapies.

INTRODUCTION

Oncogene-induced senescence (OIS) represents an essential cell-autonomous tumor-suppressive mechanism that protects cells harboring damaged genes from malignant transformation and prevents development of malignant tumors (Campisi and d'Adda di Fagagna, 2007). Activating mutations in *RAS* or *RAF* genes stimulates the mitogen-activated protein kinase (MAPK) pathway and triggers an initial burst of cell proliferation, followed by the onset of OIS (Campisi and d'Adda di Fagagna, 2007; Kuilman et al., 2010). For example, mutagenic activation of *BRAF* and OIS markers are frequently found in benign melanocytic hyperplastic lesions (nevi) in humans (Dong et al., 2003; Pollock et al., 2003). Furthermore, mice expressing $Braf^{V600E}$ mutant in melanocytes display increased proliferation of these melanocytes but rarely develop malignant melanoma (Damsky et al., 2015; Dankort et al., 2009). While senescence is the term usually reserved for a permanent cell proliferation arrest, additional genetic and epigenetic alterations can override this permanence (Souroullas and Sharpless, 2015). These additional events can restore the cycling of a genetically altered cell, leading to completion of malignant transformation and initiation of malignant tumor development. For melanocytes that harbor $Braf^{V600E}$ mutation, such events include loss of *Pten* and hyperactivation of the phosphatidylinositol 3-kinase (PI3K) pathway (Dankort et al., 2009) and/or activation of the mTOR pathway (Damsky et al., 2015).

Activation of DNA damage responses plays a central role in the development of OIS (Bartkova et al., 2006; Di Micco et al., 2006). The DNA-damage-induced production of type I interferons (IFNs, including IFNα and IFNβ) was shown to contribute to the extent of senescence in cells and tissues harboring persistent DNA damage (Yu et al., 2015). IFNs are anti-viral cytokines that bind to their cognate IFNAR1/IFNAR2 receptor to induce the signal transduction pathway involving Janus kinases and STAT1/2 proteins and culminating in expression of IFN-stimulated genes, some of which suppress cell proliferation and survival (reviewed in Platanias, 2005). IFNs can induce cell

senescence (Moiseeva et al., 2006) and, in addition to cell-autonomous effects on malignant cells, exert indirect tumor-suppressive functions, such as inhibition of angiogenesis and induction of anti-tumor immunity (Borden et al., 2007). Based on these activities, IFNs remain a standard option in the therapy of a number of cancers, including melanoma (Kirkwood et al., 2012; Tarhini et al., 2012).

However, a tumor-suppressive function of IFNs in melanoma is not fully supported by available data from mice lacking the *Ifnar1* receptor, which is required for all effects of IFNs on cells (Fuchs, 2013; Piehler et al., 2012; Uzé et al., 2007). Although growth of transplanted melanoma tumors was modestly (if at all) stimulated in *Ifnar1*$^{-/-}$ mice (Bald et al., 2014; Fuertes et al., 2011; Núñez et al., 2012), ablation of *Ifnar1* in *Hgf-Cdk4* R24C mice did not accelerate development of cutaneous melanoma induced by 7,12-dimethylbenz(a)anthracene (Bald et al., 2014). In addition, hyperactivation of either mTorc1 (Damsky et al., 2015) or PI3K-Akt (Dankort et al., 2009) pathways in mice that express *Braf*^{V600E} mutant in melanocytes could overcome OIS in *Ifnar1*$^{+/+}$ mice that do not harbor any genetic deficiency in the IFN pathway. Furthermore, *Ifnar1*$^{+/+}$ animals whose melanocytes lacked *Pten* and harbored *Braf*^{V600E} mutation developed highly aggressive metastatic melanomas (Dankort et al., 2009).

Given that the levels of IFNAR1 protein can be rapidly downregulated through a number of phosphorylation-dependent ubiquitination mechanisms (reviewed in Fuchs, 2013), we hypothesized that mobilization of these mechanisms in melanoma may override the tumor-suppressive role of IFN in this malignancy. Downregulation of IFNAR1 occurs in response to its ubiquitination by the SCF-βTrcp2/HOS E3 ubiquitin ligase (Kumar et al., 2003), which binds to IFNAR1 after its phosphorylation (on Ser535/539 in human IFNAR1 or Ser526/530 in mouse IFNAR1; Kumar et al., 2004). This phosphorylation can be induced by ligands (Zheng et al., 2011b) and additional stimuli, including inflammation (Bhattacharya et al., 2014). Melanoma cells are known to upregulate the levels of βTrcp2/HOS (Kumar et al., 2007b) and produce inflammatory cytokines that stimulate IFNAR1 ubiquitination and suppress IFN signaling (Huangfu et al., 2012).

Here we report that ablation of *Ifnar1* overcomes OIS in *Braf*^{V600E}-expressing melanocytes, leading to development of aggressive metastatic melanomas. The re-expression of IFNAR1 in melanoma cells derived from these tumors restores the expression of senescence markers and slows tumor growth; this result is consistent with a cell-autonomous tumor-suppressive role of IFN. However, data from human patients and a syngeneic mouse transplanted model also strongly support an indirect role of IFN in preventing melanoma development. Finally, mice harboring the knockin *Ifnar1*^{S526A} alleles (*Ifnar1*^{SA} that expresses the ubiquitination-deficient IFNAR1^{SA} mutant protein) displayed a notably delayed melanomagenesis in the *Braf*^{V600E}; *Pten*$^{\Delta/\Delta}$ model. This retarded melanoma development was concurrent with decreased levels of *Ifnar1*^{SA} protein and mRNA in mouse skin and was characterized by dramatically suppressed metastatic disease and increased responsiveness to molecularly targeted therapy or immunotherapy. These data characterize IFN signaling as a tumor-suppressive pathway in melanoma and provide the foundation for targeting IFNAR1 downregulation to improve therapeutic outcomes.

RESULTS

Suppression of IFN Signaling Is Sufficient to Overcome OIS and Promote Melanoma Development upon Braf Activation

Human diploid fibroblasts transduced with oncogenic *H-Ras*^{G12V} mutant exhibited an activated MAPK pathway and upregulated levels of senescence protein markers (p21$^{CIP1/WAF1}$ and HP1β) and IFN-inducible IRF7 protein (Figure 1A). Concurrent knockdown of IFNAR1 did not alter ERK phosphorylation but robustly attenuated IRF7, p21$^{CIP1/WAF1}$, and HP1β induction, suggesting a role for IFN in onset of OIS. IFNAR1 knockdown also abrogated *H-Ras*^{G12V}-induced senescence-associated β-galactosidase (SA-β-gal) activity (Figure 1B). In addition, knockout of *Ifnar1* or neutralization of endogenous IFN attenuated senescence induced by *H-Ras*^{G12V} mutant in mouse fibroblasts (Figures S1A and S1B). These data suggest that IFNs contribute to OIS in vitro.

Constitutively active *Braf*^{V600E} mutation frequently found in atypical but non-malignant melanocytic lesions (dysplastic nevi) was previously shown to induce senescence in vitro (Wajapeyee et al., 2008) and to stimulate OIS in mouse melanocytes (Damsky et al., 2015; Dankort et al., 2009; Ferguson et al., 2015). We used this inducible mouse model (*Tyr-CreERT2; Braf*$^{CA/+}$), in which melanocytes express *Braf*^{V600E} allele (Figure S1C) and activate the MAPK pathway (Figure 1C) upon topical skin treatment with 4-hydroxytamoxifen (4HT). This treatment upregulated the expression of mRNA for senescence marker *Cdkn1a* and inflammatory cytokines (*Tnfa, Il6, Ifng*; Figure S1D) known to contribute to the senescence-associated secretome (Freund et al., 2010). Activation of *Braf* also increased p21$^{CIP1/WAF1}$ protein (Figure 1C), as well as accumulation of DNA-damage-associated 53BP1 foci and SA-β-gal-positive cells (Figure 1D) characteristic of senescence. Given that all these changes were dramatically attenuated by ablation of *Ifnar1* (Figures 1C, 1D, and S1D), it is likely that IFN plays an important role in melanocytes OIS in vivo.

Braf^{V600E}; *Ifnar1*$^{-/-}$ mice displayed a greater cell proliferation as judged from Ki67 analysis in the skin and the appearance of pigment globules suggestive of melanocytes abundance (SA-β-gal staining; Figure 1D). Histological analysis of these samples reflected a massive hyperproliferation of melanocytes (Figure S1E) that macroscopically resulted in notable skin pigmentation (Figure 1E). These data suggest that IFN signaling mediates cell senescence and restricts proliferation of melanocytes harboring activated *Braf*.

We continued to observe the cohorts of *Braf*^{V600E}; *Ifnar1*$^{+/+}$ and *Braf*^{V600E}; *Ifnar1*$^{-/-}$ mice over 14 months after 4HT treatment. Consistent with a previous report (Dankort et al., 2009), *Braf*^{V600E}; *Ifnar1*$^{+/+}$ mice developed few melanomas during this time frame. Conversely, all *Braf*^{V600E}; *Ifnar1*$^{-/-}$ mice had developed tumor lesions (Figure 2A) that exhibited histopathological characteristics of melanoma (Figure 2B). Melanoma development in *Ifnar1*-deficient mice occurred with 100% penetrance (Figure 2C). Upon development, aggressively growing tumor lesions significantly shortened the lifespan of these animals

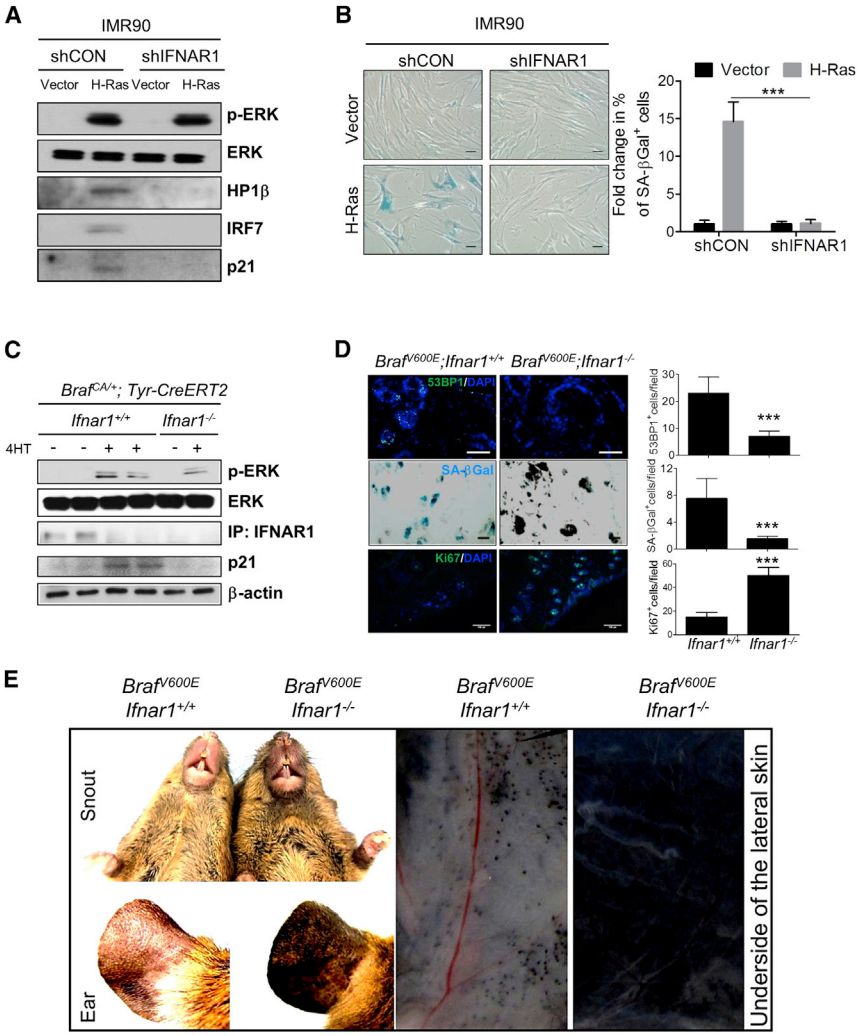


Figure 1. IFNs Contribute to the OIS and Restrict Proliferation in Melanocytes that Harbor Activated *Braf*

(A) Immunoblot analysis of human diploid fibroblast (HDF) IMR90 cells stably expressing shCON or shIFNAR1 and transduced with control vector or vector expressing *H-Ras*^{G12V}. Cell lysates were prepared 5 days after transduction. Levels of markers of senescence (HP1β, p21) and IFN signaling (IRF7) were analyzed; total ERK levels were used as a loading control.

(B) Representative SA-β-gal staining of IMR90 stably expressing shCON or shIFNAR1 and transduced with control vector or vector expressing *H-Ras* 5 days after transduction (left panel). Quantification of the number of SA-β-gal-positive cells per field (right panel).

(C) Immunoblot analysis of IFNAR1 from the whole skin lysates of adult mice at day 30 after topical administration of 4HT is shown. Levels of phospho-ERK (to indicate activation of BRAF) are also shown. Levels of total ERK and β-actin in whole lysates of corresponding samples were used for loading control (lower panels). The expression of senescent marker p21 was also evaluated.

(D) Representative immunofluorescent analysis and quantification of 53BP1-positive foci and SA-β-gal- and Ki67-positive cells in the skin of adult mice of indicated genotypes at day 30 after topical administration of 4HT. Scale bar represents 100 μm. At least 20 fields randomly chosen from three independent experiments were quantified.

(E) Representative images of the appearance of the snout and ear and the underside of the lateral skin from adult mice of indicated genotypes at day 30 after topical administration of 4HT.

Data are shown as mean ± SD; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Scale bar represents 100 μm. At least 20 fields randomly chosen from three independent experiments were quantified. See also Figure S1.

(Figure 2C) and were accompanied by numerous metastatic lesions in lymph nodes and lungs (Figures 2A and 2D). Similar results with yet shorter latency were obtained in *Ifnar1*-null animals harboring two copies of activated *Braf*^{V600E} (Figure S2). These results indicate that inactivation of IFN signaling is sufficient for *Braf*-driven melanomagenesis.

IFN Prevents Melanoma Development and Progression via Cell-Autonomous and Non-autonomous Mechanisms

Animals harboring *Braf*^{V600E}; *Ifnar1*$^{-/-}$ alleles lacked IFN signaling in all tissues. To determine whether the protective role of IFN against melanoma development is cell-autonomous and/or depends on effects of IFN in cell types other than melanocytes, we established a cell line called PVMM (Penn Vet mouse melanoma) from a spontaneous skin lesion. These cells grew in vitro and were characterized by constitutively hyperactive MAPK signaling (which was sensitive to vemurafenib treatment) and lack of IFNAR1 expression (Figure S3A). Furthermore, subcutaneous (s.c.) transplantation of these cells into the flank of syngeneic mice led to development of melanoma tumors that grew aggressively and metastasized into the lungs (Figure S3B).

We sought to determine the cell-autonomous effects of IFN signaling by restoring IFNAR1 expression selectively in melanoma cells. Given that hyperactive *BRAF* accelerates the ubiquitination-driven downregulation of IFNAR1 (Kumar et al., 2007b), we chose to use *Ifnar1*^{SA}, which cannot undergo the phosphorylation required for interacting with βTrcp2/HOS E3 ubiquitin ligase and subsequent ubiquitination, endocytosis, and degradation of IFNAR1 (Kumar et al., 2003, 2004, 2007a). PVMM cells were transduced with vectors expressing GFP alone or GFP with *Ifnar1*^{SA} (Figure S3C). Compared with expression of GFP alone, delivery of the ubiquitination-deficient IFNAR1 did not affect MAPK activation yet led to upregulation of IRF7 and p21 proteins (Figure 3A), increased number of SA-β-gal-positive cells when grown in vitro or in vivo (Figure 3B), and in the latter settings, retarded growth of tumors in syngeneic mice (Figure 3C). These results suggest that restoration of the cell-autonomous IFN signaling in melanoma cells can trigger senescence and attenuate melanoma progression.

However, analysis of melanoma samples harvested from patients before treatment with high-dose IFN adjuvant therapy showed only a trend linking low IFNAR1 levels with clinical

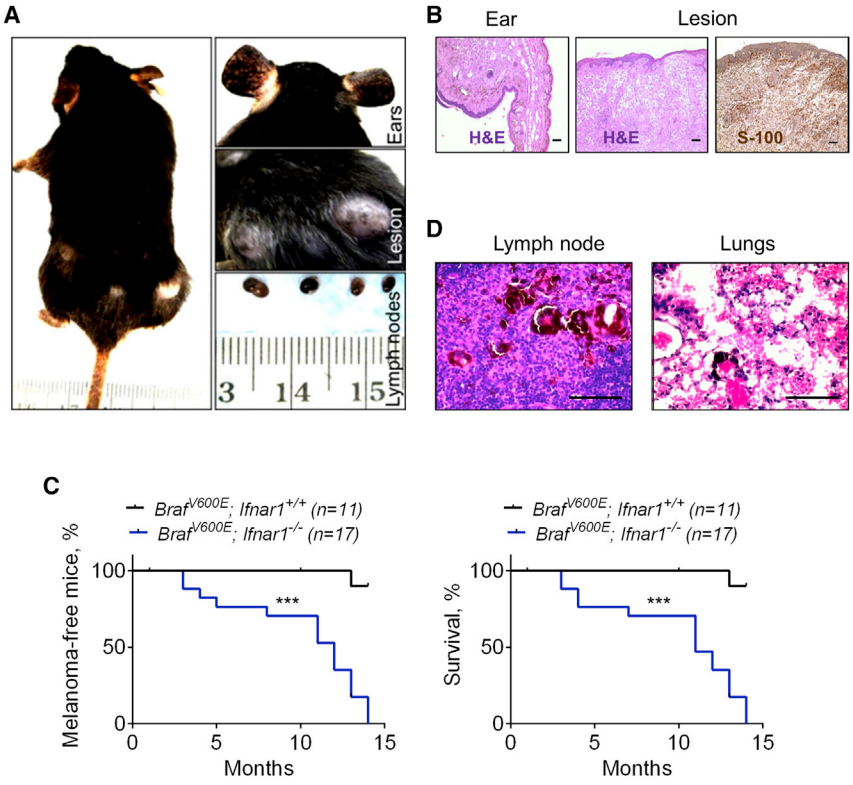


Figure 2. Suppression of IFN Signaling Is Sufficient for $Braf^{V600E}$-Driven Melanoma Development

(A) Macroscopic appearance of tumor lesions and sentinel lymph nodes developing in 4HT-treated $Braf^{CA/+}$; $Ifnar1^{-/-}$ mice.

(B) H&E staining of ear (left panel) and lesions (middle panel) and immunohistochemistry staining of S100 in the lesions (right panel) from $Braf^{V600E}$; $Ifnar1^{-/-}$ mice. Scale bar represents 100 μm.

(C) Incidence of malignant melanomas (left panel) and Kaplan-Meier analysis of survival (right panel) of mice of indicated genotypes at the indicated time after 4HT treatment.

(D) H&E staining of lymph node and lung tissues from $Braf^{V600E}$; $Ifnar1^{-/-}$ mice.

Scale bar represents 100 μm. See also Figure S2.

outcomes of relapse or mortality of these patients (Figure 3D). This trend did not reach statistical significance, suggesting that the status of IFNAR1 in the benign tissues may also contribute to the role of IFN signaling in melanoma progression. To corroborate this possibility, we transplanted unmodified *Ifnar1*-null PVMM tumor cells into syngeneic mice that harbored either wild-type *Ifnar1* or the knockin ubiquitination-deficient $Ifnar1^{SA}$ allele (described in Bhattacharya et al., 2014).

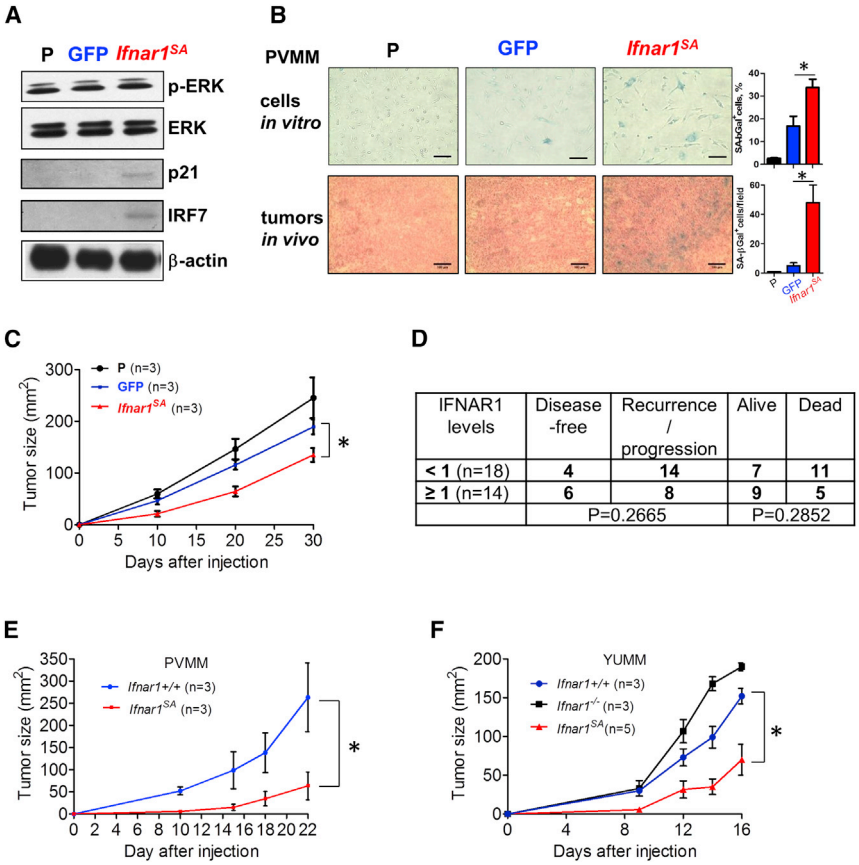
PVMM developed noticeably smaller tumors (Figure 3E) and did not produce lung metastases in $Ifnar1^{SA}$ mice. A similar result was obtained in these mice inoculated with $Ifnar1^{+/+}$ YUMM (Yale University mouse melanoma) cells (Figure 3F). Moreover, YUMM cells grew faster in $Ifnar1^{-/-}$ mice, further supporting the importance of IFN signaling in the stroma. Finally, a similar growth delay in C3H/Hej $Ifnar1^{SA}$ mice was observed for syngeneic SW1 mouse melanoma tumors (Figure S3D). In all, these results suggest that IFNs counteract melanoma development and progression through both cell-autonomous and non-cell-autonomous mechanisms.

Suppression of the IFN Pathway beyond a Specific Threshold Promotes Melanoma Development, Progression, and Metastasis and Decreases the Efficacy of Molecularly Targeted Treatment and Immunotherapy

Decreased levels of IFNAR1 were observed in human melanomas compared to normal melanocytes from matched patients samples (Huangfu et al., 2012). Accelerated degradation of IFNAR1 and attenuation of IFN responses can be promoted by the stimuli associated with tumor development, including unfolded protein response (Bhattacharya et al., 2010, 2011; Liu et al., 2009), integrated stress (Bhattacharya et al., 2013), vascular endothelial growth factor (Zheng et al., 2011a), and inflammation (Bhattacharya et al., 2014; Huangfu et al., 2012; Qian et al., 2011). Given that accelerated degradation of IFNAR1 induced by oncogene and/or inflammation or tumor stress in both melanocytes and stromal cells may mask the importance of this receptor in melanoma development and progression, we sought to test the role of IFN signaling using a combination of the $Ifnar1^{SA}$ allele with the powerful inducible *Tyr-CreERT2;* $Braf^{CA/+}$; $Pten^{f/f}$ mouse model that combines melanocyte-specific activation of *Braf* with ablation of *Pten* (Figure S4A) and ensuing activation of PI3K-Akt pathway (Damsky et al., 2011; Dankort et al., 2009).

Concurrent activation of *Braf* and PI3K-Akt pathways led to a noticeable decrease in IFNAR1 levels in the skin of $Ifnar1^{+/+}$ mice; a lesser downregulation was observed in $Ifnar1^{SA}$ tissues (Figure S4B). Accordingly, both gene array expression and qPCR analyses demonstrated suppressed IFN signaling signatures in the skin of $Ifnar1^{+/+}$ animals compared to $Ifnar1^{SA}$ skin (Figures S4C and S4D). Expression of $Braf^{V600E}$ alleles, along with *Pten* inactivation, in $Ifnar1^{+/+}$ mice led to a rapid development of aggressive metastatic melanomas within 20 days of tamoxifen treatment (Figures 4A–4D). At this time point, the analogous $Ifnar1^{SA}$ skin tissues displayed increased numbers of SA-β-gal-positive cells (Figure 4B) and only mild melanocyte proliferation, without any sign of melanoma development or metastatic spread. Melanomas in $Ifnar1^{SA}$ mice developed later (Figures 4A–4D). Similar data were obtained in mice harboring a single copy of activated *Braf* (Figures S4E and S4F). These results suggest that development of melanoma is delayed in animals that maintain a higher level of IFN signaling. Conversely, downregulation of IFNAR1 and suppression of IFN responses in wild-type mice may accelerate development of melanoma.

The melanoma-induced mortality was also delayed in $Ifnar1^{SA}$ mice (Figure 4E). This mortality was associated with the primary



IFNAR1 levels	Disease -free	Recurrence / progression	Alive	Dead
< 1 (n=18)	4	14	7	11
≥ 1 (n=14)	6	8	9	5
	P=0.2665		P=0.2852	

Figure 3. IFNs Prevent Melanoma Development and Progression via Cell-Autonomous and Non-autonomous Mechanisms

(A) Immunoblot analysis of indicated proteins in lysates from parental (P) PVMM cells and derivatives (transduced for expressing GFP alone or with IFNAR1^{SA}) 4 days after transduction.

(B) SA-β-gal staining (blue) and percentage of SA-β-gal-positive PVMM cells and their derivatives described in (A). Cells were grown in vitro (upper panels) or injected to form tumors (lower panels, counterstained with fast red). Data are shown as mean ± SD from three independent experiments (at least 30 randomly chosen fields per experiment).

(C) Tumor growth curves of PVMM cells (P) and their derivatives expressing GFP (GFP) or GFP with IFNAR1^{SA} mutant (IFNAR1^{SA}) after s.c. injection of 1 × 10^6 cells into *Ifnar1*$^{+/+}$ mice. Similar results were obtained in at least two additional independent experiments.

(D) Fisher's exact test analysis of relationship between IFNAR1 levels (analyzed by immunohistochemistry in samples harvested from melanoma patients before treatment with high-dose IFN) and therapy outcome.

(E) PVMM tumor growth in *Ifnar1*$^{+/+}$ (blue) or *Ifnar1*^{SA} (red) mice after s.c. injection of 1 × 10^6 PVMM cells. Similar results were obtained in at least two additional independent experiments.

(F) Average tumor growth of YUMM in *Ifnar1*$^{+/+}$ (blue), *Ifnar1*^{SA} (red), or *Ifnar1*$^{-/-}$ (black) mice after s.c. injection of 1 × 10^6 YUMM cells. Similar results were obtained in at least two additional independent experiments.

*p < 0.05. Scale bar represents 100 μm. See also Figure S3.

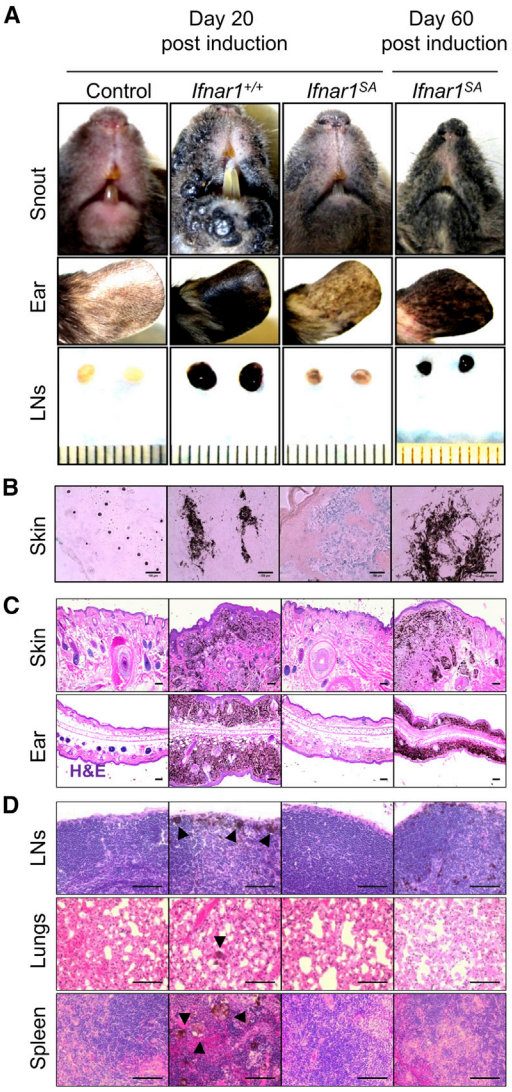
tumor load and tumor size that approached the limits of humane experimentation. While this tumor burden in euthanized animals was identical in *Ifnar1*$^{+/+}$ animals (~day 20 after tamoxifen treatment) and in *Ifnar1*^{SA} mice (~day 60 after tamoxifen treatment), it is critical that the latter animals lacked distant metastases (Figure 4D). Furthermore, despite a substantial primary tumor growth, *Ifnar1*^{SA} mice displayed a significantly reduced number of local lymph node metastases (Figure 4F). These results suggest that IFN signaling is even more critical for suppressing the metastatic disease than for inhibiting primary melanoma growth.

To delineate the mechanisms of melanoma development in *Ifnar1*^{SA} mice, we compared skin from these animals obtained 20 and 60 days after tamoxifen treatment by global gene expression analysis. A notable suppression of expression of IFN-inducible genes was observed at the latter time point (Figures 5A and S5). Subsequent qPCR analysis of expression of *Ifnb* and individual IFN-stimulated genes also revealed that IFN signaling is suppressed in the skin of *Braf*^{VE}, *Pten*$^{\Delta/\Delta}$, *Ifnar1*$^{+/+}$ type mice within 20 days (Figure 5B). However, in *Ifnar1*^{SA} at day 20, the levels of expression of *Ifnb*, *Isg15*, and *Irf7* mice were still substantially higher than those in *Ifnar1*$^{+/+}$ animals. At this time, a spike in *Ifnb* expression corresponding to increase in IRF7 and p21 proteins (Figure 5C), consistent with increased SA-β-gal staining (Figure 4B), was observed, further suggesting the importance of IFN signaling in melanocyte senescence.

Expression of IFN-inducible genes continued to decline by the time of melanoma development in *Ifnar1*^{SA} mice at day 60 (Figure 5B). Expression of IRF7 and p21 proteins followed similar kinetics (Figure 5C). These results indicate a putative link between levels of IFN signaling and melanoma development.

This decrease in IRF7 and p21$^{CIP1/WAF1}$ levels at day 60 in *Ifnar1*^{SA} skins coincided with a notable downregulation of the levels of IFNAR1 protein and mRNA that nearly reached those observed in animals harboring wild-type *Ifnar1* (Figure 5C). These results indicate an activation of additional mechanisms that act to downregulate the ubiquitination-deficient IFNAR1^{SA} protein. In all, these data support a concept that such additional mechanisms are aimed at decreasing IFN signaling beyond the threshold that is capable of preventing melanomagenesis. Accordingly, efficient inactivation of IFN signaling in the skin appears to be important for melanoma development and progression.

In lieu of data pointing to the importance of IFN signaling in non-melanocytic tissues (Figure 3), we sought to determine whether degradation of IFNAR1 plays a role in regulating the sensitivity of melanoma to molecular or immunotherapy. To this end, we initiated melanoma development by topical application of 4HT onto the skins of the *Tyr-CreERT2; Braf*$^{CA/+}$; *Pten*$^{f/f}$ mice that harbored wild-type or mutant (*Ifnar1*^{SA}) IFN receptor. Upon reaching an approximately 50 mm^2 tumor size, which required on average 21 days for *Ifnar1*$^{+/+}$ and 43 days



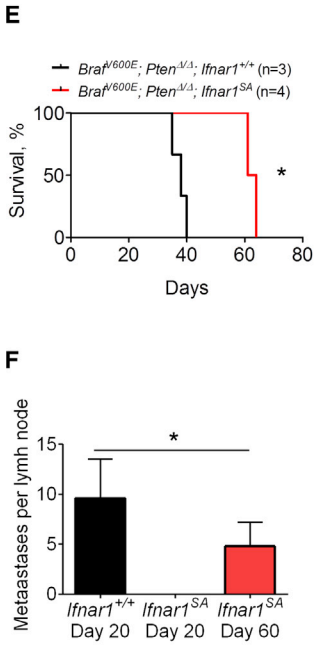


Figure 4. Downregulation of the IFN Receptor Promotes Melanoma Development, Progression, and Metastasis

(A) Macroscopic appearance of the sentinel lymph nodes and tumor lesions on the ear and snout from *Braf*^{CA}; *Pten*$^{\Delta/\Delta}$; *Ifnar1*$^{+/+}$ and *Braf*^{CA}; *Pten*$^{\Delta/\Delta}$; *Ifnar1*^{SA} mice at day 20 and day 60 after generalized induction of recombination by tamoxifen.

(B) SA-β-gal/fast red staining of skin tissues from corresponding mice.

(C) H&E staining of the ear and skin tissues from corresponding mice.

(D) H&E staining of organs from corresponding mice. Metastatic lesions are indicated by arrows.

(E) Kaplan-Meier analysis of survival in animal cohorts described in (A)–(C).

(F) Quantification of metastatic lesions in the lymph node from mice of indicated genotypes. Data are shown as mean ± SD (n = 5–6 per group); *p < 0.05. Scale bar represents 100 μm. See also Figure S4.

for *Ifnar1*^{SA} animals, we started treatment of mice with either BRAF inhibitor vemurafenib or anti-PD-1 antibody. The latter treatment modestly retarded subsequent tumor growth in *Ifnar1*$^{+/+}$ mice but was significantly more efficacious in *Ifnar1*^{SA} animals (Figure 5D). Similarly, whereas melanomas harboring wild-type IFN receptor responded to vemurafenib by merely slowing tumor progression, at the least, stable disease was observed in *Ifnar1*^{SA} mice (Figure 5E). These results suggest that downregulation of IFNAR1 limits the efficacy of both molecular anti-tumor and immunomodulatory therapies for melanomas that harbor activated *Braf* and inactivated *Pten*.

DISCUSSION

IFN Signaling as a Tumor-Suppressive Pathway in Melanoma

The long reported suppressive effects of IFN on growth and survival of established melanoma cells in vitro have been used to provide a foundation for recombinant IFN therapies (Bonnem and Spiegel, 1984; Fisher and Grant, 1985; Kirkwood and Ernstoff, 1984, 1985; Sikora, 1980). However, the concept of endogenous IFN signaling as a tumor-suppressive pathway that inhibits melanoma development has been underappreciated. Findings of suppression of the IFN pathway in melanoma patients' leukocytes (Critchley-Thorne et al., 2007, 2009) and tumors, where it inversely correlates with relapse-free survival (Bald et al., 2014), link the endogenous IFN pathway with melanoma progression. Here we provide an animal model-based mechanistic link between endogenous IFN and inhibition of melanomagenesis and demonstrate that IFN signaling plays a tumor-suppressive role in melanoma.

Our work used genetically engineered and transplanted mouse melanoma models to show that ablation of *Ifnar1* overcomes OIS, including melanocyte senescence induced by activated *Braf* (Figure 1). Furthermore, inactivation of IFN signaling in this context is sufficient to promote the development of aggressive metastatic melanomas (Figure 2). Whereas expression of IFNAR1^{SA} in *Ifnar1*-null melanoma cells can induce senescence and suppress melanoma growth in vivo, data from human melanoma patients treated in the adjuvant setting demonstrate that levels of IFNAR1 in melanoma cells do not alone reliably predict clinical outcomes with high-dose IFN. These findings suggest that IFNAR1 status in benign cells of non-melanocytic origin plays an important role in tumor-suppressive and anti-metastatic functions of endogenous IFN, in addition to melanocytes OIS. These conclusions, supported by results in transplantable models grown in *Ifnar1*^{SA} mice (Figure 3), are in line with the previously described role of IFN signaling in immune responses against melanoma (Bald et al., 2014; Fuertes et al., 2011). The putative role of IFNAR1-dependent restriction

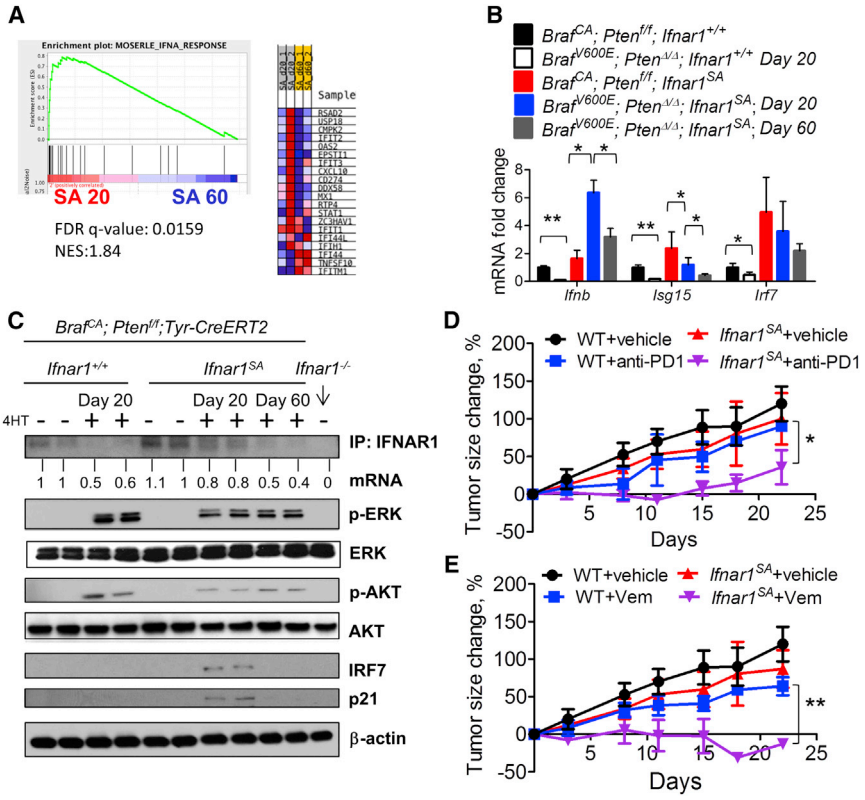


Figure 5. Inactivation of the IFN Pathway during Melanoma Development Decreases the Efficacy of Melanoma Treatment

(A) Gene set enrichment analysis (Moserle IFN response, left panel) and heatmap (right panel) of IFNα/β signaling pathway genes of the transcriptome profiles of the skin tissues from *Braf*^{CA}; *Pten*$^{\Delta/\Delta}$; *Ifnar1*^{SA} mice collected at day 20 and day 60 after tamoxifen treatment. NES, normalized enrichment score; FDR, false discovery rate.

(B) qPCR analysis of the expression of IFN-stimulated genes in skin tissues from mice of indicated genotypes at day 20 and day 60 after tamoxifen administration. Untreated mice of the same genotypes were used as a control. Data are shown as mean ± SD (n = 5–6 per group).

(C) Immunoblot analysis of indicated protein levels in the skin tissues lysates from mice of indicated genotypes treated as indicated (two samples per group). The *Ifnar1*$^{-/-}$ mice skin sample serves as a negative control for IFNAR1 detection (analyzed by immunoprecipitation-immunoblotting [IP-IB]). Relative levels of *Ifnar1* mRNA (compared with untreated skin in *Ifnar1*$^{+/+}$ animals) are shown below the IFNAR1 protein panel.

(D) Relative local melanoma tumor volume change in mice of indicated genotypes treated with control or anti-PD-1 antibody. Tumors were induced by topical application of 4HT. Tumor size in each of the treatment groups was measured every other day and plotted as the percentage change in tumor size compared to the starting size (50 mm^2). Data are shown as mean ± SD (n = 5–6 per group).

(E) Relative local melanoma tumor volume change in mice treated with a vehicle control or vemurafenib (Vem). Tumors were induced by topical application of 4HT. Tumor size in each of the treatment groups was measured every other day and plotted as the percentage change in tumor size compared to the starting size (50 mm^2). Data are shown as mean ± SD (n = 5–6 per group).

*p < 0.05; **p < 0.01. See also Figure S5.

of the inflammatory secretome (Figure S4D) in suppressing the distant metastases in *Ifnar1*^{SA} mice is under investigation.

Mechanisms of Inactivation of the IFN Pathway in Melanoma

Alterations in the recruitment of dendritic cells (known for their potent ability to produce IFN (Zitvogel et al., 2015) and lacking in melanomas characterized by an activated β-catenin pathway (Spranger et al., 2015) may affect overall expression of IFN ligands in the tumors. Furthermore, results from experiments that used the expression of IFNAR1^{SA} mutant in vitro and in vivo (Figures 3 and 4) suggest that specific serine phosphorylation-dependent ubiquitination and degradation of IFNAR1 represents an important mechanism underlying downregulation of IFNAR1 in transformed melanocytes and/or stromal cells during the development of melanoma. While activation of *Braf* alone can stimulate partial IFNAR1 downregulation (Figure 1A), additional signaling pathways activated in developing melanoma are likely to further eliminate IFNAR1, thereby depressing IFN signaling beyond the threshold at which it can sustain the tumor-suppressive role. Robust melanoma development in *Braf*^{V600E} mice (Figure 2) upon complete ablation of IFNAR1 strongly supports this hypothesis. Accordingly, a delay of melanoma development in *Ifnar1*^{SA} mice indicates that the ability of cells to maintain a certain level of IFNAR1 expression (despite a decrease in *Ifnar1* mRNA; Figure 5C) is important for counteracting the effects of oncogenic pathways and stimulation of melanoma development and progression.

Furthermore, eventual development of melanoma in *Ifnar1*^{SA} mice coincides with a decrease in IFNAR1^{SA} mRNA and protein levels and expression of IFN-stimulated genes detected in affected mouse skin (Figure 5). Because IFNAR1^{SA} mutant protein cannot interact with βTrcp2/HOS (Kumar et al., 2003, 2004), these findings indicate that activation of a yet-to-be-characterized βTrcp2/HOS-independent pathway occurs in malignant melanocytes and/or stromal cells to downregulate the levels of IFNAR1 and suppress IFN signaling. A decrease of *Ifnar1* mRNA levels seen in these tumors (Figure 5C) is indicative of transcriptional suppression and/or accelerated *Ifnar1* mRNA decay that can be a consequence of hyperactive MAPK and PI3K signaling. Upregulation of histone methyltransferase EZH2 and H3K27 methylation-driven suppression of several tumor suppressor genes was found in *Braf*^{V600E}-expressing melanomas (Hou et al., 2012). Use of an EZH2 inhibitor GSK503 (capable of upregulating the levels of *Ifnar1* in YUMM cells; data not shown) was found to prevent metastatic disease in the *Tyr::N-Ras*^{Q61K}, *Ink4a*$^{-/-}$-driven mouse melanoma model (Zingg et al., 2015)—a phenotype reminiscent of suppressed metastatic spread in *Ifnar1*^{SA} mice (Figure 4). In addition to the mechanisms conferring a decrease in *Ifnar1* mRNA, we cannot

rule out a role for βTrcp2/HOS-independent proteolysis of IFNAR1 that may involve activation of extracellular proteases previously shown to be upregulated in human and mouse melanomas (Hofmann et al., 2000, 2005; Ramont et al., 2003).

Putative Therapeutic Importance of Re-activation of IFN Signaling in Melanoma

Given that IFN therapy prevents relapse and death in up to 33% of melanoma patients, there is a pressing need to identify means to enhance this benefit and to stratifying melanoma patients to apply this modality to those patients who can benefit most from IFN treatment (Eggermont et al., 2014; Thompson et al., 2009). While a number of parameters can be used as predictors of response to IFN therapy (Gogas and Kirkwood, 2009), our data in melanoma patients suggest that relative IFNAR1 protein levels in melanoma cells are unlikely to be sufficiently informative (Figure 3D). Given a demonstrated role of IFN signaling in the benign cells of the tumor microenvironment (Figures 3E and 3F), and potentially predictive value of IFN responses in peripheral blood lymphocytes (Simons et al., 2011), future studies should focus on defining IFNAR1 levels and other parameters of IFN signaling in both melanoma and stromal cells.

Ifnar1^{SA} mice maintaining some degree of IFN signaling largely suppressed distant metastases of PVMM or *Braf*^{V600E}/*Pten*$^{\Delta/\Delta}$ melanomas (Figure 4). Given that distant metastases ultimately determine the survival of melanoma patients, these results suggest that the benefit of IFN therapy may be found in its ability to inhibit the process of metastatic dissemination. Conversely, the presence of distant metastases points to evidence of de facto inactivation of endogenous IFN pathway functions and predicts limited survival benefits of pharmacological interventions with IFN. This hypothesis is supported by clinical data from several available reports (Eggermont et al., 2014; Gogas and Kirkwood, 2009; Kirkwood et al., 2012; Tarhini et al., 2012; Thompson et al., 2009).

Additional data suggest that preserving at least some IFN signaling can be beneficial because it increases the responsiveness to BRAF inhibitors and immunotherapy (Figures 4 and 5). The latter results are in line with data reported in a different melanoma model, in which administration of IFN inducers improved the effect of immune checkpoint inhibitors (Bald et al., 2014). Efforts aimed at stabilization of IFNAR1 (via use of inhibitors of kinases involved in its phosphorylation or of SCF-βTrcp2/HOS ligases such as MLN4924) alone or in combination with agents increasing the levels of *Ifnar1* mRNA will be undertaken in future studies.

EXPERIMENTAL PROCEDURES

Melanoma Patients' Samples and Analysis

Melanoma patients with primary and/or nodal tumors banked at the University of Pittsburgh Cancer Institute (UPCI) under tissue/blood biomarker protocol UPCI 96-099 were retrieved. The detailed patient information is provided in Table S1.

Mice

All experiments with animals were carried out under protocol 803995 approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Mice were maintained in a specific pathogen-free facility in accordance with American Association for Laboratory Animal Science guidelines. All mice had water ad libitum and were fed regular chow.

C57BL/6 (*Ifnar1*$^{+/+}$, *Ifnar1*^{SA}, or *Ifnar1*$^{-/-}$; Bhattacharya et al., 2014) and *Braf*^{CA}, *Tyr-CreERT2;* and *Braf*^{CA}, *Tyr-CreERT2, Pten*$^{f/f}$ mice were previously described (Dankort et al., 2009). These mice were crossed to obtain needed genotypes, and the littermates differing in their *Ifnar1* status were used for experiments. C3H/Hej *Ifnar1*$^{+/+}$ mice were purchased from Jackson Laboratory and crossed with C57BL/6 *Ifnar1*^{SA} for at least ten generations to obtain the C3H/Hej littermates (*Ifnar1*^{SA} or *Ifnar1*$^{+/+}$) that were used in experiments on the SW1 melanoma cell line.

Topical administration of 4HT of *Braf*^{CA}*; Tyr-CreERT2; Ifnar1*$^{-/-}$ or *Braf*^{CA}*; Tyr-CreERT2; Ifnar1*$^{+/+}$ mice was performed by preparing a 25–50 mg/ml (65–130 mM) solution of 4HT (70% Z-isomer; Sigma-Aldrich) in dimethylsulphoxide and applying enough solution to wet the right ear, right flank, and tail with a small paintbrush on postnatal days 2, 3, and 4.

For localized melanoma induction, adult (6–8 weeks of age) *Braf*^{CA}*, Tyr-CreERT2, Pten*$^{f/f}$*, Ifnar1*^{SA} or *Braf*^{CA}*, Tyr-CreERT2, Pten*$^{f/f}$*, Ifnar1*$^{+/+}$ mice were treated topically with 1–2 μl of 1.9 mg/ml (5 mM) 4HT on the back skin. Generalized induction in adult *Braf*^{CA}*, Tyr-CreERT2, Pten*$^{f/f}$*, Ifnar1*^{SA} or *Braf*^{CA}*, Tyr-CreERT2, Pten*$^{f/f}$*, Ifnar1*$^{+/+}$ mice was performed by intraperitoneal injection of 25 mg/kg tamoxifen on three consecutive days. In this case, tamoxifen was prepared as a 10 mg/ml suspension in peanut oil.

Cell Lines, Culture Conditions, and Viral Infection

Braf^{CA}*; Pten*^{null}*; Cdkn2a*^{null} (YUMM, a gift from M. Bosenberg and A. Weeraratna), SW1, 293T (provided by Z. Ronai, The Burnham Institute), and *Braf*^{CA}*; Tyr-CreERT2; Ifnar1*$^{-/-}$ (PVMM) cell lines and normal human fibroblasts (IMR90) stably expressing control shRNA (shCON) or shRNA against IFNAR1 (shIFNAR1) were maintained at 37°C with 5% CO_2 in DMEM supplemented with 10% heat-inactivated fetal calf serum, penicillin, streptomycin, and L-glutamine. For lentiviral transduction, 293T cells were transfected with pCIG plasmids encoding EGFP or mouse ubiquitination-deficient IFNAR1^{SA} mutant, together with EGFP (described in Huangfu et al., 2012) using Lipofectamine Plus (Invitrogen). After 24–48 hr, supernatants were passed through a 0.45 μm nylon filter, mixed with Polybrene (8 μg/ml; Santa Cruz), and transferred to plated cancer cells. Transduction efficiency was verified by fluorescence-activated cell sorting analysis and usually was more than 80%. Similarly, 293T cell producing empty virus or virus expressing *H-Ras*^{G12V} oncogene were used for retroviral transduction of IMR90 cells.

Cancer Cell Transplantation and Organ Harvest

Cancer cells were inoculated subcutaneously in the right flank at 1 × 10^6 cells, 100 μl Dulbecco's phosphate-buffered saline (DPBS). Tumors were measured by caliper, and size was calculated as (length × width). Mice were euthanized when the tumor reached 250–300 mm^2, and tumors were dissected and analyzed.

Immunofluorescence and Immunohistochemistry

Skin samples harvested from mice were frozen in Tissue-Tek O.C.T. compound and cryosectioned in Leica CM3050 S Cryostats, fixed in acetone, washed and blocked with PBS containing 5% goat serum, and incubated with primary antibody against 53BP1 (A300-272A; Bethyl Laboratories) or anti-IFNAR1 antibody (Sino Biologicals). Levels of human IFNAR1 in melanoma cells were determined by immunohistochemistry as previously described (Huangfu et al., 2012) and the staining was scored by a qualified dermatopathologist in a double-blind manner.

Melanoma Treatment

When the tumors reached 50 mm^2, animals were gavaged daily with 40 mg/kg vemurafenib (PLX4032, S1267; Selleckchem) in 2% hydroxypropylcellulose (Klucel). For the PD-1 blockade experiment, 100 μg anti-PD-1 (RMP1-14; Bio⧹XCell) or control Ab (2A3; BioXCell) were administered intraperitoneally to mice every 4 days starting at day 8 for a total of six times.

Microarray Analyses with Illumina Whole-Genome Arrays

Total RNA was isolated with the miRNeasy mini kit (QIAGEN) from skin or tumor samples of *Braf*^{CA}*, Tyr-CreERT2, Pten*$^{f/f}$*, Ifnar1*^{SA} mice at day 20 and

day 60 after generalized induction with tamoxifen. Biotin-labeled cRNA preparations were obtained using the TargetAmp-Nano Labeling Kit (Epicenter) as recommended by the manufacturer. Thereafter, 0.75 μg cRNA was hybridized to Illumina Sentrix Mouse-6 v.1 BeadChips, which were scanned with an Illumina BeadStation 500 (both from Applied Biosystems). Data were collected with Illumina BeadStudio 3.1.1.0 software, and statistical analyses were conducted on the IlluminaGUI R-package. Gene sets from microarray data were analyzed for overlap with curated datasets (C5, H) in MSigDB using the web interface available at http://www.broadinstitute.org/gsea/msigdb.

Statistical Analyses

Data are presented as mean ± SD. Statistical analysis was performed using Microsoft Excel. Statistical significance was calculated using a two-tailed Student's t test. A p value < 0.05 was considered significant.

ACCESSION NUMBERS

The accession number for the raw data reported in this paper is GEO: GSE76842.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.03.006.

AUTHOR CONTRIBUTIONS

S.Y.F., K.V.K., J.M.K., H.R., X.W., C.K., and J.A.D. designed the research; Y.V.K., K.V.K., Q.Y., A.O., D.P.B., A.B., D.D., C.S., and H.R. performed the experiments and interpreted the data; and S.Y.F., K.V.K., J.M.K., H.R., C.K., and J.A.D. wrote the manuscript with the help of all authors.

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