Early differential responses elicited by BRAFV600E in adult mouse models

Giuseppe Bosso1, Pablo Lanuza-Gracia1,2, Sergio Piñeiro-Hermida1,2, Merve Yilmaz1, Rosa Serrano1 and Maria A. Blasco1,2

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The BRAF gene is frequently mutated in cancer. The most common genetic mutation is a single nucleotide transition which gives rise to a constitutively active BRAF kinase (BRAFV600E) which in turn sustains continuous cell proliferation. The study of BRAFV600E murine models has been mainly focused on the role of BRAFV600E in tumor development but little is known on the early molecular impact of BRAFV600E expression in vivo. Here, we study the immediate effects of acute ubiquitous BRAFV600E activation in vivo. We find that BRAFV600E elicits a rapid DNA damage response in the liver, spleen, lungs but not in thyroids. This DNA damage response does not occur at telomeres and is accompanied by activation of the senescence marker p21CIP1 only in lungs but not in liver or spleen. Moreover, in lungs, BRAFV600E provokes an acute inflammatory state with a tissue-specific recruitment of neutrophils in the alveolar parenchyma and macrophages in bronchi/bronchioles, as well as bronchial/bronchiolar epithelium transdifferentiation and development of adenomas. Furthermore, whereas in non-tumor alveolar type II (ATII)s pneumocytes, acute BRAFV600E induction elicits rapid p53-independent p21CIP1 activation, adenoma ATIIIs express p53 without resulting in p21CIP1 gene activation. Conversely, albeit in Club cells BRAFV600E-mediated proliferative cue is more exacerbated compared to that occurring in ATIIIs, such oncogenic stimuli culminates with p21CIP1-mediated cell cycle arrest and apoptosis. Our findings indicate that acute BRAFV600E expression drives an immediate induction of DNA damage response in vivo. More importantly, it also results in rapid differential responses of cell cycle and senescence-associated proteins in lung epithelia, thus revealing the early molecular changes emerging in BRAFV600E-challenged cells during tumorigenesis in vivo.

INTRODUCTION

The BRAF gene, encoding a master kinase of RAS-activated RAF-MEK-ERK (RAS-) pathway, is frequently mutated in human malignancies [1–6]. More than 90% of these mutations affect codon 600 of the BRAF protein, and out of these, ~90% represent the 1799T > A nucleotide transition, which results in a constitutively active [7] BRAF variant (BRAFV600E) which indefinitely sustains cell proliferation.

The investigation of BRAFV600E genetically engineered mouse models (GEMMS) has been focused on the role of BRAFV600E in cancer in diverse tissues/organs [8–15]. BRAFV600E expression in vivo triggers an early hyperplastic growth which culminates in a proliferative arrest known as oncogene-induced senescence (OIS) [9, 11, 15], which is driven by p53/p21CIP1 and retinoblastoma protein (Rb)/p16INK4a pathways [16, 17].

Albeit the above-mentioned GEMMS, where BRAFV600E expression relies on tissue-specific promoters, allowed to dissect the function of BRAFV600E in cancer, such an approach carries the limitation of lacking the global view of potential early effects induced by this oncogene. Indeed, apart from the initial wave of hyperplasia, the instant consequences on BRAFV600E-activation in vivo remain unexplored. Here we study the early events following acute expression of BRAFV600E in vivo.

RESULTS

Ubiquitous acute conditional activation of BRAFV600E allele is lethal in adult mice

To analyze the immediate impact of ubiquitous expression of BRAFV600E in vivo, we generated UbiCreER T2+/−;BRAFSL_V600E/+ (BRAFV600E) mice harboring UbiCreER T2 allele [18], expressing the conditionally active CreER T2 recombinase gene under the control of the human ubiquitin promoter (UbiCreER T2), combined with BRAFSL_V600E allele [9]. First, we checked the viability of BRAFV600E mice in the absence of tamoxifen treatments. Albeit until the age of 9 weeks all the mice appeared healthy, starting from 10 weeks from birth they showed weight loss, locomotion alteration, bad shape, papillomatous skin lesions and all of them died between 12–18 weeks from birth (Supplementary Fig. 1A, B), a phenotype which is most likely due to the effects of a spontaneous Cre-mediated recombination of BRAFV600E allele over time. Consistently, PCR analysis of spontaneous papilloma-like lesions arisen in some of the tamoxifen-untreated BRAFV600E mice revealed Cre-mediated activation of BRAFV600E allele (Supplementary Fig. 1C).

To induce an acute BRAFV600E activation, 7–8 weeks old mice were administered tamoxifen intraperitoneally. The BRAFV600E mice, but not the UbiCreER T2+/− (control) strain, started to appear sick 2–3 days post-injection and needed to be euthanatized after...
3–5 days (Supplementary Fig. 2A, B). Macroscopic analysis revealed that BRAFV600E mice had pale livers, which may be indicative of hepatic steatosis (HS) (Supplementary Fig. 2C). PCR analysis confirmed Cre-mediated rearrangement of BRAFV600E allele upon tamoxifen administration, resulting in BRAFV600E expression in all the tissues/organs analyzed (Supplementary Fig. 2D).

Early effects of BRAFV600E expression in thyroids, liver and spleen
First, we analyzed the early effects of BRAFV600E expression in thyroids. Hematoxylin eosin (H&E) staining revealed no morphological alterations between BRAFV600E and control thyroids at 4–5 days after Cre-induction (Supplementary Fig. 2E). Phosphorylation analysis of the downstream effector ERK kinase (ppERK) revealed an increase in ppERK-positive cells in BRAFV600E thyroids compared to control, thus confirming that BRAFV600E is induced in thyroid glands and is stimulating the RAS-pathway (Supplementary Fig. 2F). However, BRAFV600E thyroids displayed no changes in apoptosis, as determined by caspase 3 (CC3), in senescence as determined by p21CIP1, or in DNA damage as indicated by the DNA damage protein γH2AX (Supplementary Fig. 2G–I) compared to controls, indicating that acute BRAFV600E expression in thyroids does not have an apparent impact on cellular viability programs.

Next, we checked the immediate effects of BRAFV600E expression in the liver. In agreement with pale livers present in BRAFV600E mice at their end-point, H&E staining revealed the presence of microvesicular HS which was not present in age-matched controls (Fig. 1A) (see Discussion). BRAFV600E livers showed an enrichment in ppERK-positive cells, thus confirming RAS-pathway activation (Fig. 1B). Concomitantly, albeit γH2AX-positive BRAFV600E hepatocytes were increased 2-fold compared to controls, neither CC3-nor p21CIP1-positive cells were significantly altered (Fig. 1C–E), thus suggesting that after 3–5 days of acute expression, although BRAFV600E induces HS and DNA damage, it elicits neither apoptosis nor alterations in p21CIP1 expression in the liver.

We next investigated the effects of BRAFV600E activation in the spleen. Histopathology analyses revealed a substantial loss of cellularity in both white and red pulp zones as well as the appearance of apoptotic bodies in BRAFV600E spleens but not in controls (Fig. 1F). Only BRAFV600E spleens displayed loss of a clear boundary between the white and red pulp (Fig. 1F), which is indicative of a general cell depletion [19]. Although we found ppERK activation in BRAFV600E spleens (Fig. 1G), Ki67-positive proliferating BRAFV600E cells were significantly reduced (Supplementary Fig. 3), which is in agreement with the observed loss of cellularity. Coherently, CC3-positive cells were increased compared to controls, suggesting that acute BRAFV600E expression in the spleen results in lower proliferation and apoptosis induction (Fig. 1H). Interestingly, γH2AX-positive cells in BRAFV600E spleens were increased by 7-fold compared to controls suggestive of enhanced DNA damage following BRAFV600E expression (Fig. 1I). In contrast, we observed no accumulation of p21CIP1 [20] (Fig. 1J). Collectively, in the spleen acute BRAFV600E induction induces DNA damage without eliciting p21CIP1 expression.

Early effects of BRAFV600E expression in lung alveolar parenchyma
To investigate the immediate effects of BRAFV600E expression in lungs, we first confirmed that BRAFV600E was indeed expressed in lungs at the protein level by immunoblot analysis (Supplementary Fig. 4). Next, we found that BRAFV600E alveolar parenchyma showed alveolar wall thickening and adenomas (Fig. 2A–C). An increase in ppERK- and Ki67-positive cells confirmed that BRAFV600E leads to RAS-pathway activation and consequent proliferation in lungs (Fig. 2D, E). We found no differences in alveolar CC3-positive cells between BRAFV600E and control mice (Fig. 2F). Interestingly, BRAFV600E expression elicited a significant increment in γH2AX and p21CIP1 protein levels, as well as in the number cells positive for such markers (Supplementary Fig. 4, Fig. 2G, H). Moreover, telomere-induc ed foci (TIF) analysis revealed that BRAFV600E elicited DNA damage is not telomeric (Supplementary Fig. 5A). However, we found no changes in the frequency of cells expressing either p53, or p16INK4a and p19ARF, which are all expressed in senescent BRAFV600E-driven lung adenomas [21] (Fig. 2I–K).

Altogether, these data suggest that in the alveolar parenchyma BRAFV600E elicits rapid DNA damage and p53-independent p21CIP1 activation without inducing other classical hallmarks of senescence.

Effects of BRAFV600E expression in lung adenomas
The finding that BRAFV600E mice displayed adenomas (Fig. 2A–C) staining positive for the prosurfactant protein C (SPC), a marker of alveolar type II cells (ATIs), and negative for CC10, a protein specifically expressed by Club cells (CCs), confirmed the evidence that BRAFV600E-induced adenomas display the properties of ATIs [9, 17] (Fig. 3A, B). Although the adenomas showed high levels of ppERK and Ki67, which are indicative of the BRAFV600E-driven proliferative wave (Fig. 3C, D), they also showed increased DNA damage compared to controls (Fig. 3E). Notably, as occurred in normal alveoli, even in the adenomas, BRAFV600E-induced DNA damage is not telomeric (Supplementary Fig. 5B). Tumors also showed increased expression of CC3 and p21CIP1, whereas p53 cells were increased by 500-fold compared to control, thus suggesting apoptosis, p21 and p53 induction in the adenomas (Fig. 3F–H). Moreover, we detected enriched protein levels of the senescent markers p15INK4b, p16INK4a and p27KIP1 (Supplementary Fig. 4), as well as in the occurrence of p16INK4a- and p19ARF-positive cells (Fig. 3I, J), therefore indicating that senescence is taking place in some of the adenoma cells. Collectively, these findings indicate that BRAFV600E-driven adenomas are composed by both proliferating and senescent cells. 

Albeit in BRAFV600E mice both non-tumor alveolar parenchyma and adenomas showed increased p21CIP1 levels compared to controls (Fig. 2H, Fig. 3G, Supplementary Fig. 4), neither smaller nor larger adenomas show significant changes in p21CIP1 levels compared to non-tumor BRAFV600E alveolar zones (Supplementary Fig. 6A). Nevertheless, only adenomas showed p53, p16INK4a, and p19ARF upregulation (Fig. 2I–K, Fig. 3H–J). Thus, we hypothesized that p21CIP1 may be activated via p53 only in adenomas and that alternative mechanisms might be employed to induce p21CIP1 in BRAFV600E-challenged non-tumor alveoli which might not reflect the activation of classical OIS.

BRAFV600E is known to activate p21CIP1 expression via E2F transcription factors in vitro, upon cyclin-dependent kinases (CDKs)-mediated Rb inactivation [22, 23]. Nevertheless, no alterations in the percentage of cells showing the inactivated phosphorylated version of Rb was observed either between BRAFV600E non-tumor areas and adenomas or between BRAFV600E and control mice (Supplementary Fig. 6B), thus suggesting that BRAFV600E-mediated p21CIP1 activation in the alveolar parenchyma may not rely on pRb/E2F axis.

The RAS-pathway also induces small mother against decapentaplegic-3 (SMAD3) [24, 25] and STAT3 [26, 27], two transcriptions factors whose phosphorylated versions (pSMAD3, pSTAT3) activate p21CIP1 gene [28]. Unexpectedly, we found no changes in the percentage of pSMAD3-positive cells among the lung and potentially suggesting that p21CIP1 may be induced via STAT3 in BRAFV600E lung parenchyma.
Fig. 1 Early effects of ubiquitous expression of BRAF<sup>V600E</sup> in liver and spleen. A–E Representative images and quantifications showing A H&E staining, B ppERK, C γH2AX, D CC3 and E p21<sup>CIP1</sup> immunostainings in liver sections from BRAF<sup>V600E</sup> and control mice. F–J Representative images and quantifications showing F H&E staining, G PPERK, H CC3, I γH2AX and J p21<sup>CIP1</sup> immunostainings in spleen sections from BRAF<sup>V600E</sup> and control mice. Quantifications were performed on five different areas of the sections in a random way. Data are expressed as mean ± SEM; n = animals per group. *P < 0.05; **P < 0.01; ***P < 0.001, ns = not significant. (T Student’s test unpaired). Arrows point to selected positive cells for the indicated marker. Legend for figure F: WP = White Pulp, RD = Red Pulp; the white dashed line marks the boundary between white and red pulps. The arrows point to apoptotic bodies. Insets: magnifications of areas inside dashed squares.
Fig. 2  Early effects of BRAFV600E expression in the alveolar parenchyma. Representative images and quantifications showing A–C H&E staining, D ppERK, E Ki67, F CC3, G γH2AX, H p21CIP1, I p53, J p16INK4a, K p19ARF immunostainings in lung sections from BRAFV600E and control mice. Quantifications were performed on five different areas of the sections in a random way. Data are expressed as mean ± SEM; n = animals per group. *P < 0.05; **P < 0.01; ***P < 0.001, ns = not significant. (T Student’s test unpaired). Arrows point to selected positive cells for the indicated marker. Legend for figure A: Br: bronchus, Av: Alveolus, Nd: hyperplastic nodule, Hp: hyperplastic alveolar epithelium. Legend for I: the arrows point to neutrophils. Figures B and C represent the quantifications of the nodules shown in A. Insets: magnifications of areas inside dashed squares.
Fig. 3  Effects of expression of BRAFV600E in hyperplastic nodules. Representative images (top) and quantifications (bottom) showing H&E staining, A SPC, B CC10, C ppERK, D Ki67, E γH2AX, F CC3, G p21CIP1, H p53, I p16INK4, J p19ARF immunostainings in lung sections from BRAFV600E and control mice. Quantifications were performed on four different random areas of at least four hyperplastic nodules sections and four different random areas of uninuced alveolar parenchyma. Data are expressed as mean ± SEM; n represents respectively the number of animals per group in “normal epithelium” samples and the number of tumors in the “hyperplastic nodules” samples. For each condition at least 4 mice were used. *P < 0.05; **P < 0.01; ***P < 0.001, ns = not significant (T Student’s test unpaired). Arrows point to selected positive cells for the indicated marker. Insets: magnifications of areas inside dashed squares.
Molecular differences between BRAF<sup>V600E</sup>-challenged tumor and non-tumor ATIIIs
To better analyze the differential response elicited by BRAF<sup>V600E</sup> in ATIIIs, we checked whether BRAF<sup>V600E</sup> might affect the proliferation index of non-tumor and adenoma ATIIIs. Double immunohistochemistry stainings with SPC and Ki67 markers revealed that upon BRAF<sup>V600E</sup> activation, albeit both non-tumor and tumor ATIIIs from BRAF<sup>V600E</sup> mice tended to display a higher proliferation index, we found no significant changes in the number of SPC<sup>+</sup> Ki67<sup>+</sup> cells (Fig. 4A). Consistently, the percentage
of ATII expressing pRb, another hallmark of cell cycle progression, among adenomas and non-tumor alveolar areas from both BRAFV600E and control mice was not affected (Fig. 4B), therefore enforcing the evidence that acute BRAFV600E activation does not result in drastic changes in the expression of proliferative markers in ATII. Interestingly, both non-tumor and tumor BRAFV600E ATII displayed increased DNA damage (Fig. 4C). Remarkably, only non-tumor BRAFV600E ATII showed an increment in p21CIP1 expression compared to both controls and adenoma ATII (Fig. 4D), thus indicating that the majority of p21CIP1-positive cells in adenomas are not SPC+ ATII and that the oncogenic challenge elicits a rapid p21CIP1 activation in ATII before they can give rise to adenomas (Supplementary Fig. 6A). Surprisingly, whereas there was no alteration in p53 expression in non-tumor BRAFV600E ATII, we observed a robust p53 induction in adenomas (Fig. 4E), thus suggesting that p21CIP1 activation in non-tumor ATII does not rely on p53 and that p53 induction in adenoma ATII promptly results in p21CIP1 expression. Moreover, double staining experiments by using SPC marker combined with either pSMAD3 (Supplementary Fig. 7) or pSTAT3 (Fig. 4F) showed a drastic increment in pSTAT3, but not in pSMAD3, in BRAFV600E mice compared to control, thus confirming that 1) the BRAFV600E-dependent increase of pSTAT3 in the lungs (Supplementary Fig. 6D) is ascribable to pSTAT3 enrichment in ATII and 2) arguing for the possibility that pSTAT3 activation may be uncoupled from cell proliferation in BRAFV600E-challenged ATII, at least at the specific stages of oncogene-induced cell transformation analyzed (Fig. 4A, B, F).

Altogether, these findings indicate that albeit BRAFV600E induces DNA damage as well as pSTAT3 in ATII outside and inside the adenomas, it results in differential p21CIP1 and p53 expression in non-tumor and adenoma ATII.

**Early effects of BRAFV600E in bronchial/bronchiolar epithelium and Club cells**

We next studied the early effects of BRAFV600E expression in bronchi/bronchioles. Following BRAFV600E activation, bronchial/bronchiolar cells showed a significant loss of cells positive for the specific CC marker CC10 (Club cell secretory protein 10KDa) (Fig. 5A). Furthermore, intensity of CC10 staining was also significantly reduced (Supplementary Fig. 8A). Concomitantly, SPC+ intrabronchial cells were drastically increased (Fig. 5B), thus suggesting that BRAFV600E induces transdifferentiation of CCs into ATII. Moreover, an increment in Ki67-positive cells (Fig. 5C) was accompanied by respectively a 2-fold and 8-fold increase in the intensity of cyclin D1 staining and in the number of pRb-positive cells in BRAFV600E bronchi/bronchioles compared to controls (Fig. 5D, E), therefore indicating a dramatic stimulation of cell cycle progression. Simultaneously, BRAFV600E mice displayed a 2-fold increase of γH2AX-positive cells (Fig. 5F) and although they tended to display more p53+ cells, such an increase did not reach statistical significance. Nevertheless, p21CIP1- and CC3-positive bronchial/bronchiolar BRAFV600E cells were enriched by 15-fold and 2-fold respectively compared to controls (Fig. 5H, I), thus suggesting that the dramatic proliferative cues observed upon BRAFV600E challenge culminates in a robust p53-independent p21-mediated cell cycle arrest and cell death. Intriguingly, there were no changes in the frequency of p16INK4a- and p19ARF+ positive cells (Fig. 5J, K), thus suggesting that p21CIP1 increase is not coupled to other bronchi/bronchiolar senescence markers [32].

To investigate the early BRAFV600E-driven responses in CCs, we first checked the proliferation index of BRAFV600E CCs. Contrary to ATII, double immunohistochemistry staining with CC10 and Ki67 markers revealed that BRAFV600E CCs showed a dramatic 20-fold and 15-fold increase respectively in Ki67 and pRb (Fig. 6A, B), thus confirming the finding that CCs are sensitive to BRAFV600E-mediated proliferation stimulation. However, additional double immunostainings with CC10, γH2AX and p21CIP1 markers revealed that BRAFV600E CCs showed respectively a 5.4-fold and 10-fold increase in γH2AX- and p21-positive cells compared to controls (Fig. 6C, D), therefore enforcing the evidence that BRAFV600E also elicits a dramatic induction of DNA damage and p21CIP1 in CCs.

Interestingly, we found the same expression pattern described above also in SPC+ CCs. Indeed, transdifferentiating BRAFV600E CCs were positive for Ki67 and pRb (Fig. 6E, F), as well as for γH2AX and p21CIP1 markers (Fig. 6G, H), thus indicating that the BRAFV600E-mediated proliferation stimulation coexists with the cytotoxic response during the early steps of CC-to-ATII transdifferentiation. It is worth pointing out that bronchial/bronchiolar cells staining positive or negative for SPC show the same frequency in p21CIP1-, γH2AX-, Ki67-, pRb-positive cells (Supplementary Fig. 8B–E), thus suggesting that the increment respectively in p21CIP1 expression, DNA damage and proliferation may not affect the onset of the transdifferentiation process.

Altogether, these findings unveil that upon BRAFV600E-challenge CCs transdifferentiate and massively activate a robust cell cycle progression signaling which rapidly culminates in cell cycle inhibition and apoptosis.

**p21CIP1 activation in lungs is the consequence of acute tamoxifen-mediated BRAFV600E induction**

Next, we ruled out the possibility that p53-independent p21CIP1 activation in bronchi/bronchioles and in non-tumor alveolar parenchyma might be ascribable to a prolonged effect of BRAFV600E chronic activation, which may be spontaneously occurred at some earlier time-points before tamoxifen injections. For this purpose, concomitantly with tamoxifen-treated BRAFV600E mice, we also analyzed the lungs of 10–11 weeks old BRAFV600E mice without previous tamoxifen treatment. Remarkably, although such untreated mice showed spontaneous lung adenomas expressing high levels of p21CIP1 and p53, we found no differences in both these proteins in non-tumor alveolar parenchyma of BRAFV600E mice compared to untreated age-matched controls (Supplementary Fig. 9A, B). Similaly, we observed no change in either Ki67 or p21CIP1 in bronchi/bronchioles compared to controls (Supplementary Fig. 9C, D), thus enforcing the evidence that the proliferation induction and p21CIP1 activation in non-tumor alveolar parenchyma and in bronchi/bronchioles observed in tamoxifen-treated BRAFV600E mice is an immediate and acute effect of BRAFV600E expression rather than a cumulative effect over time of random events of Cre-dependent recombination of...
Fig. 5  Analysis of the early molecular response of BRAFV600E induction in bronchial/bronchiolar epithelium. A–K Representative images (top) and quantifications (bottom) showing bronchial/bronchiolar epithelial cells staining positive for A CC10, B SPC, C Ki67, D cyclin D1, E pRb, F γH2AX, G p53, H p21^{CIP1}, I CC3, J p16^{INK4a}, K p19^{ARF} immunostainings in lung sections from BRAFV600E and control mice. Quantifications were performed on at least five different areas of the lung sections in a random way. Data are expressed as mean ± SEM; n = animals per group. *P < 0.05; **P < 0.01; ***P < 0.001, ns = not significant. (T Student’s test unpaired). Arrows point to selected positive cells for the indicated marker. Insets: magnifications of areas inside dashed squares. Asterisks in G indicate an unspecific signal of the antibody.
Fig. 6  Analysis of the early molecular response of BRAF\textsuperscript{V600E} induction in Club cells. A–D  Representative images (top) and quantifications (bottom) showing CC10\textsuperscript{+} cells staining positive for A Ki67, B pRb, C γH2AX, D p21\textsuperscript{CIP1} double immunostainings in lung sections from BRAF\textsuperscript{V600E} and control mice. E–H  Representative images (top) and quantifications (bottom) showing bronchial/bronchiolar SPC\textsuperscript{+} cells staining positive for E Ki67, F pRb, G γH2AX, H p21\textsuperscript{CIP1} double immunostainings in lung sections from BRAF\textsuperscript{V600E} and control mice. Quantifications were performed on at least five different areas of the lung sections in a random way. Data are expressed as mean ± SEM; n = animals per group. *P < 0.05; **P < 0.01; ***P < 0.001, ns = not significant. (T Student’s test unpaired). Arrows point to selected positive cells for the indicated marker. Insets: magnifications of areas inside dashed squares.
**Fig. 7**  **BRAF**<sup>V600E</sup> induction results in an overall M2-like macrophages increase in the lung. **A–C** Representative images (on the left) and quantifications (on the right) showing F4/80<sup>+</sup> cells staining positive for **A** pSTAT3, **B** PPARγ, **C** c-MYC immunostainings in alveolar parenchyma and adenoma sections from **BRAF**<sup>V600E</sup> and control mice. **D–F** Representative images (top) and quantifications (bottom) showing F4/80<sup>+</sup> cells staining positive for **D** pSTAT3, **E** PPARγ, **F** c-MYC double immunostainings in bronchi/bronchiolar parenchyma of lung sections from **BRAF**<sup>V600E</sup> and control mice. Quantifications were performed on at least five different areas of the lung sections in a random way. Data are expressed as mean ± SEM; *n* = animals per group. *P* < 0.05; **P** < 0.01; ***P*** < 0.001, ns = not significant. (ANOVA test with Dunnet post-hoc correction (A, B, C), T Student’s test unpaired (D, E, F)). Arrows point to selected positive cells for the indicated marker. Insets: magnifications of areas inside dashed squares.
Fig. 8  **BRAFV600E induction results in ROS generation in the spleen but not in the lungs, liver or thyroids.** Representative images (top) and quantifications (bottom) showing stainings positive for **A–D** 4-hydroxy-2-nonenal and **E–H** 8-hydroxy-2′-deoxyguanosine respectively in spleen (**A, E**), lung (**B, F**), liver (**C, G**) and thyroids (**D, H**) sections from **BRAFV600E** and control mice. Quantifications were performed on at least five different areas of tissue sections in a random way. Data are expressed as mean ± SEM; n = animals per group. *P < 0.05; **P < 0.01; ***P < 0.001, ns = not significant. (T Student’s test unpaired). Arrows point to selected positive cells for the indicated marker. Insets: magnifications of areas inside dashed squares.
BRAFV600E allele which may lastly result in a basal/chronic BRAFV600E-activation.

Early effects of BRAFV600E expression on leukocytes in lungs

We also checked whether BRAFV600E might elicit acute lung inflammation. Interestingly, 4 days after BRAFV600E-induction, we found a dramatic infiltration of neutrophils, harboring the characteristic multilobed nuclei and staining positive for the neutrophil marker myeloperoxidase (MPO) (Supplementary Fig. 10A, B) in the alveolar parenchyma of BRAFV600E mice but not in controls. Nevertheless, we found no enrichment of cells positive for the monocyte/macrophagic marker F4/80 in the alveoli (Supplementary Fig. 10C). Furthermore, the occurrence of CD4+ T-lymphocytes in BRAFV600E mice was reduced twice compared to control (Supplementary Fig. 10D), thus indicating that BRAFV600E ubiquitous expression induces an immediate alveolar infiltration of neutrophils as well as a loss in CD4+ lymphocytes without affecting monocyctic/macrophagic lineage.

Conversely, albeit F4/80+ cells were increased in BRAFV600E bronchi/bronchioles compared to controls (Supplementary Fig. 10E), neither neutrophils nor CD4+ lymphocytes frequencies were perturbed (Supplementary Fig. 10F, G), thus suggesting that BRAFV600E triggers an immediate bronchial infiltration specifically of F4/80+ cells. Similarly, also adenosins displayed a specific enrichment in F4/80+ cells (Supplementary Fig. 10H), but not in either MPO- or CD4-positive cells (Supplementary Fig. 10I, J), thus possibly indicating that BRAFV600E-driven adenosins may preferentially recruit macrophages rather than neutrophils or lymphocytes.

Furthermore, double immunostainings revealed that the percentages of F4/80+ cells positive for the anti-inflammatory M2 macrophagic markers pSTAT3 [33–35] peroxisome-activated proliferator receptor-γ (PPARγ) [33, 36, 37] and c-MYC [33, 38, 39] were globally enhanced in both bronchial/bronchiolar and alveolar parenchyma of mutant mice as well as in adenosins compared to control (Fig. 7A–F), thus indicating a BRAFV600E-mediated overall increase in pro-tumoral macrophages in lungs. Conversely, F4/80+ cells positive for the pro-inflammatory M1 macrophagic marker hypoxia inducible factor-1α (HIF1α) [35, 40–42] were significantly increased only in adenosins, but not in either alveolar or bronchial/bronchiolar parenchyma of mutant mice compared to controls (Supplementary Fig. 11A, B), thus giving further confirmation on the ability of BRAFV600E to orchestrate the immediate recruitment of specific leukocytes in different pulmonary epithelia.

BRAFV600E-expression induces ROS generation in vivo

We finally checked whether BRAFV600E might result in reactive oxygen species (ROS) production in vivo. Interestingly, immunostaining experiments revealed increased levels of the ROS markers 4-hydroxy-2-nonenal [43–46] and 8-hydroxy-2’-deoxyguanosine [43, 47–50] in the spleen but not in lungs, liver, or thyroids of BRAFV600E mice compared to controls, thus suggesting that, albeit the BRAFV600E-induced DNA damage may be ROS-dependent in vivo as well as in vitro [51], additional mechanisms might be involved in BRAFV600E-dependent DNA damage induction in lungs and liver (Fig. 8A–H).

DISCUSSION

Here we reported that ubiquitous acute BRAFV600E expression leads to a rapidly lethal sickness characterized by general weakness and weight loss. This outcome may be partially ascribable to lung acute inflammation and to a rapid energetic depletion likely attributable to BRAFV600E-triggered lipolysis, a process which is mediated by activated RAS-pathway [52]. We also observed that BRAFV600E activation induces microvesicular HS, a condition where an excess of fatty acids is accumulated into hepatocytes. Thus, it is conceivable that the BRAFV600E-triggered hepatic fat accumulation may be ascribable to a likely increase of serum fatty acids consequent to the RAS-pathway-driven lipolysis in adipocytes [52].

Our findings provided the first evidence in vivo that acute BRAFV600E expression elicits instant DNA damage in an organ-specific fashion. p21CIP1 [53], which may be activated by p53 upon genotoxic insults [54] and by oncogene activation via pRb/E2F [22], promotes cell cycle arrest and senescence [22] by inhibiting CDKs [22]. Nevertheless, despite BRAFV600E induces both DNA damage and p21CIP1 activation in vitro [51, 55] as well as in senescent lung adenosins [17], we found no differences in p21CIP1 levels either in liver or spleen upon BRAFV600E expression. Thus, we unveiled that, in the organs where BRAFV600E rapidly induces robust DNA damage, an immediate p21CIP1 activation does not occur in a generalized manner. Such observations suggest that p21CIP1 may be activated only at later time points in the presence of a constant oncogenic stimulus, or that BRAFV600E ability to induce DNA damage in certain tissues/organisms may be uncoupled from p21CIP1 activation.

We also uncovered that BRAFV600E expression yields a differential response of cell cycle/senescence-associated proteins in ATIs. Indeed, albeit all the BRAFV600E-challenged ATIs showed increased DNA damage, while non-transformed ATIs express p21CIP1 in the absence of p53 activation, tumorogenic ATIs displayed enhanced p53 expression coupled with a significant p21CIP1 reduction compared to non-tumor cells. The strike differences in such expression patterns argue for the possibility that non-tumor ATIs may represent an early stage of tumor development in which a rapid p53-independent-p21CIP1 induction might be an immediate barrier to cancer initiation which, at a certain point, may be repressed thus allowing cell proliferation. Alternatively, some ATIs might be naturally more refractory to an immediate BRAFV600E-dependent p21CIP1 activation and be more prone to give rise to adenosins, which lastly, during the onset of senescence, will activate p53. Thus, the evidence that p53 induction in adenoma ATIs is accompanied by no alteration in p21 expression might be due to the fact the p53 activation is at an initial stage and therefore it has not reached yet the threshold necessary for an efficient p21CIP1 gene activation.

Albeit it has been established that BRAFV600E promotes senescence or apoptosis without yielding any previous proliferative stimulation in vitro [51, 56], BRAFV600E expression in vivo results in an initial hyperplasic wave lastly culminating in the onset of tumor senescence, characterized by DNA damage [17], and p21CIP1 [17], p16INK4a [9], p19ARF [21] and p53 [57] expression. Nevertheless, we unveiled that in BRAFV600E mice, non-tumor alveolar parenchyma showed rapid p21CIP1 induction, which is not accompanied by activation of any among the well-known proteins associated to OIS in lungs [9, 17], thus arguing for the possibility that such immediate tumor-suppression response may differ from classical OIS. Indeed, such p53/pRb-independent p21CIP1 activation in non-tumor alveolar parenchyma may reflect a rapid BRAFV600E-mediated cytotoxic response reminiscent to that observed in CCs (see below), thus suggesting that albeit BRAFV600E ATIs are prone to give rise to adenosins, yet there is an immediate cell cycle arrest in a minority of the challenged ATIs.

In contrast to the extensive research work conducted in ATIs, very little is known about the early molecular effects of BRAFV600E in bronchi/bronchioles. Here we unveiled that BRAFV600E initiates CCs transdifferentiation into ATIs. Concomitantly, we observed a proliferation stimulation resulting in DNA damage, cell cycle arrest and cell death. Both proliferative and cytotoxic responses are much more exacerbated in CCs compared to ATIs, which can account for the well-known characteristic of CCs to be recalcitrant to RAS-pathway stimulation [58, 59].
We also uncovered that BRAFV600E rapidly elicits an acute inflammatory response in lungs by differentially recruiting neutrophils in the alveoli and F4/80-positive cells in bronchi/bronchioles and adenomas. The generation of GEMMs in which BRAFV600E expression is driven specifically in neutrophils and alveolar macrophages will provide helpful insights into the mechanisms underlying the BRAFV600E pleiotropic effect on leukocytes in lungs.

**MATERIALS AND METHODS**

**Murine models**

BRAFV600E+/- mice were described previously [9, 10, 12]. This mouse model was crossed with a mouse strain carrying ubiquitously expressed, tamoxifen-activated recombinase, UBC-CreERT2 [18], to generate UBC-CreERT2/-;BRAFV600E+/- mice. These mice received intraperitoneal injections of 4-hydroxy tamoxifen (Sigma H6278) (1 mg/injection, 3–4 injections, 1 injection per day for 3 or 4 consecutive days).

All mice were maintained at the Spanish National Cancer Research Centre under specific pathogen-free conditions in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA). All animal experiments were approved by our Institutional Animal Care and Use Committee (IACUC) and by the Ethical Committee for animal experimentation (CEbyBA) (PROEX 106/72/20). We followed the Reporting in Vivo Experiments (ARRIVE) guidelines developed by the National Centre for the Replacement, Replacement & Reduction of Animals in Research (NC3Rs). Both male and female mice, with mixed background, were used for the experiments.

**Immuno-FISH**

Immuno-FISH was performed in formalin-fixed paraffin-embedded mouse lung sections to identify telomeric induced foci (TIF) as previously described [60, 61]. Immuno-FISH was performed as follows: after deparaffinization and citrate antigen retrieval, samples were permeabilized for 3 h in PBSX1-0.5% Triton, blocked for 2 h with 10% fetal bovine serum and 1 h with 5% BSA in PBSX1-0.1% Triton-10mM Glycine (PBSTG), and immunofluorescence with anti-S3BP1 rabbit antibody (Novus Biologicals NB100-304) diluted 1:500 was performed. Samples were incubated O/N at 4 °C with the primary antibody in PBSTG. Slides were further washed with PBSTG and incubated with 488-Alexa labeled secondary antibody in DAKO antibody diluent reagent (S3022). After immunofluorescence, samples were fixed for 20 min in 4% paraformaldehyde in PBSX1 and followed by FISH. Briefly, samples were washed with PBS and dehydrated in Ethanol 70, 90 and 100%. The samples were then incubated with a telomeric PNA probe labeled with CY3 (Panagene) in 50% formamide for 30 min, washed in the presence of 50% formamide and counterstained with DAPI. TIF were identified by colocalization of CY3 and 488-Alexa double positive spots. Confocal microscopy was performed at room temperature with a laser-scanning fluorescence microscope (TCS SP5; Leica) using a Plan Apo 63A-1.40 NA oil immersion objective (HCX; Leica). Maximal projection of Z-stack images generated using advanced fluorescence software (LAS) was analyzed with the Definiens XD software package. The DAPI images were used to detect signals inside the nuclei.

**Immunohistochemistry analyses in tissue sections**

Tissues were fixed in 10% buffered formalin, embedded in paraffin wax and sectioned at 5 mm. For histological examination sections were stained with hematoxylin and eosin, according to standard procedures as previously described [62]. CC3 Cleaved Caspase 3 Asp175 (Cell Signaling Technology 9661), CC10 (Santa Cruz Biotechnology sc-9772), CD4 (Cell Signaling Technology 25229), prosurfactant protein C (millipore AB3786), p21 (29G RevMAB Biosciences USA), anti-actin 1:5000 (A5441, Sigma), anti-BRAF 1:200 (F-7, sc-5284, Santa Cruz), anti-BRAFV600E 1:300 (31-1042-00 RevMAB Biosciences USA), anti-h2A2X Ser139 1:5000 (Merck 05-636), homemade rat anti-p15INK4b clone PAT65B (neat supernatant), homemade rat anti-p16INK4a clone PLO33B (neat supernatant), homemade rat anti-p19ARF clone PIL346C (neat supernatant), homemade rat anti-p27KIP1 clone HUGO921 (neat supernatant), homemade rat anti-p53 clone POE316A (neat supernatant). Antibody binding was detected after incubation with a secondary antibody coupled to horseradish peroxidase using chemiluminescence with ECL detection KIT (GE Healthcare) with Chemidoc (Biorad). For the quantification, protein-band intensities were quantitated by densitometric analysis with ImageLab software (Biorad). The total levels of each protein analyzed have been normalized versus actin and the mean of the specific protein/actin ratio deriving from at least 3 different replicates has been used to generate the chart as previously described [63].

**Quantification and statistical analysis**

Immunohistochemistry quantifications were performed by direct cell counting by using Zen1.1 Zeiss and Image J softwares. Immuno-FISH quantitations were carried out by direct counting of cells and 53BP1 foci on single planes of each z-stack by using LAS X software (Leica). Unpaired Student’s t-test (two-tailed), ANOVA followed by Tukey’s post-hoc correction, Log Rank test were used to determine statistical significance. P-values of less than 0.05 were considered significant. *p < 0.05, **p < 0.01, ***p < 0.001. Statistical analysis was performed using Microsoft Excel 2016 and GraphPad/PRISM8. For animal studies no blinding/randomization was done/used. The number of mice per each experiment as well as the size of the experiments were obtained by performing power analysis.

**DATA AVAILABILITY**

The datasets and other information that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS
MAB conceived the idea. MAB and GB designed the experiments. GB, PL, SPH and MY performed the experiments. MAB and GB wrote the manuscript. RS aided with mice treatments.

COMPETING INTERESTS
The authors declare no competing interests.

ETHICS
All authors approved and directly participated in the planning and/or execution of the experiments and/or analysis of the data presented herein. The animal studies were conducted in accordance with the Animal Use Protocol approved by the Institutional Animal Care and Use Committee (IACUC) and by the Ethical Committee for animal experimentation (CEHyBA) (PROEX 106.7/20).

CONSENT FOR PUBLICATION
All authors have provided their consent for publication.

ADDITIONAL INFORMATION
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Correspondence and requests for materials should be addressed to Maria A. Blasco.

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