Identification of a novel *PHIP::BRAF* gene fusion in infantile fibrosarcoma

Authors: Boutaina Boulouadnine[1], Laurence de Villenfagne[1], Christine Galant[2], Raphael Sciot[3], Bénédicte Brichard[4], Jean-Baptiste Demoulin[1]*

Affiliations:

1. de Duve Institute, Université catholique de Louvain, Brussels, Belgium.

2. Department of Pathology, Cliniques Universitaires Saint-Luc and Université catholique de Louvain, Brussels, BE-1200, Belgium.

3. Department of Pathology, University Hospital Leuven and KULeuven, Leuven, BE-3000, Belgium.

4. Department of Pediatric Hematology and Oncology, Cliniques Universitaires Saint-Luc and Université catholique de Louvain, Brussels, BE-1200, Belgium.

***Corresponding Author:** Professor Jean-Baptiste Demoulin, de Duve Institute, UCLouvain, Avenue Hippocrate 75, Box B1.74.05, Brussels 1200, Belgium ; e-mail: jb.demoulin@uclouvain.be.

Conflict of interest

None declared.

Abbreviated title: *PHIP::BRAF* fusion in infantile fibrosarcoma

Keywords: Infantile fibrosarcoma, BRAF gene fusion, trametinib, tyrosine kinase inhibitor

ABSTRACT

Introduction

The *ETV6::NTRK3* fusion is the most common gene alteration in infantile fibrosarcoma, a soft tissue tumor affecting patients under two years of age. Less frequently, these tumors harbor fusions of genes encoding other kinases, such as *BRAF*, which activates MEK in the mitogen-activated protein kinase pathway. The identification and characterization of these oncogenes is crucial to facilitate diagnosis, validate new treatments and better understand the pathophysiology of these neoplasms.

Methods

Herein, we analyzed an *ETV6::NTRK3*-negative infantile fibrosarcoma from a 5-day-old patient by RNA-sequencing to identify new fusion transcripts. Functional exploration of the fusion of interest was performed by *in vitro* assays to study its activity, oncogenicity and sensitivity to the MEK inhibitor trametinib.

Results

We identified a novel fusion involving the *PHIP* and *BRAF* genes. The corresponding fusion protein constitutively activated the mitogen-activated protein kinase pathway, resulting in fibroblast transformation. Treatment of transfected cells with trametinib effectively inhibited signaling by *PHIP::BRAF*.

Conclusion

PHIP::BRAF is a novel fusion oncogene that can be targeted by trametinib in infantile fibrosarcoma.

INTRODUCTION

Infantile fibrosarcoma (IFS) is a malignant fibroblastic tumor that affects children under 2 years of age.[1] IFS most commonly presents as a tumor mass in the extremities, but also in the trunk, head and neck.[1] The first-line therapy is surgical resection, which may be supplemented or replaced by chemotherapy when resection is incomplete or impossible. The risk of metastasis is low, but recurrence occurs in up to 40% of cases.[2]

Seventy percent of IFS harbor the canonical t(12;15)(p13;q25) translocation inducing the production of an ETV6::NTRK3 fusion protein, which triggers the mitogen-activated protein kinase (MAPK) pathway and promotes cell proliferation.[3] A subset of IFS and mesenchymal tumors with IFS-like morphology are driven by *NTRK3* fusion to other partners or by fusions involving other protein kinases associated with the MAPK cascade, such as *NTRK1*, *NTRK2*, *MET*, *BRAF*, and *RAF1*.[4]

The identification of these fusion kinases opened the possibility of targeted therapies for IFS. Solid tumors harboring *NTRK1*, *NTRK2* and *NTRK3* fusions can be targeted with the FDA-approved kinase inhibitors larotrectinib and entrectinib.[5] Similarly, an IFS-like tumor with a fusion involving the *MET* receptor tyrosine kinase was effectively treated with cabozantinib.[6] Inhibition of the MAPK pathway can be achieved through trametinib, an allosteric MEK inhibitor that was approved by FDA for the treatment of melanoma and metastatic non-small cell lung cancer harboring BRAF mutations. In the setting of relapsing undifferentiated sarcoma, the use of trametinib was also shown to be effective.[7]

Here, we report a novel fusion gene involving *PHIP* and *BRAF* with oncogenic activity in fibrosarcoma.

MATERIAL AND METHODS

Study design

This study was approved by the institutional ethical review board of Université catholique de Louvain. Sample analysis was performed after informed consent of the parents.

Transcriptome analysis and fusion identification

Extraction of total RNA from optimal cutting temperature compound-embedded tissue was performed using TriPure (Roche, Switzerland). The library was constructed using TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA) and paired-end sequenced on an Illumina platform to produce 100 million reads of 150 bp length (Macrogen-Europe). The verification of the raw RNA-seq read quality was performed on FastQC (version 0.11.9). Reads were then aligned to the GRCh37 reference genome via STAR aligner (version 2.7.2d) in a two-mode process as described.[8] Fusion genes were called using STAR-Fusion (version 1.9.0) and FusionCatcher (version 1.10).[9,10] The selected gene fusion was detected by both softwares and had at least one JunctionReadCount and one SpanningFragCount. After reverse transcription of RNA, the presence of the gene fusion was validated by PCR amplification and Sanger sequencing of the breakpoint using the primers 5'-TCACCAAAGAAAAAGAAGCCC-3' and 5'-GTTAGTGAGCCAGGTAATGAGGC-3'.

Reagents and antibodies

The HA-tagged *PHIP::BRAF* coding sequence was introduced in pCDNA3 (GenScript, NJ, USA). The pCMV3-*BRAF* vector (HG11632-UT) was provided by Sino Biological (Beijing, China). Trametinib was purchased from Selleck Chemicals (Houston, TX, USA). Anti-BRAF antibody (clone 28E1) was purchased from

Proteintech (Chicago, IL, USA). Anti-HA Tag (clone C29F4) and anti-phospho-ERK1/2 (Thr202/Tyr204) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-ERK2 (EET) antibody was previously described.[11] Anti-β-actin antibody (clone AC-15) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and luciferase reporter assay

Human embryonic kidney (HEK) 293T and porcine aortic endothelial (PAE) cells were cultured in Dulbecco's modified Eagle medium (Lonza Bioscience) supplemented with fetal calf serum (10%), 50 U/mL penicillin and 50 µg/mL streptomycin.

PAE cells (50 000/well) were seeded in a 24-well plate. After 24 hours, cells were co-transfected with 3 plasmids: *PHIP::BRAF* or a control (250 ng), pEF1-βGal (250 ng), and pSRE-Luc (250 ng) using TurboFect Transfection Reagent (Thermo Fisher Scientific). After 4 hours, trametinib was added to the appropriate wells. Cells were lysed 24 hours after transfection, followed by luciferase activity measurement as previously described.[12] The luminescence intensity was divided by the β-galactosidase activity, used as an internal control.

Western blot

HEK293T (500 000 cells/well) were seeded in a 6-well plate. The next day, cells were transfected with *PHIP::BRAF* using the calcium phosphate method as previously described.[13] Four hours after transfection, cells were washed and fresh medium was added. The next day, trametinib was added to the appropriate conditions for 150 minutes. Cells were lysed and lysates were prepared as described elsewhere.[12] Membranes were revealed using the SuperSignal™ West Pico PLUS

Chemiluminescent Substrate (Thermo Fisher Scientific). Membrane reprobing was performed after stripping in 0.4 M NaOH for 7 minutes and 30 seconds.

Focus forming assay

NIH3T3 cells were transfected with 500 ng of empty vector or *PHIP::BRAF* with Lipofectamine 2000 (Thermo Fisher Scientific). Samples were prepared as previously described.[14] Foci were quantified using the Bio1D software (Vilber Lourmat, Marnela-Vallée, France).

RESULTS

Case presentation

A 5-day-old male patient was admitted to the hospital with a dorsal left paravertebral mass discovered at birth. He was the first child of a couple with no particular medical history. His birth weight was 2.5 kg and he had no dysmorphia. The lesion was stable in size since birth. The ultrasonography showed a subcutaneous nodule with regular contours, slightly lateralized to the left, measuring 3.8 x 3.5 x 1 cm, and developed at the expense of a left paravertebral muscle head. It did not extend into the spinal canal. MRI confirmed the dorsal soft tissue mass, showing a T2 hypersignal and a T1 isosignal, excluding a fatty mass. The initial diagnostic hypothesis was myofibromatosis or infantile fibrosarcoma. Complete surgical resection was performed at 7 days of life. At the time of submission of this report, the child was 24 months old and doing well with no sign of recurrence.

Histologic and molecular analyses

Histologic examination revealed a hypercellular lesion, characterized by a proliferation of small spindle cells with large nuclei (Figure 1A). Some mitoses were noted. Immunohistochemical examination was positive for alpha smooth muscle actin and BAF47, and negative for muscle markers. The morphological and immunohistochemical appearance was consistent with an infantile fibrosarcoma. FISH testing for an *ETV6* rearrangement was negative.

RNA sequencing was performed on total RNA extracted from the patient's tumor. Fusion calling identified a novel gene fusion, *PHIP::BRAF*, involving chromosomes 6 and 7 where the *PHIP* and *BRAF* genes are located, respectively. The predicted breakpoints were intronic and occurs after exon 24 of *PHIP* (NM_017934.7) and before exon 9 of *BRAF* (NM_004333.6), resulting in an in-frame fusion. The WD40 domain of PHIP and the kinase domain of BRAF were both retained in the fusion (Figure 1B). The presence of *PHIP::BRAF* was validated by RT-PCR amplification and Sanger sequencing of the breakpoint (Figure 1C and 1D).

To functionally characterize the fusion, the corresponding open reading frame was inserted into an expression vector. The fusion protein was expressed in HEK293T cells and was detected at the expected size of 153 kDa by Western blot (Figure 2A). We studied the ability of the fusion to activate the MAPK pathway by analyzing the phosphorylation of ERK-1 and -2 by Western blot. As shown in Figure 2A, the fusion induced constitutive phosphorylation of ERKs. Trametinib inhibited this phosphorylation at a 100 nM concentration. We also tested the signaling activity of the fusion by a luciferase reporter assay that is sensitive to serum response factor activation by MAPK. This assay confirmed MAPK activation and the effect of trametinib (Figure 2B).

Finally, we tested the ability of the fusion to transform mouse NIH3T3 fibroblasts in a classical foci-forming assay. Figure 2C shows that *PHIP::BRAF* induced the formation of foci in a statistically significant manner compared to the empty vector, suggesting that it acts as a *bona fide* oncogene.

DISCUSSION

In this report, we identified a new *PHIP::BRAF* gene fusion in a case of infantile fibrosarcoma. We demonstrated that the fusion is an oncogene that activates the MAPK pathway and transforms fibroblasts, supporting a role as a cancer driver gene in fibrosarcoma. Fusions involving *BRAF* as a 3' partner gene are found in a wide range of solid tumors.[15] They have been functionally characterized and their sensitivity to inhibitors was studied in cancers such as melanoma, thyroid cancer and pediatric pilocytic astrocytoma.[16-19] In these fusions, the BRAF kinase domain is activated mainly through the loss of its N-terminal CR1 autoinhibitory domain.[20] In IFS, several fusions involving BRAF have been reported, but not functionally characterized.[4,21,22] In addition, Wegert and colleagues identified intragenic rearrangements of *BRAF* involving a deletion of the CR1 domain associated with a duplication of exon 2.[23] The CR1 domain contains the RAS-binding domain that negatively regulates BRAF dimerization in the inactive state. Loss of this domain results in RAS-independent dimerization of BRAF through a motif located in the protein C-terminus.[19] The 5' fusion partner may also contain an oligomerization motif.[19] The PHIP protein is a proposed biomarker and promoter of metastasis in BRAF-wild-type melanoma.[24] The contribution, if any, of PHIP to the oncogenic effect of the fusion remains to be elucidated.

The routine clinical use of high-throughput sequencing technologies could improve the management of patients with IFS. Molecular biology and the search for fusions

could help in the differential diagnosis with related tumors, such as myofibromas. Furthermore, it could help validating a more specific treatment in cases that are surgically difficult to treat by identifying the genomic alteration at the origin of the disease, as an alternative to chemo- and radiotherapy. Although BRAF fusions are not responsive to first-generation BRAF inhibitors, such as vemurafenib and dabrafenib, targeting BRAF may be possible with pan-RAF inhibitors, such as LY3009120, which showed effectiveness in preclinical models.[25] As an alternative, we tested the sensitivity of the *PHIP::BRAF* fusion to trametinib which was effective in inhibiting the fusion activity. This inhibitor may be a therapeutic option for cases harboring BRAF fusions refractory to surgery, when complete resection of the tumor cannot be achieved.

Acknowledgements

This work was supported by a grant from the Belgian Foundation against Cancer. B.B. was supported by a fellowship from the Fonds de la Recherche Scientifique - FNRS (Belgium).

References

1. Chung EB, Enzinger FM. Infantile fibrosarcoma. *Cancer*. Aug 1976;38(2):729-39. doi:10.1002/1097-0142(197608)38:2<729::aid-cncr2820380216>3.0.co;2-z
2. Farmakis SG, Herman TE, Siegel MJ. Congenital infantile fibrosarcoma. *J Perinatol*. Apr 2014;34(4):329-30. doi:10.1038/jp.2013.164
3. Sheng WQ, Hisaoka M, Okamoto S, et al. Congenital-infantile fibrosarcoma. A clinicopathologic study of 10 cases and molecular detection of the ETV6-NTRK3 fusion transcripts using paraffin-embedded tissues. *Am J Clin Pathol*. Mar 2001;115(3):348-55. doi:10.1309/3H24-E7T7-V37G-AKKQ
4. Penning AJ, Al-Ibraheemi A, Michal M, et al. Novel BRAF gene fusions and activating point mutations in spindle cell sarcomas with histologic overlap with infantile fibrosarcoma. *Mod Pathol*. Aug 2021;34(8):1530-1540. doi:10.1038/s41379-021-00806-w
5. Orbach D, Sparber-Sauer M, Laetsch TW, et al. Spotlight on the treatment of infantile fibrosarcoma in the era of neurotrophic tropomyosin receptor kinase inhibitors: International consensus and remaining controversies. *Eur J Cancer*. Sep 2020;137:183-192. doi:10.1016/j.ejca.2020.06.028
6. Gupta A, Belsky JA, Schieffer KM, et al. Infantile fibrosarcoma-like tumor driven by novel RBPMS-MET fusion consolidated with cabozantinib. *Cold Spring Harb Mol Case Stud*. Oct 2020;6(5)doi:10.1101/mcs.a005645
7. Vairy S, Jouan L, Bilodeau M, et al. Novel PDE10A-BRAF Fusion With Concomitant NF1 Mutation Identified in an Undifferentiated Sarcoma of Infancy With Sustained Response to Trametinib. *JCO Precis Oncol*. Nov 2018;2:1-13. doi:10.1200/PO.18.00007
8. Dachy G, Fraitag S, Boulouadnine B, Cordi S, Demoulin JB. Novel COL4A1-VEGFD gene fusion in myofibroma. *J Cell Mol Med*. May 2021;25(9):4387-4394. doi:10.1111/jcmm.16502
9. Haas BJ, Dobin A, Li B, Stransky N, Pochet N, Regev A. Accuracy assessment of fusion transcript detection via read-mapping and de novo fusion transcript assembly-based methods. *Genome Biol*. Oct 21 2019;20(1):213. doi:10.1186/s13059-019-1842-9
10. Nicorici D, Şatalan M, Edgren H, et al. FusionCatcher – a tool for finding somatic fusion genes in paired-end RNA-sequencing data. *bioRxiv*. 2014-01-01 00:00:00 2014;
11. Lennartsson J, Blume-Jensen P, Hermanson M, Ponten E, Carlberg M, Ronnstrand L. Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-kit mediated activation of the Ras/MAP kinase pathway and c-fos induction. *Oncogene*. Sep 30 1999;18(40):5546-53. doi:10.1038/sj.onc.1202929
12. Arts FA, Velghe AI, Stevens M, Renauld JC, Essaghir A, Demoulin JB. Idiopathic basal ganglia calcification-associated PDGFRB mutations impair the receptor signalling. *J Cell Mol Med*. Jan 2015;19(1):239-48. doi:10.1111/jcmm.12443
13. Toffalini F, Kallin A, Vandenberghe P, et al. The fusion proteins TEL-PDGFRbeta and FIP1L1-PDGFRalpha escape ubiquitination and degradation. *Haematologica*. Aug 2009;94(8):1085-93. doi:10.3324/haematol.2008.001149
14. Arts FA, Chand D, Pecquet C, et al. PDGFRB mutants found in patients with familial infantile myofibromatosis or overgrowth syndrome are oncogenic and sensitive to imatinib. *Oncogene*. Jun 23 2016;35(25):3239-48. doi:10.1038/onc.2015.383
15. Ross JS, Wang K, Chmielecki J, et al. The distribution of BRAF gene fusions in solid tumors and response to targeted therapy. *Int J Cancer*. Feb 15 2016;138(4):881-90. doi:10.1002/ijc.29825
16. Sievert AJ, Lang SS, Boucher KL, et al. Paradoxical activation and RAF inhibitor resistance of BRAF protein kinase fusions characterizing pediatric astrocytomas. *Proc Natl Acad Sci U S A*. Apr 9 2013;110(15):5957-62. doi:10.1073/pnas.1219232110
17. Ricarte-Filho JC, Li S, Garcia-Rendueles ME, et al. Identification of kinase fusion oncogenes in post-Chernobyl radiation-induced thyroid cancers. *J Clin Invest*. Nov 2013;123(11):4935-44. doi:10.1172/JCI69766

18. Botton T, Talevich E, Mishra VK, et al. Genetic Heterogeneity of BRAF Fusion Kinases in Melanoma Affects Drug Responses. *Cell Rep*. Oct 15 2019;29(3):573-588 e7. doi:10.1016/j.celrep.2019.09.009
19. Hutchinson KE, Lipson D, Stephens PJ, et al. BRAF fusions define a distinct molecular subset of melanomas with potential sensitivity to MEK inhibition. *Clin Cancer Res*. Dec 15 2013;19(24):6696-702. doi:10.1158/1078-0432.CCR-13-1746
20. Botton T, Yeh I, Nelson T, et al. Recurrent BRAF kinase fusions in melanocytic tumors offer an opportunity for targeted therapy. *Pigment Cell Melanoma Res*. Nov 2013;26(6):845-51. doi:10.1111/pcmr.12148
21. Kao YC, Fletcher CDM, Alaggio R, et al. Recurrent BRAF Gene Fusions in a Subset of Pediatric Spindle Cell Sarcomas: Expanding the Genetic Spectrum of Tumors With Overlapping Features With Infantile Fibrosarcoma. *Am J Surg Pathol*. Jan 2018;42(1):28-38. doi:10.1097/PAS.0000000000000938
22. Hughes CE, Correa H, Benedetti DJ, Smith B, Sumegi J, Bridge J. Second Report of PDE10A-BRAF Fusion in Pediatric Spindle Cell Sarcoma With Infantile Fibrosarcoma-Like Morphology Suggesting PDE10A-BRAF Fusion Is a Recurrent Event. *Pediatr Dev Pathol*. Nov-Dec 2021;24(6):554-558. doi:10.1177/10935266211012186
23. Wegert J, Vokuhl C, Collord G, et al. Recurrent intragenic rearrangements of EGFR and BRAF in soft tissue tumors of infants. *Nat Commun*. Jun 18 2018;9(1):2378. doi:10.1038/s41467-018-04650-6
24. De Semir D, Nosrati M, Bezrookove V, et al. Pleckstrin homology domain-interacting protein (PHIP) as a marker and mediator of melanoma metastasis. *Proc Natl Acad Sci U S A*. May 1 2012;109(18):7067-72. doi:10.1073/pnas.1119949109
25. Jain P, Surrey LF, Straka J, et al. BRAF fusions in pediatric histiocytic neoplasms define distinct therapeutic responsiveness to RAF paradox breakers. *Pediatr Blood Cancer*. Jun 2021;68(6):e28933. doi:10.1002/pbc.28933

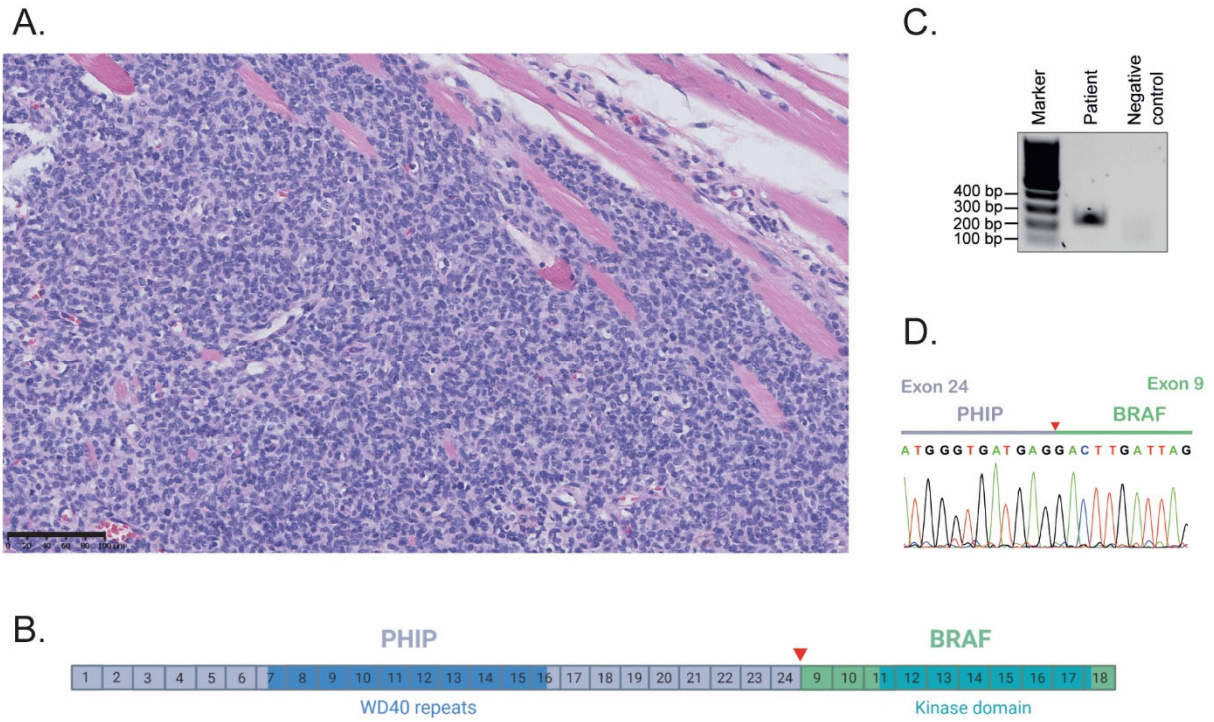

Figure 1. Identification of a novel *PHIP::BRAF* gene fusion in infantile fibrosarcoma. (**A**) HE staining of a tumor section, showing cell proliferation without particular architectural arrangement, pleomorphism and atypia. The lesion infiltrates the surrounding muscle (scale: 100 µm). (**B**) Schematic representation of the *PHIP::BRAF* transcript with its exon structure and key protein domains. The red arrow depicts the breakpoint. **(C**) PCR amplification of the *PHIP::BRAF* breakpoint (251 bp). The negative control consisted in cDNA from the HT1080 fibrosarcoma cell line. (**D**) Sanger sequencing results highlighting the breakpoint.

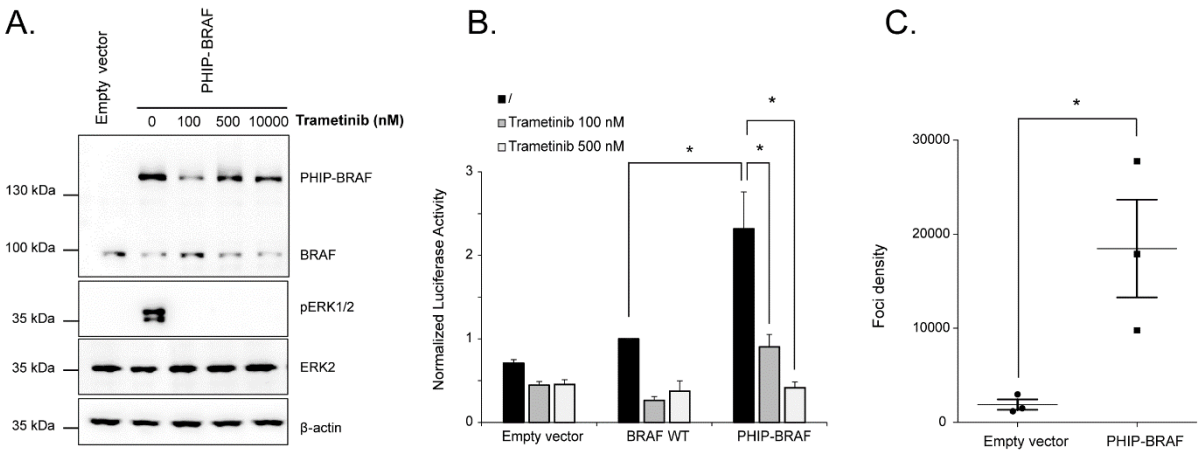

Figure 2. *PHIP::BRAF* activates the MAPK pathway and transforms fibroblasts. (**A**) HEK293T cells were transiently transfected with an empty vector or *PHIP::BRAF*. Cell lysates were analyzed by immunoblotting using antibodies as indicated. One representative experiment out of three is shown. (**B)** PAE cells were co-transfected with an empty vector, wild-type (WT) *BRAF* or *PHIP::BRAF* and with a luciferase reporter plasmid responding to the MAPK pathway. The histograms represent the mean of three independent experiments with S.E.M. (Student's t-test; *P < 0.05). Values were normalized to the WT condition. (**C)** NIH3T3 were transfected with an empty vector or *PHIP::BRAF* and treated with G418. After approximately two weeks, cells were fixed in methanol and colored with crystal violet. Results (mean of 3 independent experiments) with S.E.M. are shown in the scatter dot plot (Student's t-test; *P < 0.05). The dots indicate the value obtained in the individual experiments.