

The expression of the tumour suppressor HBP1 is down-regulated by growth factors via the PI3K/PKB/FOXO pathway

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Growth factors inactivate the FOXO (forkhead box O) transcription factors through PI3K (phosphoinositide 3-kinase) and PKB (protein kinase B). By comparing microarray data from multiple model systems, we identified HBP1 (high-mobility group-box protein 1) as a novel downstream target of this pathway. *HBP1* mRNA was down-regulated by PDGF (platelet-derived growth factor), FGF (fibroblast growth factor), PI3K and PKB, whereas it was up-regulated by FOXO factors. This observation was confirmed in human and murine fibroblasts as well as in cell lines derived from leukaemia, breast adenocarcinoma and colon carcinoma. Bioinformatics analysis led to the identification of a conserved consensus FOXO-binding site in the *HBP1* promoter. By luciferase activity assay and ChIP, we demonstrated that

FOXO bound to this site and regulated the *HBP1* promoter activity in a PI3K-dependent manner. Silencing of *HBP1* by shRNA increased the proliferation of human fibroblasts in response to growth factors, suggesting that HBP1 limits cell growth. Finally, by analysing a transcriptomics dataset from The Cancer Genome Atlas, we observed that HBP1 expression was lower in breast tumours that had lost FOXO expression. In conclusion, HBP1 is a novel target of the PI3K/FOXO pathway and controls cell proliferation in response to growth factors.

Key words: Akt/protein kinase B (PKB), cell proliferation, forkhead box O (FOXO), growth factor, high-mobility group-box protein 1 (HBP1), transcriptional regulation.

INTRODUCTION

Growth factors, such as PDGF (platelet-derived growth factor), induce cell proliferation, migration and differentiation through the activation of different signalling mediators such as the PI3K (phosphoinositide 3-kinase)/PKB (protein kinase B, also known as Akt) pathway [1]. Downstream of growth factor receptors, PKB inhibits the activity of the forkhead box O transcription factors FOXO1, FOXO3, FOXO4 and FOXO6 through phosphorylation on conserved residues (for example Thr³², Ser²⁵³ and Ser³¹⁵ of FOXO3) [2,3]. The phosphorylation of FOXO by PKB promotes the binding of 14-3-3 protein to FOXO and its export from the nucleus to the cytosol where it is sequestered or ubiquitinated and then degraded by proteasomes [3,4]. In addition, in a previous study, we showed that growth factors not only induce the phosphorylation and inactivation of FOXO by PKB, but also repress FOXO expression at the mRNA level [5].

In the absence of growth factor stimulation, FOXO proteins are localized in the nucleus where they can bind DNA and regulate transcription. They regulate the expression of many genes promoting cell cycle arrest (induction of p21^{Cip1} and p27^{Kip1} and repression of cyclin D1 and D2), DNA damage repair [induction of Gadd45 (growth-arrest and DNA-damage-inducible protein 45)], apoptosis [induction of Fas ligand, Bim and TRAIL (tumour-necrosis-factor-related apoptosis-inducing ligand)] and the response to oxidative stress (induction of catalase and superoxide dismutase) [2,6].

FOXO factors are considered as tumour suppressors [7] and are dysregulated in breast cancer and in other types of tumours including rhabdomyosarcoma, leukaemia, glioblastoma and prostate cancer [8]. In more than 70 % of breast cancers, mutations and/or amplifications are found in genes that control the PI3K pathway such as receptor tyrosine kinases (for example *ERBB2*, *FGFR1*), the PI3K catalytic subunits p110 α and p110 β (*PIK3CA* and *PIK3CB*), the PI3K regulatory subunit p85 α (*PIK3RI*), *PKB*, *KRAS*, *PDK1* (phosphoinositide-dependent kinase 1) and *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) [9]. Constitutive PI3K activation in these cells is expected to inactivate FOXO. Other mechanisms of FOXO dysregulation in breast cancers have been demonstrated. It has been shown that FOXO3 is targeted by *miR-96* and FOXO1 by *miR-27a*, *miR-96* and *miR-182* in this cancer [10,11]. Another study suggested that IKK [I κ B (inhibitor of nuclear factor κ B) kinase] is often constitutively activated in breast cancer and can phosphorylate FOXO3 causing its degradation through the ubiquitin-dependent proteasome pathway. FOXO3 down-regulation by IKK promotes cell proliferation and tumorigenesis [12].

In the present study we focused on the identification of novel genes regulated by the PI3K/PKB/FOXO pathway downstream of growth factor stimulation. By combining microarray data from multiple experiments, we identified HBP1 [HMG (high-mobility group)-box protein 1] as a novel FOXO target, which was down-regulated by the growth factors PI3K and PKB. In addition,

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ER, oestrogen receptor; FGF, fibroblast growth factor; FOXO, forkhead box O; GEO, Gene Expression Omnibus; HBP1, HMG-box protein 1; HEK, human embryonic kidney; HMG, high-mobility group; IGF-1, insulin-like growth factor 1; IKK, I κ B (inhibitor of nuclear factor κ B) kinase; IL-3, interleukin 3; 4-OHT, 4-hydroxytamoxifen; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; RNAPII, RNA polymerase II; TCGA, The Cancer Genome Atlas; WST1, water-soluble tetrazolium salt 1.

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we showed that *HBPI* knockdown was enough to increase cell proliferation.

HBPI is a transcription factor that belongs to the family of HMG proteins, which share a common DNA-binding domain, the HMG-box domain [13]. *HBPI* was first identified in the rat for its ability to reverse a potassium transport-defective phenotype [14]. This factor is ubiquitously expressed and can act either as a transcriptional repressor or activator. It promotes cell cycle arrest by controlling the expression of genes involved in cell cycle regulation such as cyclin D1 and N-Myc (neuroblastoma-derived Myc) [15–19]. *HBPI* was shown to play a role in cell differentiation through the up-regulation of MPO (myeloperoxidase) in haemopoietic cells [20] or the down-regulation of MyoD during terminal differentiation of muscle cells [21] for example. *HBPI* has also been implicated in premature senescence induced by an oncogene through the up-regulation of the cell cycle inhibitor p16^{INK4A} and the down-regulation of DNMT1 (DNA methyltransferase 1) [22–24]. The activity of *HBPI* is modulated through various mechanisms including its interaction with the retinoblastoma protein pRb and p130 [15,22,25], acetylation by p300/CBP [26] and phosphorylation by the MAPK (mitogen-activated protein kinase) p38 [18]. By contrast, little is known about transcriptional regulation of *HBPI* expression, which is the topic of the present study.

EXPERIMENTAL

Cells and reagents

AG01518 human foreskin fibroblasts (Coriell Institute for Medical Research, Camden, NJ, U.S.A.) were grown in Quantum 333 medium (A&E Scientific). HEK (human embryonic kidney)-293T cells (A.T.C.C., Manassas, VA, U.S.A.), MCF7 human breast adenocarcinoma cells and Ba/F3 mouse lymphocytic cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Thermo Scientific) with 10% FBS and 1% penicillin/streptomycin (Invitrogen), in the presence of IL-3 (interleukin 3; 100 units/ml) for Ba/F3 cells, as described previously [27,28]. IL-3 was produced in our laboratory by transfected CHO (Chinese-hamster ovary) cells (a gift from Professor A. Burgess, Ludwig Institute, Melbourne, Australia). NIH 3T3 cells (DSMZ, Braunschweig, Germany) were cultured in DMEM with 10% FBS and antibiotics. EOL-1 eosinophilic leukaemia cells (DSMZ) were maintained in Iscove's modified Dulbecco's medium (Invitrogen) with 20% FBS, antibiotics and non-essential amino acids. DLD1 and DLD1-F3 colon carcinoma cells were grown in RPMI 1640 medium with 10% FBS and standard supplements. PDGF-BB, FGF-4 and IGF-1 (insulin-like growth factor 1) were obtained from PeproTech. Imatinib and LY294002 were purchased from LC Laboratories and Calbiochem respectively. PKB inhibitor VIII was obtained from Santa Cruz Biotechnology and MK-2206 was from Selleckchem. pcDNA3-FOXO3-A3-ER, pcDNA3-HA-FOXO4-A3-ER, SR α -myr110 α -ER and SR α -myrPKB-ER have been described previously [29–31]. The absence of *Mycoplasma* infection was regularly checked for all cell lines.

Lentivirus-mediated shRNA silencing

A set of five shRNA-specific clones for human *HBPI* were obtained from Thermo Scientific, with an additional two from Sigma, and a set of five shRNA-specific clones for human FOXO3 were obtained from Sigma. All of these shRNAs were expressed in pLKO.1 lentiviral vector. On the basis of their efficiency,

two constructs for *HBPI* [TRCN0000015274 (shHBPI-74) and TRCN0000229982 (shHBPI-2)] and two for *FOXO3* [TRCN0000040101 (shFOXO3-31) and TRCN0000040102 (shFOXO3-32)] were used for further studies. They were all packaged for viral production and infection and tested for target knockdown. A negative 'scrambled' control was obtained from Addgene (catalogue number 1864). The production of virus and the infection were performed as described in [5].

RNA extraction and RT-qPCR

Total RNA was extracted using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. RNA (1 μ g) was subjected to reverse transcription using MMLV (Moloney murine leukaemia virus) reverse transcriptase enzyme (Invitrogen). Quantitative PCR analysis was performed as described in [32,33]. The oligonucleotides used are described in Supplementary Table S1 (at <http://www.biochemj.org/bj/460/bj4600025add.htm>).

Microarray analysis

Gene expression analysis of human fibroblasts treated with growth factors and Ba/F3 cells expressing activated PI3K pathway components have been described previously [GEO (Gene Expression Omnibus) accession numbers GSE14256 and GSE35705 [5,29]]. Ba/F3 cells expressing Fip1L1-PDGFR α (PDGF receptor α) or TEL-PDGFR β [34] were treated with imatinib or control medium for 4h. RNA was extracted as described above and used to hybridize Affymetrix gene chips (GEO accession number GSE44810) as described in [35].

Western blot experiments and antibodies

Cells were lysed in lysis buffer (25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 6 mM EDTA, 10% glycerol and 1% Triton X-100) containing protease inhibitors (1 mM Pefabloc[®] and 1.7 μ g/ml aprotinin) and incubated on ice for 15 min. Extracts were cleared by centrifugation for 10 min at 10000 *g* at 4°C and protein concentration was determined using the BCA Protein Assay Kit (Pierce). Protein extracts (30–50 μ g) were loaded on to SDS/PAGE (8–10% gel) and transferred on to a PVDF membrane. Western blots were performed with anti-HBPI (Santa Cruz Biotechnology A-5, sc-376831), anti-FLAG M5 (Sigma, F-4042), anti-FOXO3 (Millipore, 07-702 and Cell Signaling Technology, 2497), anti-pAkt (Ser⁴⁷³) (Cell Signaling Technology, 9271) and anti- β -actin (Sigma, A-5441) antibodies. We used secondary antibodies coupled to horseradish peroxidase (Cell Signaling Technology).

Luciferase assays

The *HBPI* promoter (500 bp) was amplified by nested PCR using human genomic DNA as a template. The oligonucleotides used are described in Supplementary Table S1. The promoter was subcloned into pGL3-Basic luciferase vector using NheI and XhoI restriction sites. The mutant was obtained by QuikChange[®] mutagenesis (Stratagene) using oligonucleotides described in Supplementary Table S1. All of the constructs were sequenced. The luciferase assay was performed as described previously [5,32] (see the Supplementary Experimental section at <http://www.biochemj.org/bj/460/bj4600025add.htm>).

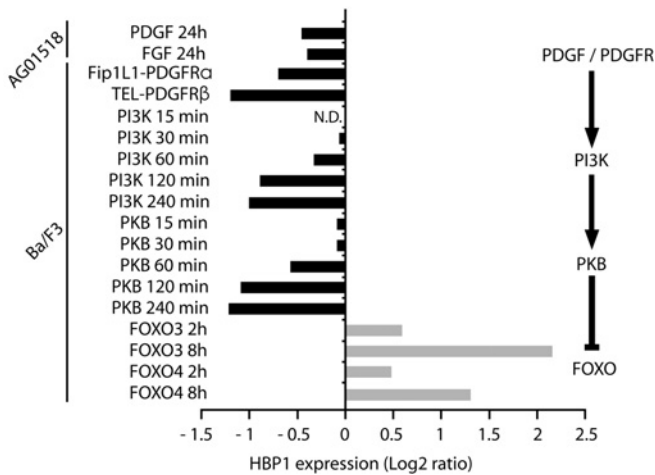


Figure 1 Microarray data identify HBP1 downstream of the growth factor/PI3K/PKB/FOXO pathway

Microarray data from different experiments were compared to identify new target genes downstream of the growth factor/PI3K/PKB/FOXO pathway. AG01518 human fibroblasts were treated for 24 h with PDGF-BB or basic FGF. Ba/F3 cells that express Fip1L1-PDGFR α or TEL-PDGFR β were treated with imatinib for 4 h or left untreated. Ba/F3 cells that express myr-PI3K-ER or myr-PKB-ER were treated with 4-OHT for different periods of time. Ba/F3 cells expressing FOXO3-A3-ER or FOXO4-A3-ER were treated with 4-OHT for 2 h or 8 h. After RNA extraction, microarray analyses were performed as described previously [29,35]. N.D., not detected.

ChIP assay

For ER (oestrogen receptor)-fused and endogenous FOXO3 ChIP-qPCR, 10^7 DLD1-F3 and 4×10^7 DLD1 cells were used respectively. DLD1 cells were treated with 10 μ M LY294002 (Enzo Life Sciences) or left untreated and DLD1-F3 cells

expressing FOXO3-A3-ER were treated or not with 1 μ M 4-OHT (4-hydroxytamoxifen; Sigma). ChIP and sequencing were performed as described previously [36] using anti-FOXO3 (Santa Cruz Biotechnology, H144), anti-ER (Santa Cruz Biotechnology, MC-20) and normal rabbit IgG (Santa Cruz Biotechnology) antibodies. RT-qPCR analyses were performed with IQ SYBR-Green mix (Bio-Rad Laboratories) using oligonucleotides described in Supplementary Table S1.

Proliferation assay

Proliferation measurement was performed by thymidine incorporation, as described previously [5] and by WST1 (water-soluble tetrazolium salt 1) assay (Roche). Briefly, AG01518 human fibroblasts were seeded in quadruplicate in a 96-well plate in complete medium (5000 cells/well). For the thymidine incorporation assay, cells were starved in serum-free minimum Eagle's medium for 48 h. FBS (10%), PDGF-BB (25 ng/ml) and FGF-4 (10 ng/ml with 10 μ g/ml heparin) were then added with [3 H]thymidine (0.5 μ Ci/well; GE Healthcare) for 24 h. Microtitre plates were harvested using a cell harvester (PerkinElmer Life Sciences). We used a TopCount instrument (PerkinElmer Life Sciences) to quantify the radioactivity incorporated into DNA. For the WST1 test, cells were starved and treated with FBS, PDGF-BB and FGF-4 (same concentrations) for three days before the addition of WST1 reagent (10 μ l, 1:10 final dilution) and absorbance was measured at 450 nm and 650 nm as recommended by the manufacturer (Roche).

Statistical analysis

Statistical significance was calculated according to Student's *t* test (* P < 0.05; ** P < 0.01; *** P < 0.001). All experiments were performed at least three times unless otherwise stated.

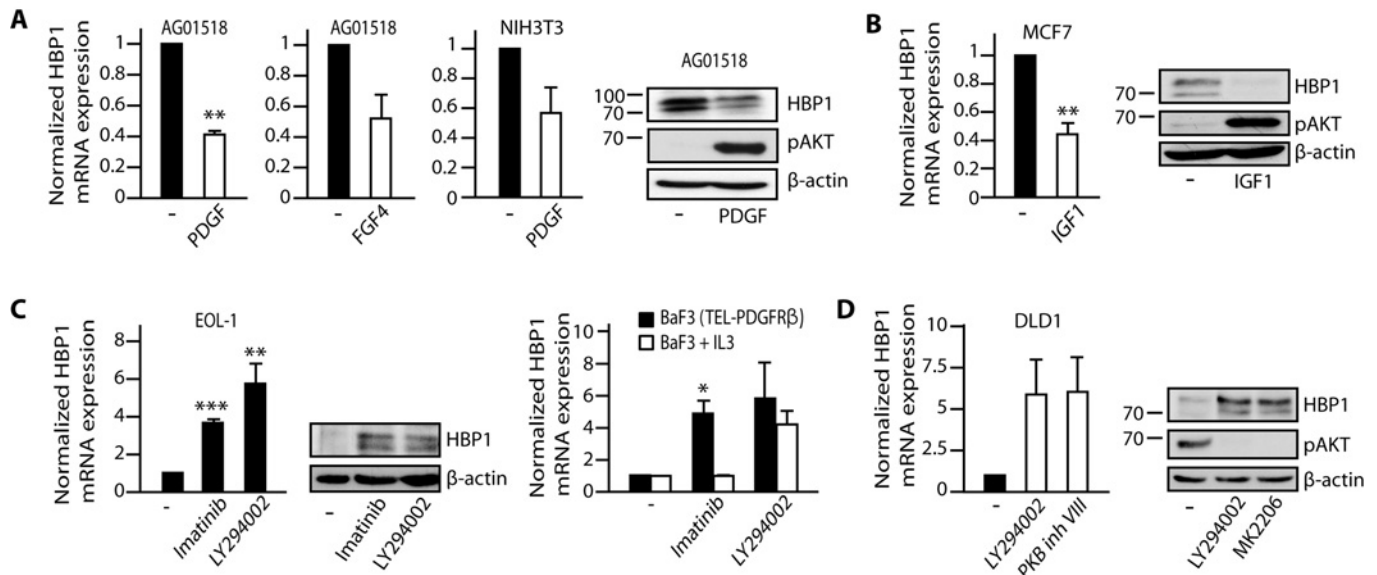


Figure 2 HBP1 expression is down-regulated by growth factors through the PI3K/PKB pathway

(A) Human fibroblasts (AG01518) and murine fibroblasts (NIH 3T3) were serum-starved for 24 h and treated with PDGF-BB (25 ng/ml) or FGF4 (10 ng/ml with 10 μ g/ml heparin) for 24 h before RNA or protein extraction. (B) MCF7 breast cancer cells were serum-starved for 24 h and treated with IGF-1 (50 ng/ml) for 24 h before RNA or protein extraction. (C) EOL-1 cells were treated with imatinib (100 nM) for 4 h or LY294002 (20 μ M) for 24 h before RNA or protein extraction. Ba/F3 cells (TEL-PDGFR β or IL-3) were treated with imatinib (100 nM) or LY294002 (20 μ M) for 4 h before RNA extraction. Ba/F3 cells cultured with IL-3 were used as a control. (D) DLD1 colon carcinoma cells were treated for 8 h with LY294002 (10 μ M) or PKB inhibitor (inh) VIII (10 μ M) before RNA extraction and for 8 h with LY294002 (10 μ M) or MK2206 (5 μ M) before protein extraction. In all conditions, the expression of HBP1 was measured by RT-qPCR and divided by the expression of a housekeeping gene [*RPLP0* (ribosomal protein, large, P0), except for DLD1 and mouse cells in which β -actin was used]. The untreated control condition was set to 1. The results are means $\pm$ S.E.M. for three independent experiments. * P < 0.05, ** P < 0.01 and *** P < 0.001. The HBP1 protein was detected by Western blot using an anti-HBP1 antibody. Molecular masses are shown in kDa.

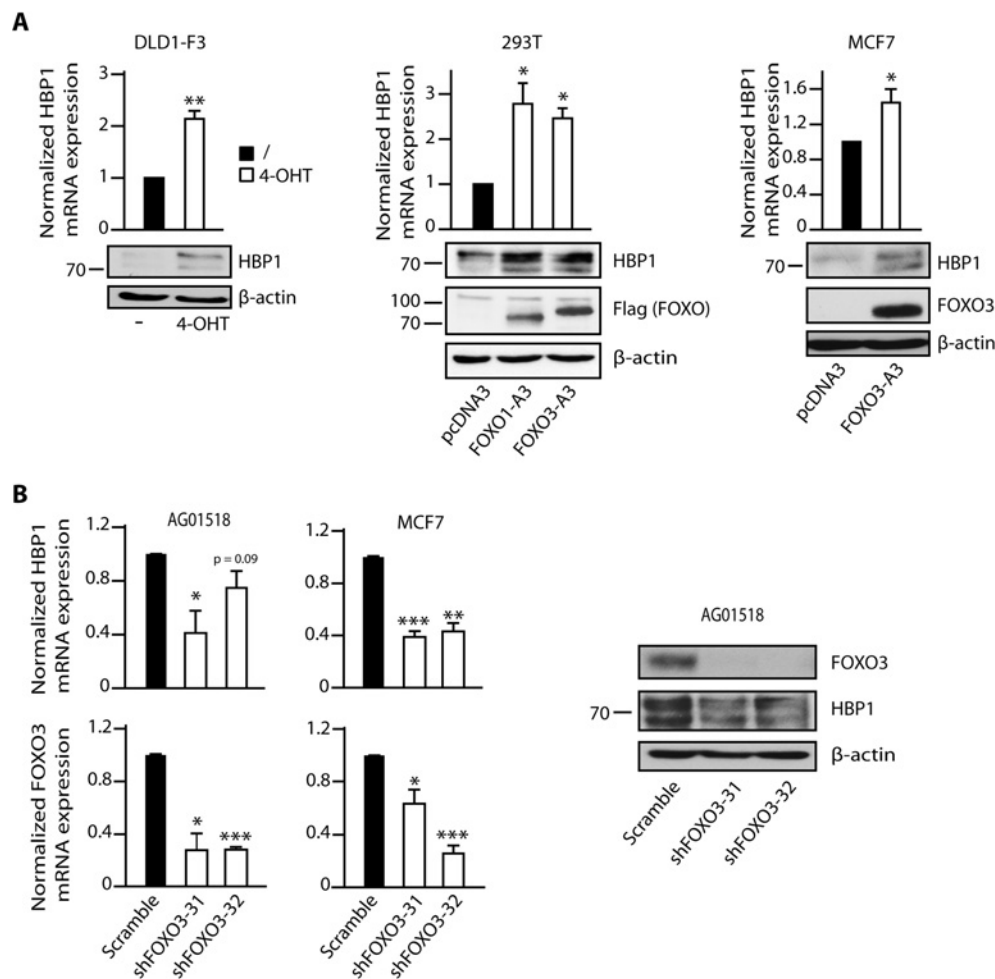


Figure 3 HBP1 expression is up-regulated by FOXO activation

(A) DLD1-F3 cells were treated for 8 h with 4-OHT (1 μ M), HEK-293T cells were transfected for 24 h with FLAG-tagged FOXO1-A3 or FOXO3-A3 constructs compared with pcDNA3 as a control and MCF7 cells were transfected with pcDNA3 or FOXO3-A3. In all conditions, RNA or proteins were extracted. (B) AG01518 and MCF7 were infected with lentiviral particles that express one of the two FOXO3 shRNA (shFOXO3-31 or shFOXO3-32) and RNA or proteins were extracted. In all conditions, the expression of HBP1 or FOXO3 was measured by RT-qPCR and divided by the expression of a housekeeping gene [*RPLP0* (ribosomal protein, large, P0), except for DLD1-F3 in which β -actin was used]. The untreated control condition was set to 1. The results are means $\pm$ S.E.M. for three independent experiments. A unilateral Student's *t* test was performed for the shRNA experiment. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. Western blot experiments were performed using anti-HBP1, anti-FOXO3 and anti-FLAG antibodies. Molecular masses are shown in kDa.

RESULTS

HBP1 is a novel target gene regulated by growth factors, PI3K, PKB and FOXO

To identify genes regulated by growth factors through the PI3K/PKB/FOXO axis, we combined multiple microarray data. We have previously reported the regulation of genes in AG01518 normal human fibroblasts treated with PDGF or FGF [5]. We also analysed Ba/F3 cells that expressed a constitutively active form of PDGFR α or PDGFR β (Fip1L1-PDGFR α or TEL-PDGFR β) [34]. In this case, untreated cells were compared with cells treated with imatinib (a potent PDGFR inhibitor) for 4 h. Finally, we included expression data from Ba/F3 cells that expressed a conditionally activated variant of the key components of the PI3K pathway: myr-PI3K, myr-PKB, FOXO3-A3 or FOXO4-A3 (A3: mutation of the three PKB phosphorylation sites to alanine) fused to the hormone-binding domain of the ER. These cells have been extensively described previously [29]. Coupling of these proteins to the ER domain induces the recruitment of heat-shock

proteins that maintain them in an inactive state. The binding of 4-OHT to the ER domain allows the dissociation of this complex and the rapid activation of the proteins of interest [29]. The myr tag corresponds to a myristoylation domain, which was fused to the p110 α subunit of PI3K or to PKB α and targets them to the plasma membrane where they become rapidly activated. We focused on genes that were down-regulated by growth factors, by the constitutively active forms of PDGFR, by PI3K and PKB, but up-regulated by FOXO3 and FOXO4 or vice versa. Collectively, these data revealed that HBP1 is a potential transcriptional target of this pathway. Indeed, its expression was decreased by PDGF, FGF, PI3K and PKB and increased by FOXO factors (Figure 1).

HBP1 expression is down-regulated by growth factors through the PI3K pathway

First, to confirm that HBP1 is transcriptionally regulated by growth factors, we used AG01518 human fibroblasts treated with

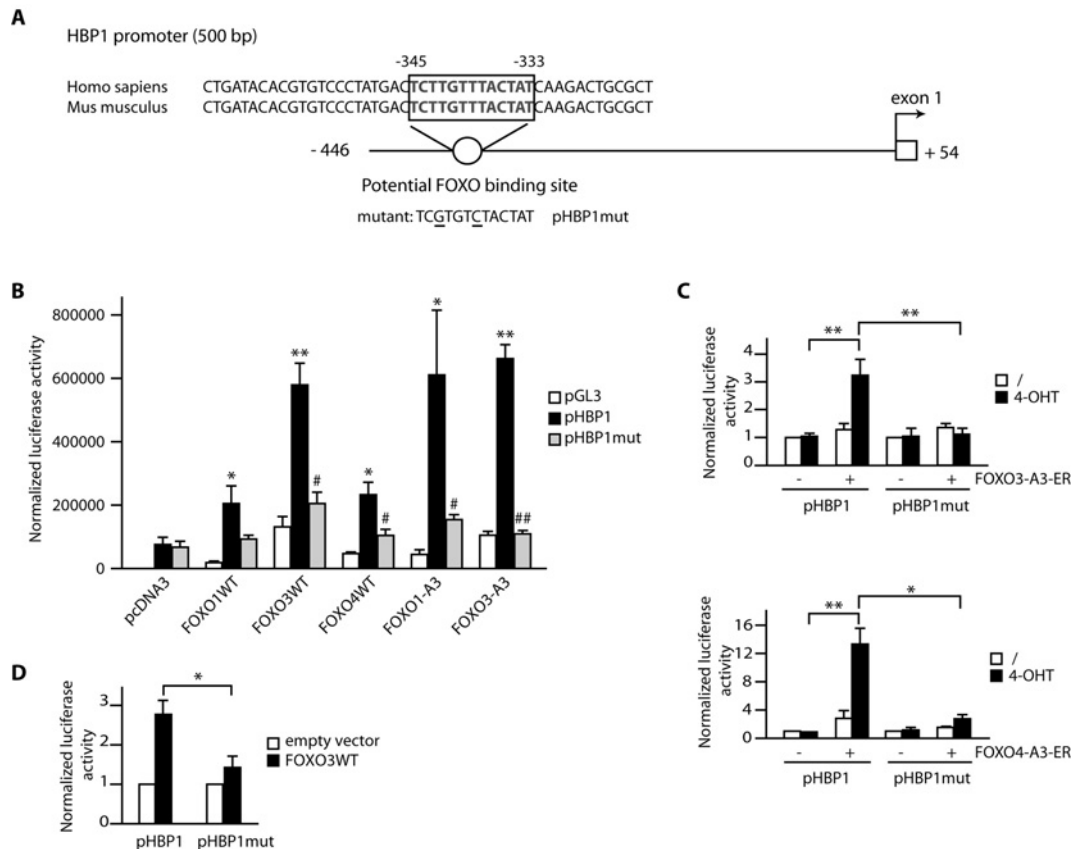


Figure 4 Identification of a FOXO-binding site in the *HBP1* promoter

(A) A fragment of the *HBP1* promoter (500 bp) that contains a conserved FOXO-binding site was cloned upstream of the luciferase reporter gene (pHBP1). A mutant (pHBP1mut) was obtained by changing two nucleotides in the FOXO-binding site. (B) HEK-293T cells were co-transfected with *HBP1* promoter constructs, with pcDNA3, FOXO1WT, FOXO3WT, FOXO4WT, FOXO1-A3 or FOXO3-A3 and with pE1F- β -galactosidase for 24 h. One representative experiment out of three is shown. pHBP1 activity was compared with pGL3 (* P < 0.05 and ** P < 0.01) and the activity of the mutant promoter was compared with the wild-type *HBP1* promoter (# P < 0.05 and ## P < 0.01). (C) HEK-293T cells were co-transfected with *HBP1* promoter constructs, FOXO3-A3-ER or FOXO4-A3-ER and pE1F- β -galactosidase and were treated with 100 nM 4-OHT for 24 h. (D) NIH 3T3 fibroblasts were co-transfected with *HBP1* promoter constructs, FOXO3WT or pcDNA3 control vector and pE1F- β -galactosidase for 24 h. In all experiments, luciferase and β -galactosidase activities were measured and the luciferase activity was normalized to the β -galactosidase activity. The results are means $\pm$ S.E.M. * P < 0.05 and ** P < 0.01.

PDGF-BB or FGF4 and NIH 3T3 murine fibroblasts treated with PDGF-BB. We observed that the mRNA expression of *HBP1* was repressed approximately 2-fold after stimulation of AG01518 and NIH 3T3 with these growth factors for 24 h (Figure 2A). We confirmed the effect of PDGF on HBP1 expression at the protein level by Western blot (Figure 2A). Further inspection of experiments available in the GEO database revealed that HBP1 is down-regulated in primary cultures of astrocytes and aortic smooth muscle cells in response to growth factors (Supplementary Table S2 at <http://www.biochemj.org/bj/460/bj4600025add.htm>). These results confirmed that HBP1 is negatively regulated by growth factors in normal mammalian cells.

Next, we tested whether growth factors also regulated HBP1 expression in cancer cells. The treatment of MCF7 human breast adenocarcinoma cells with IGF-1 reduced the expression of HBP1 at the mRNA and protein levels (Figure 2B). Data from the GEO database confirmed this result and revealed that HBP1 is also down-regulated in HeLa cervical carcinoma cells treated with EGF (epidermal growth factor; Supplementary Table S2).

To determine whether oncogenic variants of growth factor receptors could also regulate HBP1 expression, we next used EOL-1 cells that endogenously express the fusion protein Fip1L1-

PDGFR α and Ba/F3 cells transfected with TEL-PDGFR β . As expected, blocking PDGFR constitutive signalling with imatinib enhanced HBP1 expression in these cells, but not in normal Ba/F3 cells, which do not express PDGFR (Figure 2C). These results showed that oncogenic growth factor receptors down-regulate the expression of HBP1, similar to wild-type receptors.

Finally, to confirm that this regulation is mediated by the PI3K/PKB pathway, we treated cells with the PI3K inhibitor LY294002 or with a PKB inhibitor (PKB inhibitor VIII or MK-2206). We observed that the treatment of EOL-1 cells and Ba/F3 cells expressing TEL-PDGFR β with LY294002 up-regulated HBP1 (Figure 2C). LY294002 also increased the expression of HBP1 in Ba/F3 cells that were cultured in the presence of IL-3, which is known to activate PI3K and PKB [37]. In DLD1 colon carcinoma cells treated with LY294002 or with a PKB inhibitor, HBP1 expression was strongly induced (Figure 2D). These results confirmed that the PI3K/PKB pathway is responsible for the repression of HBP1 expression, as expected from the microarray data.

Taken together, these results suggest that HBP1 is negatively regulated by growth factors through PI3K activation in normal and tumour cells.

HBP1 expression is up-regulated by FOXO activation

We next investigated whether the regulation of HBP1 was mediated by the FOXO transcription factors using several approaches. First, we used DLD1 cells stably transfected with FOXO3-A3-ER [36,38] which we will refer to as DLD1-F3 cells. Upon activation of FOXO3-A3-ER with 4-OHT, HBP1 expression was up-regulated at the mRNA and protein levels in DLD1-F3 cells (Figure 3A). Similar results were obtained in human fibroblasts that expressed FOXO3-A3-ER (results not shown). HBP1 expression was also increased by transfecting constitutively active forms of FOXO1 and FOXO3 (FOXO1-A3 and FOXO3-A3) in HEK-293T and MCF7 cells (Figure 3A). Conversely, the knockdown of endogenous FOXO3 with two specific shRNAs in AG01518 and MCF7 cells reduced HBP1 expression (Figure 3B). In control experiments, these two shRNAs reduced FOXO3 expression in both cell lines (Figure 3B). Altogether, these results demonstrate that the activated FOXO transcription factors positively regulate the expression of HBP1.

HBP1 promoter contains a functional FOXO-binding site

We next determined whether FOXO proteins could bind directly to the promoter of *HBP1*. For this purpose, we performed a bioinformatics analysis to identify conserved transcription-factor-binding sites (<http://bioit.dmbur.ugent.be/contrav2/>). We identified a putative FOXO-binding site in the promoter of *HBP1* at position –345 to –333 bp upstream of the transcription initiation site. This sequence was perfectly conserved between human and mouse (Figure 4A). A 500 bp fragment of the *HBP1* promoter containing this FOXO-binding site was cloned in front of a luciferase reporter gene (Figure 4A). In HEK-293T cells, the *HBP1* promoter activity was significantly increased by co-transfection of the wild-type FOXO-1, FOXO-3 and FOXO-4 (Figure 4B). The stimulation was maximal when constitutively activated FOXO1-A3 and FOXO3-A3 were transfected (Figure 4B). We confirmed the positive effect of FOXO3 and FOXO4 by using the 4-OHT-inducible system in transfected HEK-293T cells (Figure 4C). In NIH 3T3 cells, wild-type FOXO3 also induced the *HBP1* promoter activity (Figure 4D). We next mutated two nucleotides of the identified FOXO-binding site (Figure 4A). These mutations greatly reduced the effect of all FOXO factors on the activity of the *HBP1* promoter (Figure 4). In conclusion, we identified a FOXO-binding site in the proximal promoter of *HBP1*, which is regulated by the three ubiquitous FOXO isoforms.

FOXO directly binds to the HBP1 promoter in a PI3K-dependent manner

To investigate further whether FOXO transcription factors directly bind to the proximal promoter of *HBP1*, we first analysed data from ChIP combined with deep-sequencing (ChIP-seq), which were published recently [36]. In this experiment, DLD1-F3 and DLD1 cells were treated with 4-OHT and the exogenous FOXO3-A3-ER fusion protein was immunoprecipitated with an anti-ER antibody. We observed an increased binding of FOXO3 to the proximal promoter of *HBP1* when DLD1-F3 cells were treated with 4-OHT compared with DLD1 cells that do not express the fusion protein (Figure 5A, upper panels). To test the binding of endogenous FOXO3, DLD1 cells were treated with PKB inhibitor VIII before immunoprecipitation with an anti-FOXO3 antibody. As expected, FOXO3 bound to the *HBP1* promoter when PKB was inhibited (Figure 5A, lower panels). To demonstrate that FOXO3 bound to the site that we had previously identified (Figure 4), we performed ChIP followed

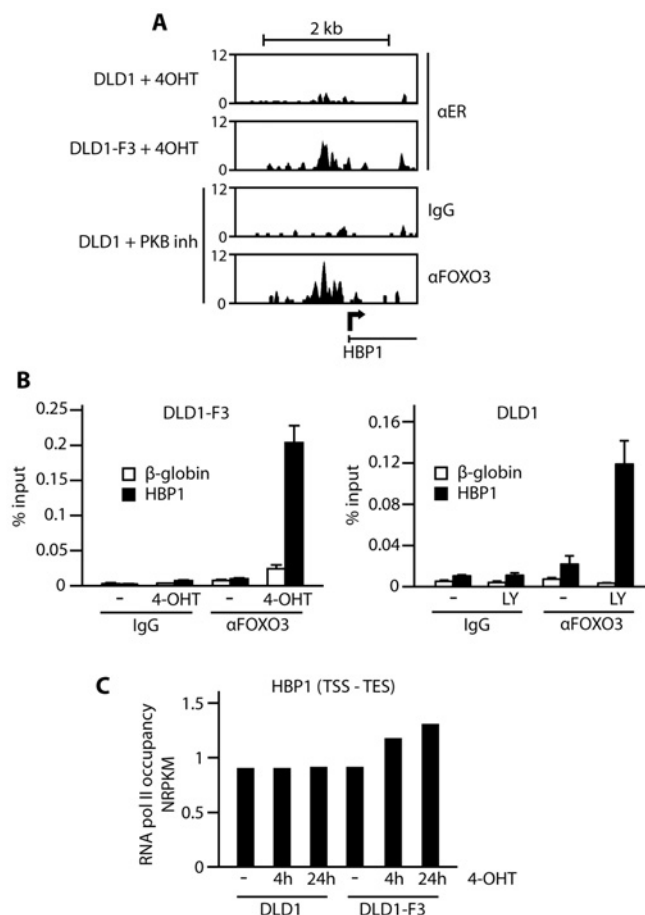


Figure 5 FOXO binds to *HBP1* promoter in a PI3K-dependent manner

(A) DLD1 and DLD1-F3 cells were treated with 4-OHT (1 μ M) for 1 h and immunoprecipitation was performed using an anti-ER antibody. Alternatively, DLD1 cells were treated with PKB inhibitor VIII (inh) (10 μ M) for 1 h and immunoprecipitation was performed with an anti-FOXO3 antibody or control IgG. Bound DNA was sequenced. The y-axis values represent tag coverage per base per 10⁶ sequenced reads [36]. (B) DLD1-F3 cells were treated with 4-OHT (1 μ M) over 1 h and DLD1 cells were treated with LY294002 (10 μ M) for 1 h. ChIP experiments were performed using an anti-FOXO3 antibody and IgG control. Specific binding to the *HBP1* promoter was measured by RT-qPCR on bound DNA and percentage of input was determined. An amplicon within the β -globin locus was used as a negative control for specific DNA binding. For DLD1-F3 cells, one representative experiment out of two independent biological replicates is shown, and for DLD1 cells, one out of three independent experiments. (C) RNAPII ChIP-seq was performed in DLD1 and DLD1-F3 cells without 4-OHT or after treatment with 4-OHT for 4 h or 24 h [36]. RNAPII occupancy of the *HBP1* gene (TSS, transcriptional start site, to TES, transcriptional end site) was determined (NRPKM, normalized read counts per kb of transcript and million sequencing tags).

by RT-qPCR. A 112-bp DNA fragment that contained the FOXO-binding site of the *HBP1* promoter was amplified. In DLD1-F3 cells, FOXO3 binding to the *HBP1* promoter increased when the cells were treated with 4-OHT compared with untreated cells (Figure 5B). We also showed that the binding of endogenous FOXO3 to the *HBP1* promoter was increased in DLD1 cells treated with LY294002, in line with the ChIP-seq data (Figure 5B). Finally, to evaluate whether FOXO-induced up-regulation of HBP1 expression correlated with increased transcription, we determined RNAPII (RNA polymerase II) occupancy from the ChIP-seq data [36]. After treatment of DLD1-F3 cells with 4-OHT for 4 h or 24 h, we observed increased RNAPII occupancy in the *HBP1* gene. In DLD1 cells, used as a control, there was no change (Figure 5C). Taken together, these results show a direct

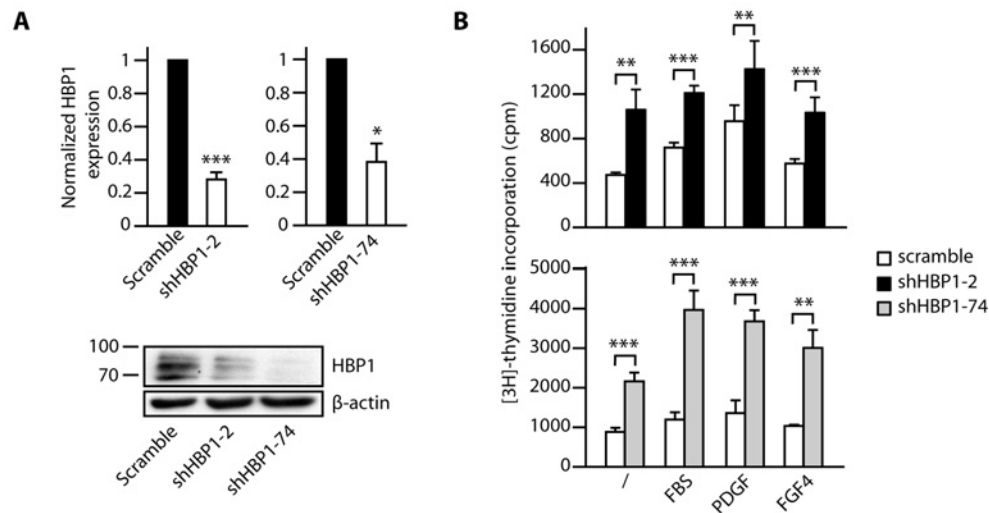


Figure 6 *HBP1* knockdown enhances cell proliferation

Human fibroblasts (AG01518) were infected with lentiviral particles that express one of the two *HBP1* shRNAs (shHBP1-2 or shHBP1-74) or control shRNA (scramble). **(A)** RNA or proteins were extracted 72 h after infection and *HBP1* expression was measured by RT-qPCR or by Western blot. Molecular masses are shown in kDa. **(B)** Serum-starved fibroblasts were treated with FBS (10 %), PDGF (25 ng/ml) or FGF4 (10 ng/ml with 10 μ g/ml heparin) for 24 h and [3 H]thymidine incorporation was measured. The results are representative of three independent experiments. * P < 0.05, ** P < 0.01 and *** P < 0.001.

regulation of *HBP1* gene expression by FOXO3 through *HBP1* promoter binding and increased transcription.

HBP1 knockdown enhances cell proliferation

Several reports have suggested that *HBP1* is a negative regulator of the cell cycle [15–17]. To evaluate the role of *HBP1* in cell proliferation in response to growth factors, we performed a thymidine incorporation assay with fibroblasts that expressed shRNA targeting *HBP1* compared with shRNA control. First, we selected two shRNAs that efficiently targeted *HBP1* (shHBP1-2 and shHBP1-74) in a set of validation experiments in AG01518 human fibroblasts. We observed that the two shHBP1 shRNAs significantly reduced *HBP1* expression at the mRNA and protein levels (Figure 6A). The thymidine incorporation assay showed that, in the presence of growth factors, *HBP1* knockdown significantly increased cell proliferation. Moreover, this increase was also observed in the absence of stimulation, indicating that the loss of *HBP1* expression provided a growth advantage to the cells (Figure 6B). We confirmed these results by assessing the effect of *HBP1* knockdown on the number of viable cells using the WST1 reagent (Supplementary Figure S1 at <http://www.biochemj.org/bj/460/bj4600025add.htm>). These data suggest that *HBP1* down-regulation may significantly contribute to growth factor-induced proliferation.

HBP1 and FOXO1 expression are correlated in breast tumours

It is already known that both FOXO and *HBP1* are down-regulated in breast tumours [10–12,39,40]. Since the results of the present study identified FOXO as a key transcriptional regulator of *HBP1*, we tested whether FOXO down-regulation was responsible for *HBP1* repression in breast cancers. For this purpose, we analysed microarray data from TCGA (The Cancer Genome Atlas). This database includes gene expression data for 542 breast cancer samples and 63 normal breast tissues. As expected, *HBP1*

and FOXO1 expression were significantly decreased in tumour samples (Figure 7A). We have previously shown that FOXO factors induce the expression of the FOXO1 isoform in a feedback loop [5]. Hence the expression of FOXO1 can be considered as a surrogate marker for FOXO activation. Using the Cbioportal web site, we interrogated the same TCGA dataset restricted to patients with complete data in terms of transcriptomics, proteomics and genomics measurements. This dataset gathers 453 patients, from which we selected 61 having a decreased FOXO1 or FOXO3 expression at the mRNA or protein level. Although the difference was modest, *HBP1* expression was significantly lower in patients with decreased FOXO expression (Figure 7B). Taking FOXO4 into account did not affect the result (results not shown). We also observed this effect by analysing RNA-seq data from the same patients (Figure 7B). Taken together, these results indicate that the down-regulation of *HBP1* in breast cancers is associated with a lower FOXO expression, as expected if the *HBP1* gene is transcriptionally regulated by FOXO.

DISCUSSION

In the present study we identified the *HBP1* gene as a novel transcriptional target of FOXO transcription factors, which are described as crucial cell cycle regulators that prevent tumour development. We showed by comparative microarray analysis that *HBP1* expression was down-regulated by PDGF and FGF stimulation, PI3K and PKB activation, and up-regulated by FOXO. We confirmed these results in a broad range of cell types, including normal and cancer cells, in humans and mice. We also showed by luciferase assay and ChIP that FOXO directly bound to the *HBP1* promoter to regulate its activity.

We showed that the three proteins FOXO1, FOXO3 and FOXO4 all similarly induce the activity of the *HBP1* promoter, indicating that the regulation of *HBP1* is not specific for one FOXO factor. Redundancy among the FOXO family members, which share a common DNA-binding domain, is well established [2]. For

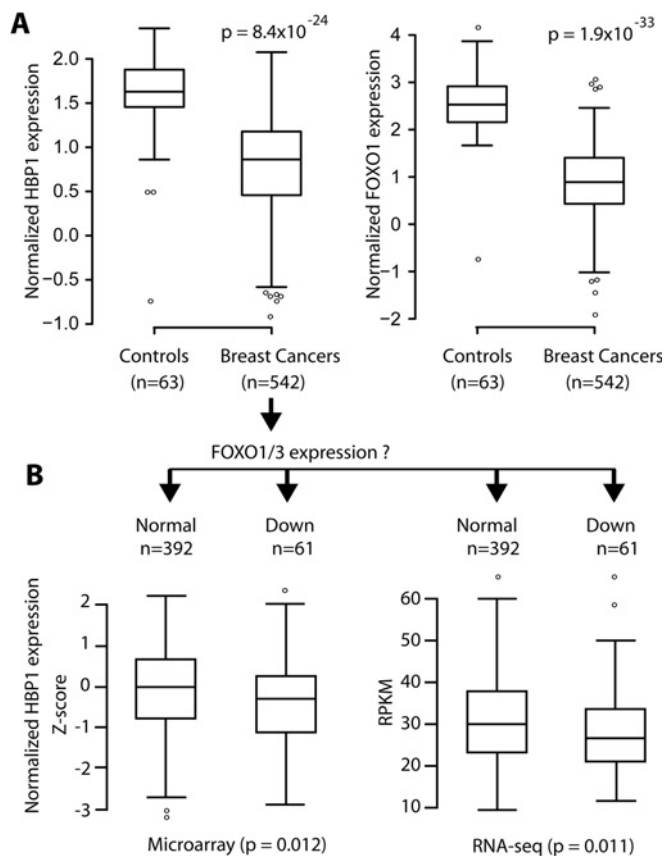


Figure 7 HBP1 and FOXO1 expression are correlated in breast cancers

(A) The expression of HBP1 and FOXO1 was analysed in data from the breast cancer patient cohort of TCGA. (B) Using the Cbioportal web site and querying the complete tumour samples dataset (453 patients), we identified tumour patients with down-regulation of FOXO3 or FOXO1 at the protein or gene expression levels ($n = 61$). We then compared *HBP1* gene expression from microarray data (left-hand panel) and RNA-seq (right-hand panel) in these 61 patients compared with patients with normal FOXO expression ($n = 392$). See the Supplementary Experimental section (at <http://www.biochemj.org/bj/460/bj4600025add.htm>) for details.

example, the regulation of the cell cycle inhibitor p27^{Kip1} can be mediated by FOXO1, FOXO3 or FOXO4 [41–43]. Accordingly, we did not find much difference in gene regulation by FOXO3 compared with FOXO4 in the microarray data of Ba/F3 cells (results not shown and [29]). We cannot, however, exclude the possibility that one particular FOXO could preferentially induce HBP1 expression in a specific tissue since the regulation of a target gene by one FOXO rather than another one seems to depend, at least in part, on the tissue and the interactions with co-factors [8].

The role of HBP1 in the control of cell cycle has already been established [15–19]. Our results showed that *HBP1* knockdown was sufficient to increase fibroblast proliferation, suggesting that it maintains a proliferative barrier in these cells. A link between growth factors and FOXO has already been established because FOXO is inactivated by growth factors through its phosphorylation by PKB on the one hand [3], and on the other hand by the fact that they share common target genes that they regulate in an opposite manner to control cell proliferation. For example, growth factors impair the function of the cyclin-dependent kinase inhibitor p27^{Kip1} through its phosphorylation by PKB [44], whereas FOXO factors directly induce its expression to repress the cell cycle [41–43]. In a previous study, we also showed that FOXO1 down-regulation strongly enhanced human

fibroblast proliferation [5]. In the present study, we identified a new FOXO target gene which is also important for the control of cell proliferation downstream of growth factors. It is not clear how HBP1 limits cell proliferation in response to growth factors; however, they also share some common genes that could partially explain it. For example, the expression of c-Myc and cyclin D1 is induced by PDGF [45,46], whereas HBP1 negatively regulated c-Myc transcriptional activity [47] and down-regulated cyclin D1 expression in leukaemic myeloid cells [17]. However, we did not observe any effect of HBP1 on cyclin D1 expression (results not shown).

Future studies should analyse whether HBP1 might also regulate other growth factor functions. In addition to their role in cell proliferation, growth factors also promote cell migration and prevent apoptosis in many cell types [48–50]. In contrast, HBP1 seemed to have the opposite effect. In a breast cancer cell line, *HBP1* knockdown promotes cell migration [40]. In another study, it was found that HBP1 overexpression promoted apoptosis in leukaemic myeloid cells as shown by increased annexin-V staining and FasL mRNA [17]. Thus HBP1 could also act as a brake in the control of cell migration and cell survival in response to growth factors.

In the present study we have confirmed by a bioinformatics analysis of the large TCGA patient cohort that both HBP1 and FOXO1 are down-regulated in breast tumours compared with normal tissue. This analysis also indicated that breast tumours with decreased FOXO expression expressed significantly less HBP1. In line with these results, growth factors and FOXO regulated HBP1 in the breast cancer cell line MCF7. Therefore the regulation of HBP1 expression, which we initially observed in cell culture, may also be relevant for human breast tumours. This hypothesis is supported by the observation that breast tumours frequently harbour activating mutations in the PI3K pathway [9]. In addition, HBP1 expression may be down-regulated by *miR-17-5p* in breast cancer cell lines [40]. Interestingly, bioinformatics analysis predicted that FOXO1 could also be targeted by this miRNA [51]. Hence, in breast cancers overexpressing *miR-17-5p*, the down-regulation of HBP1 by this miRNA could be mediated directly or indirectly via FOXO1 down-regulation. Altogether, HBP1 down-regulation in breast cancers could be explained by the inactivation of FOXO subsequent to PI3K constitutive activation or miRNA. IKK, which was also shown to inactivate FOXO in breast cancer, could also play a role [12].

In conclusion, we have identified HBP1 as a novel transcriptional target of FOXO, which controls cell proliferation induced by growth factors and may be relevant for tumour development.

AUTHOR CONTRIBUTION

Alexandra Coomans de Brachène, Emeline Bollaert, Astrid Eijkelenboom and Audrey de Rocca Serra performed the experiments and analysed the results. Kristan van der Vos, Paul Coffier, Ahmed Essaghir and Jean-Baptiste Demoulin performed and interpreted the microarray experiments. Astrid Eijkelenboom and Boudewijn Burgering performed and analysed the ChIP experiments. Ahmed Essaghir analysed the TCGA data. Alexandra Coomans de Brachène and Jean-Baptiste Demoulin designed the study and wrote the paper. All authors critically reviewed the paper prior to submission.

ACKNOWLEDGEMENTS

We thank Kunliang Guan (University of California San Diego, CA, U.S.A.) and William Sellers (Novartis Institutes for Biomedical Research, Cambridge, MA, U.S.A.) for plasmids.

FUNDING

A.C.B. is the recipient of a grant from F.R.S. – FNRS (Le Fonds de la Recherche Scientifique) – Télévie [grant number 7.4583.11F]. This project was supported by grants from the Salus Sanguinis foundation, Action de Recherche Concertée (Communauté Française de Belgique) and the Dutch Scientific Organization (NOW-VIDI).

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Received 7 November 2013/30 January 2014; accepted 6 March 2014

Published on the Internet 25 April 2014, doi:10.1042/BJ20131467

SUPPLEMENTARY ONLINE DATA

The expression of the tumour suppressor HBP1 is down-regulated by growth factors via the PI3K/PKB/FOXO pathway

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EXPERIMENTAL

Luciferase assay

The different plasmids (pGL3, pHBP1 and pHBP1mut) were linearized by BamHI digestion before transfection. Sub-confluent HEK-293T cells were transfected using calcium phosphate precipitation with 0.125 µg of pGL3, 0.15 µg of pE1F-β-galactosidase, 0.5 µg of pCMV6-XL4-FOXO1WT (Origene SC118890), pCMV5-FLAG-FOXO3WT (Addgene 14937), pcDNA3-FLAG-FOXO4WT (Addgene 17549), pcDNA3-FLAG-FKHR AAA (FOXO1-A3, Addgene 13508 [1]) or pcDNA3-FLAG-FKHRL1 AAA (FOXO3-A3, Addgene 10709 [2]) (6-well plates) or 0.3 µg of pBabe-FOXO3-A3-ER or pcDNA3-FOXO4-A3-ER (12-well plates). The cells were incubated for 4 h, washed with PBS and then cultured in normal medium for 24 h. When ER-fusion proteins were transfected, cells were stimulated for 24 h with 100 nM 4-OHT. NIH 3T3 cells were transfected using Lipofectamine™ 2000 (Invitrogen) with 0.5 µg of pGL3, 1.5 µg of pCMV5-FLAG-FOXO3WT and 1 µg of pE1F-β-galactosidase in 12-well plates. After 24 h, luciferase and β-galactosidase activities were measured.

Data mining in TCGA

A dataset of normal and matched normal breast cancer patients was downloaded from the TCGA database (TCGA-BRCA: 63 controls and 542 tumours; 11/16/2012; normalized at the gene level 3). We used R scripts to plot *HBP1* and *FOXO1* gene expression in normal compared with cancer patients using Wilcoxon's test to assess statistical significance. To identify patients with decreased FOXO expression, we queried the TCGA-BRCA dataset from Cbioportal (<http://www.cbioportal.org/public-portal/>, November 2012), which includes proteomics, genomics and transcriptomics

data for 453 breast cancer samples. Using Cbioportal query language (OQL), we selected a group of patients with decreased *FOXO1* mRNA expression, decreased *FOXO3* mRNA expression or decreased FOXO3 protein expression (in each case, Z-score ≤ −2). FOXO1 protein expression was not available. Including FOXO4 in the analysis did not affect the patient lists. Using the “cgsr” R package, which gives access to the Cbioportal data, we compared *HBP1* gene expression in tumours with decreased FOXO expression (the patients that were positive to the query above) compared with the rest of patients using RNA-Seq or microarray normalized data. The significance of the comparison was assisted by Wilcoxon's test.

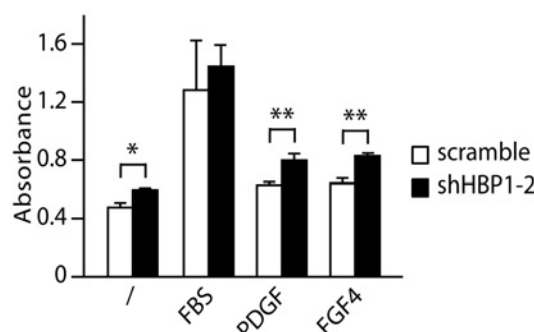


Figure S1 HBP1 knockdown enhances cell proliferation

Human fibroblasts (AG01518) were infected with lentiviral particles encoding *HBP1* shRNA or control shRNA (scramble). Cells were serum-starved and treated with FBS (10%), PDGF (25 ng/ml) or FGF4 (10 ng/ml, heparin 10 µg/ml) for 3 days. WST1 reagent was added and the absorbance was measured at 450 nm and 650 nm. The values at 650 nm were subtracted from the values at 450 nm. This results are the means ± S.E.M. for three independent experiments. **P* < 0.05 and ***P* < 0.01.

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Table S1 List of oligonucleotidesFwd, forward; prom, promoter; Rev, reverse; *RPLP0*, ribosomal protein, large, P0.

Experiment	Gene	Oligonucleotide (5' → 3')
Nested PCR for the cloning of pHBP1 in pGL3	<i>HBP1</i> prom Fwd (first PCR)	TGGGTGCTGCAAGAGGCTT
	<i>HBP1</i> prom Rev (first PCR)	AGCTCTTACTCCTCAACCGA
	<i>HBP1</i> prom Fwd (second PCR)	TATAGCTAGCGAAAAGAAGGCCCAAGC
	<i>HBP1</i> prom Rev (second PCR)	TATACTCGAGATTACCAAGTCTTCAAGCTTC
Mutagenesis for pHBP1mut	<i>HBP1</i> prom Fwd	CGTGTCCTATGACTCGTGTCTACTATCAAGACTGCGC
	<i>HBP1</i> prom Rev	GCGCAGTCTTGATAGTAGACACGAGTCATAGGGACACG
Quantitative PCR in human cells: fibroblasts, MCF7, HEK-293T and EOL1 cells	Human <i>HBP1</i> Fwd	ATGATTGGGATGGTGCAAT
	Human <i>HBP1</i> Rev	ACCATAGCCCTTGTAATGC
	Human <i>RPLP0</i> Fwd	TCGACAATGGCAGCATCTAC
	Human <i>RPLP0</i> Rev	ATCCGTCTCCACAGACAAGG
	Human <i>FOXO3</i> Fwd	TGAGAAGTCCCCAGCGACTT
	Human <i>FOXO3</i> Rev	TCCAACCCATCAGCATCCA
Quantitative PCR in mouse cells: Ba/F3 and NIH 3T3	Mouse <i>HBP1</i> Fwd	CTACCTCCTGGACACCCCTGA
	Mouse <i>HBP1</i> Rev	CAGTACCAGGTCCTCCTCCA
	Mouse β -actin Fwd	CTCTGGCTCTAGCACCATTGAAG
	Mouse β -actin Rev	GCTGGAAGGTGGACAGTGAG
Quantitative PCR in DLD1 and DLD1-F3 cells	Human <i>HBP1</i> Fwd	GTGATGAACACATGGAGCTTG
	Human <i>HBP1</i> Rev	ACATGCCAGATTGGGTAGGA
	Human β -actin Fwd	GGATGCAGAAGGAGATTACTGC
	Human β -actin Rev	CCACCGATCCACACAGAGTA
ChIP RT-qPCR	Human <i>HBP1</i> Fwd	TGTCCCAGACACCAAAACAA
	Human <i>HBP1</i> Rev	CGGGGAAGAGCTGGACTAC
	Human β -globin Fwd	ATGGGACGCTTGATGTTTTCT
	Human β -globin Rev	CCCTGTTACTTATCCCTCTCTC

Table S2 GEO reports in which HBP1 is regulated by growth factors in different cell lines

EGF, epidermal growth factor.

Cells	GEO number	Treatment	Impact on HBP1 expression	Reference
Aortic smooth muscle	GDS2704	PDGF	Down	[3]
Astrocyte	GDS2146	EGF	Down	[4]
MCF7 breast cancer	GDS3484	IGF-1	Down	[5]
HeLa cervical carcinoma	GDS2623	EGF	Down	[6]

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Received 7 November 2013/30 January 2014; accepted 6 March 2014

Published on the Internet 25 April 2014, doi:10.1042/BJ20131467