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

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INTRODUCTION

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), PGF (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.

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 p110α and p110β (PIK3CA and PIK3CB), the PI3K regulatory subunit p85α (PIK3R1), 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 mTOR and FOXO1 by miR-96 and FOXO1 by miR-27a, miR-96 and miR-182 in this cancer [10,11]. Another study suggested that IKK (IkB inhibitor of nuclear factor-kB) 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,
we showed that HBP1 knockdown was enough to increase cell proliferation.

HBP1 is a transcription factor that belongs to the family of HMG proteins, which share a common DNA-binding domain, the HMG-box domain [13]. HBP1 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]. HBP1 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. HBP1 has also been implicated in premature senescence induced by an oncogene through the up-regulation of the cell cycle inhibitor p16INK4A and the down-regulation of DNMT1 (DNA methyltransferase 1) [22–24]. The activity of HBP1 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 HBP1 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 Scientifics). HEK (human embryonic kidney)-293T cells (A.T.C.C., Manassas, V.A., 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 and reagents

DNA and standard supplements. PDGF-BB, FGF-4 and IGF-1 were obtained from Sigma. All of these shRNAs were expressed in pLKO.1 lentiviral vector. On the basis of their efficiency, two constructs for HBP1 [TRCN0000015274 (shHBP1-74) and TRCN0000229982 (shHBP1-2)] 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 μg) was subjected to reverse transcription using MMLV (Moroney 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 GSE44526 and GSE33705 [5,29]). Ba/F3 cells expressing Fip1L1–PDGFRα (PDGF receptor α) or TEL–PDGFRα [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 μg/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 μg) were loaded on to SDS/PAGE (8–10% gel) and transferred on to a PVDF membrane. Western blots were performed with anti-HBP1 (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 HBP1 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 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).
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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–PDGFRα or TEL–PDGFRβ 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, 107 DLD1-F3 and 4 × 107 DLD1 cells were used respectively. DLD1 cells were treated with 10 μM LY294002 (Enzo Life Sciences) or left untreated and DLD1-F3 cells expressing FOXO3-A3–ER or FOXO4-A3–ER were treated or not with 1 μM 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 μg/ml heparin) were then added with [3H]thymidine (0.5 μCi/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 μl, 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 μg/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 μM) for 24 h before RNA or protein extraction. Ba/F3 cells (TEL–PDGFRβ or TEL-IL3) were treated with imatinib (100 nM) or LY294002 (20 μM) 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 μM) or PKB inhibitor (inh) VIII (10 μM) before RNA extraction and for 8 h with LY294002 (10 μM) or MK2206 (5 μM) 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 ± 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.
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Figure 3  HBP1 expression is up-regulated by FOXO activation

(A) DLD1-F3 cells were treated for 8 h with 4-OHT (1 μM), 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 ± 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 PDGFRα or PDGFRβ (Fip1L1-PDGFRα or TEL-PDGFRβ) [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 p110α subunit of PI3K or to PKBα 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, FOXO3-A3-ER 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 ± 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–PDGFRα and Ba/F3 cells transfected with TEL–PDGFRβ. 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–PDGFRβ 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 transfect 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 FOXO1-3 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.dmbr.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 (4k A). 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 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 5 FOXO binds to HBP1 promoter in a PI3K-dependent manner](https://example.com/figure5.png)

(A) DLD1 and DLD1-F3 cells were treated with 4-OHT (1 μM) for 1 h and immunoprecipitation was performed using an anti-ER antibody. Alternatively, DLD1 cells were treated with PKB inhibitor VIII (inh) (10 μM) 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 pair of sequenced reads [36]. (B) DLD1-F3 cells were treated with 4-OHT (1 μM) over 1 h and DLD1 cells were treated with LY294002 (10 μM) 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).
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**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 μg/ml heparin) for 24 h and [3H]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 Chiportal 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
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 HBPI 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 HBPI in the control of cell cycle has already been established [15–19]. Our results showed that HBPI 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 [15–19]. Our results showed that HBPI knockdown was sufficient to increase 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 HBPI 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 HBPI 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 HBPI on cyclin D1 expression (results not shown).

Future studies should analyse whether HBPI 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, HBPI seemed to have the opposite effect. In a breast cancer cell line, HBPI knockdown promotes cell migration [40]. In another study, it was found that HBPI overexpression promoted apoptosis in leukaemic myeloid cells as shown by increased annexin-V staining and FasL mRNA [17]. Thus HBPI 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 HBPI 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 HBPI. In line with these results, growth factors and FOXO regulated HBPI in the breast cancer cell line MCF7. Therefore the regulation of HBPI expression, which we initially observed in breast cancer cells, 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, HBPI 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 HBPI by this miRNA could be mediated directly or indirectly via FOXO1 down-regulation. Altogether, HBPI down-regulation in breast cancers could be explained by the inactivation of FOXO1 subsequent to PI3K constitutive activation or miRNA. IKK, which was also shown to inactivate FOXO1 in breast cancer, could also play a role [12].

In conclusion, we have identified HBPI as a novel transcriptional target of FOXO1, which controls cell proliferation induced by growth factors and may be relevant for tumour development.

**AUTHOR CONTRIBUTION**

Alexandra Coomans de Brachene, Emeline Bollaert, Astrid Eijkelenboom and Audrey de Rocca Serra performed the experiments and analysed the results. Kristian van der Vos, Paul Cofer, 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.

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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 “cgdsr” 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 600 nm. The values at 650 nm were subtracted from the values at 450 nm. This results are the mean±S.E.M. for three independent experiments. *P < 0.05 and **P < 0.01.

1 These authors contributed equally to this work.
2 To whom correspondence should be addressed (email jb.demoulin@uclouvain.be).
**Table S1 List of oligonucleotides**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Gene</th>
<th>Oligonucleotide (5′→3′)</th>
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</thead>
<tbody>
<tr>
<td>Nested PCR for the cloning of pHBP1 in pGL3</td>
<td>HBP1 prom (first PCR)</td>
<td>TGGGTGCTGCAAGAGGCTT</td>
</tr>
<tr>
<td></td>
<td>HBP1 prom Rev (first PCR)</td>
<td>AGCTTTAATCCCTAACACGGA</td>
</tr>
<tr>
<td></td>
<td>HBP1 prom (second PCR)</td>
<td>TATAGCTAGGGAAAAGAAGAGGCCCAACGG</td>
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<td>HBP1 prom Fwd</td>
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<tr>
<td></td>
<td>HBP1 prom Rev</td>
<td>AGCTTTAATCCCTAACACGGA</td>
</tr>
<tr>
<td>Quantitative PCR in human cells: fibroblasts, MCF7, HEK-293T and EOL1 cells</td>
<td>Human HBP1 Fwd</td>
<td>GTGGTGGCTGCAAGAGGCTT</td>
</tr>
<tr>
<td></td>
<td>Human HBP1 Rev</td>
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<tr>
<td></td>
<td>Human RPLP0 Fwd</td>
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<td>Human RPLP0 Rev</td>
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<td>Human FOXO3 Rev</td>
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<td>Quantitative PCR in mouse cells: Ba/F3 and NIH 3T3</td>
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<td>Mouse HBP1 Rev</td>
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<tr>
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<td>Mouse β-actin Fwd</td>
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<td>Mouse β-actin Rev</td>
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<tr>
<td>Quantitative PCR in DLD1 and DLD1-F3 cells</td>
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<td>Human HBP1 Rev</td>
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**Table S2 GEO reports in which HBP1 is regulated by growth factors in different cell lines**

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<tr>
<th>Cells</th>
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<th>Treatment</th>
<th>Impact on HBP1 expression</th>
<th>Reference</th>
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<tr>
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<td>PDGF</td>
<td>Down</td>
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<tr>
<td>Astrocyte</td>
<td>GDS2146</td>
<td>EGF</td>
<td>Down</td>
<td>[4]</td>
</tr>
<tr>
<td>MCF7 breast cancer</td>
<td>GDS3484</td>
<td>IGF-1</td>
<td>Down</td>
<td>[5]</td>
</tr>
<tr>
<td>Hela cervical carcinoma</td>
<td>GDS2623</td>
<td>EGF</td>
<td>Down</td>
<td>[6]</td>
</tr>
</tbody>
</table>

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