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FOXP1 acts through a negative feedback loop to suppress FOXO-induced apoptosis

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Transcriptional activity of Forkhead box transcription factor class O (FOXO) proteins can result in a variety of cellular outcomes depending on cell type and activating stimulus. These transcription factors are negatively regulated by the phosphoinositol 3-kinase (PI3K)–protein kinase B (PKB) signaling pathway, which is thought to have a pivotal role in regulating survival of tumor cells in a variety of cancers. Recently, it has become clear that FOXO proteins can promote resistance to anti-cancer therapeutics, designed to inhibit PI3K–PKB activity, by inducing the expression of proteins that provide feedback at different levels of this pathway. We questioned whether such a feedback mechanism may also exist directly at the level of FOXO-induced transcription. To identify critical modulators of FOXO transcriptional output, we performed gene expression analyses after conditional activation of key components of the PI3K–PKB–FOXO signaling pathway and identified FOXP1 as a direct FOXO transcriptional target. Using chromatin immunoprecipitation followed by next-generation sequencing, we show that FOXP1 binds enhancers that are pre-occupied by FOXO3. By sequencing the transcriptomes of cells in which FOXO is specifically activated in the absence of FOXP1, we demonstrate that FOXP1 can modulate the expression of a specific subset of FOXO target genes, including inhibiting expression of the pro-apoptotic gene *BIK*. FOXO activation in FOXP1-knockdown cells resulted in increased cell death, demonstrating that FOXP1 prevents FOXO-induced apoptosis. We therefore propose that FOXP1 represents an important modulator of FOXO-induced transcription, promoting cellular survival.
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Forkhead box transcription factor class O (FOXO) proteins are important regulators of cell fate outcome, capable of inducing cell death as well as promoting cell survival and adaptation to environmental stress.[1] In mammals, this Forkhead subfamily consists of four members, of which the three predominant FOXO1, FOXO3 and FOXO4 display a high degree of redundancy.[2] Given their importance in modulating cellular survival, it is not surprising that FOXO activity is tightly regulated by different modes of cellular signaling. Receptor tyrosine kinase (RTK) signaling activates phosphoinositol 3-kinase (PI3K) and subsequently protein kinase B (PKB, also known as Akt), which directly phosphorylates FOXO proteins. This results in the nuclear exclusion and consequently inactivation of FOXO.[3] The PI3K–PKB–FOXO signaling module is evolutionary conserved and has been associated with regulating stress resistance and longevity in a variety of species, including the nematode *Caenorhabditis elegans*,[4] the fruit fly *Drosophila melanogaster*[5,6] and mammals.[7,8] Importantly, active PI3K–PKB signaling is a major pro-oncogenic pathway driving tumorigenesis in a diversity of tumor types.[9] Considerable effort has been directed towards the development of therapeutic agents that directly or indirectly inhibit this signal transduction pathway, such as specific PI3K and PKB, or RTK inhibitors.[10] As a consequence of this inhibition, FOXO is no longer phosphorylated by PKB, translocates to the nucleus and induces target gene expression. In addition, nuclear translocation and activation of FOXO can also be induced by various classes of chemotherapeutic compounds,[11] possibly in response to phosphorylation by p38.[12] In the nucleus, FOXO proteins induce the transcription of genes involved in a variety of biological processes, which ultimately can result in different phenotypic outcomes, depending on cell type and activating stimulus. Indeed, the chemotherapeutic agent cisplatin was shown to induce cell death through FOXO3 activation in colon carcinoma cells.[13] In contrast, leukemic cells treated with doxorubicin were reported to develop resistance through a FOXO-induced feedback mechanism by upregulation of *PIK3CA*, which ultimately result in increased PI3K–PKB activity.[14] Furthermore, whereas FOXO3 was shown to mediate breast cancer cell death in response to human epidermal growth factor receptor 2 inhibition,[15] PKB inhibition increases

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FOXO-dependent RTK signaling through upregulation of HER3.[16] These reports indicate that upon FOXO activation, cell survival mechanisms are transcriptionally induced by inducing feedback at various levels of the PI3K–PKB–FOXO pathway, including at the level of RTK signaling[16] and PI3K signaling.[14] However, to date a feedback circuit at the nuclear level has not been reported. Nevertheless, such a feedback mechanism does represent a reasonable manner for regulating the outcome of FOXO transcriptional activity, particularly as FOXO is known to have diverse nuclear interaction partners in various cellular systems.[17] For example, FOXO was reported to associate with SMAD transcription factors in regulating $p21^{Cip1}$ expression and thereby cell proliferation,[18] and with β-catenin in regulating transcriptional response to oxidative stress.[19] We speculated that if such a feedback mechanism exists, it could represent a potentially important therapeutic target given the importance of the PI3K–PKB–FOXO pathway in tumor development and in response to anti-cancer treatment. We therefore set out to identify FOXO transcriptional targets that in turn may directly modulate the transcriptional outcome of FOXO proteins. Here, we identify another Forkhead box protein, FOXP1, as a direct transcriptional target of FOXO. Importantly, we demonstrate that FOXP1 directly modulates FOXO-induced transcription by binding the same enhancers and modifying the expression of specific subsets of target genes resulting in cellular survival.

Results

FOXP1 is transcriptionally regulated by PI3K–PKB–FOXO signaling. To investigate the transcriptional outcome of PI3K–PKB–FOXO signaling, we systematically analyzed gene expression changes in the murine bone marrow-derived cell line Ba/F3, in which clones were generated that ectopically express conditionally active variants of PI3K, PKB, FOXO3 and FOXO4.[20] To selectively activate these proteins, constitutively active versions of PI3K (myrPI3K), PKB (myrPKB), FOXO3 (FOXO3A3) or FOXO4 (FOXO4A3) were fused to the hormone-binding domain (HBD) of the estrogen receptor (ER), rendering them conditionally active after addition of tamoxifen (4-OHT). Subsequently, we performed gene expression microarray analyses to identify specific pathway transcriptional targets (Figure 1a).[20] To identify target genes that potentially affect the transcriptional outcome of FOXO activation, we applied gene ontology (GO)-term analysis. Interestingly, we observed enrichment for genes involved in negative regulation of transcription (Figure 1b). Among these, we found *Mxi1* and *Cited2*, both recently described to be regulated by PI3K–PKB–FOXO activity, validating our approach.[21,22]

Using this approach, we identified the Forkhead box protein FOXP1 as a transcriptional target (Figure 1b). As members of the Forkhead box family share a highly conserved DNA-binding domain, they could therefore potentially regulate expression of overlapping target genes.[23] Independent *Foxp1* mRNA evaluation using quantitative real-time PCR (qRT-PCR) confirmed transcriptional upregulation in murine Ba/F3 cells upon FOXO3A3–ER or FOXO4A3–ER activation (Figure 1c). A well-characterized cellular model system for studying FOXO activation is the human colon carcinoma cell line DLD1, which has been engineered to express FOXO3A3–ER (DL23).[20,24] Specific FOXO3A3–ER activation in this system also increased *FOXP1* mRNA expression (Figure 1d). Importantly, wild-type cells do not increase *FOXP1* mRNA levels upon 4-OHT treatment, indicating specificity of the fusion protein in this response (Figures 1c and d). Correspondingly, activation of FOXO3(A3)–ER increased FOXP1 protein levels in various cell types, including DL23 cells (Figure 2a) and the human osteosarcoma cell line U2OS (Figure 2b), demonstrating that this observation is not restricted to a single cell type. Cells expressing only the ER HBD did not show increased FOXP1 protein expression after 4-OHT treatment, demonstrating the specificity of FOXO3 in FOXP1 upregulation (Figure 2b). Indeed, activation of endogenous FOXO by inhibition of PI3K–PKB signaling or induction of a variety of environmental stress signals similarly increased FOXP1 protein levels (Figure 2c and Supplementary Figure 1).

The rapid increase in *FOXP1* mRNA expression after specific activation of FOXO3 ($\sim$3-fold increase after 2 h of 4-OHT treatment (Figure 1d)) suggests that FOXP1 expression is most likely directly regulated. Indeed, bioinformatics analysis of the genome-wide binding profile of FOXO3 in DLD1 cells, as determined by chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq),[25] showed specific transcription factor binding to the genomic locus where the *FOXP1* gene is located after direct FOXO activation by 4-OHT treatment of DL23 cells as well as by indirect FOXO activation through PKB inhibition of DLD1 cells (Figure 3a). Importantly, 4-OHT treatment of DL23 cells also results in RNA polymerase II (RNAPII) recruitment to two transcription start sites (TSSs) of the *FOXP1* gene (Figure 3a). RNAPII occupancy is not influenced by mRNA stability and therefore a more direct measurement for transcriptional activity.[26] To validate these analyses, we evaluated FOXO3 recruitment to the *FOXP1* genomic locus in response to PKB inhibition by ChIP using a FOXO3-specific antibody followed by qPCR (ChIP-qPCR). Indeed upon PKB inhibition, we observed a significant and specific enrichment of FOXO3 binding to the genomic region that was identified by the ChIP-seq analysis (Figure 3b). A similar result was obtained upon PI3K inhibition (Supplementary Figure 2a) and also when the assay was performed in DL23 cells upon 4-OHT treatment using a specific antibody against the ER moiety of the fusion protein (Supplementary Figure 2b). Taken together, we demonstrate that activation of FOXO3 transcriptionally upregulates FOXP1 expression by direct binding of FOXO3 to a *FOXP1* enhancer region, followed by RNAPII recruitment.

FOXP1 and FOXO3 proteins bind the same enhancers. Next, we questioned whether FOXP1 could directly affect the transcriptional outcome of FOXO3 activation by binding similar regulatory elements in the genome. To identify FOXP1 genomic-binding sites, we performed ChIP-seq in DL23 cells, which were treated with 4-OHT to ensure high FOXP1 expression levels. We made use of a validated FOXP1 antibody, which has previously been used for a similar approach.[27] In total, we identified 23 050 peaks

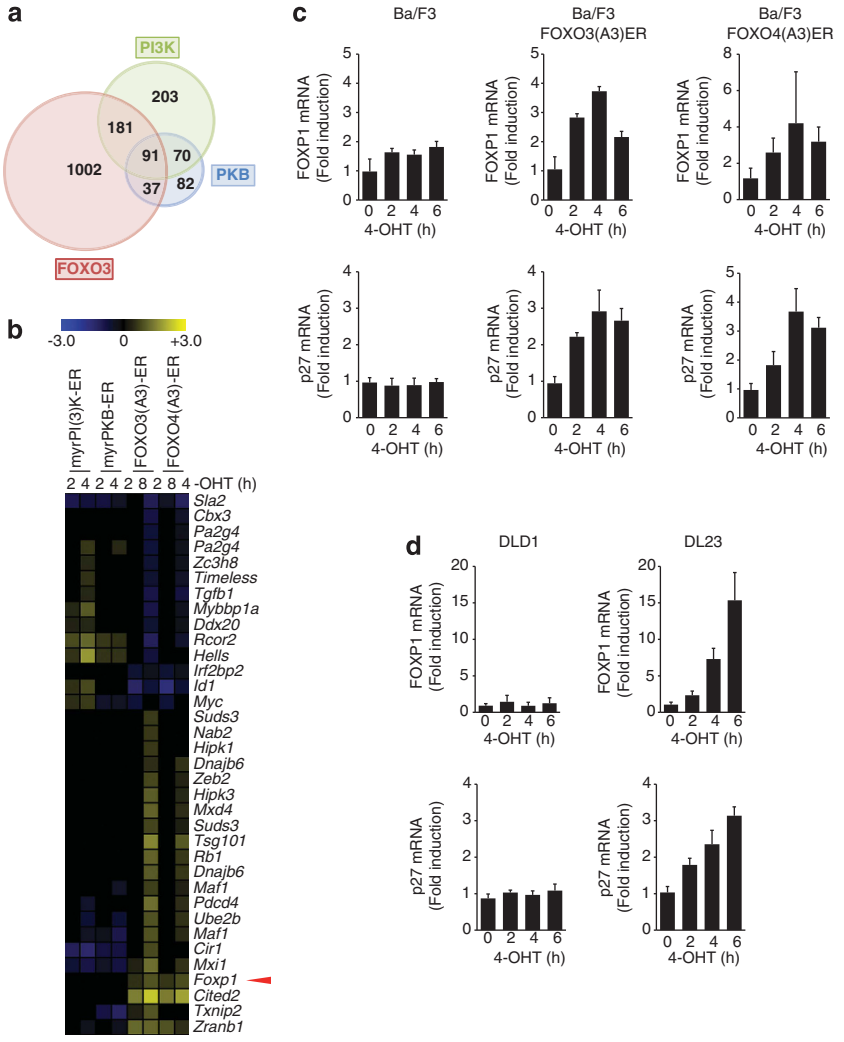

Figure 1 PI3K–PKB–FOXO signaling regulates FOXP1 mRNA expression levels. (**a**) Ba/F3 cells expressing myrPI3K–ER, myrPKB–ER or FOXO3(A3)–ER were stimulated with 100 nM 4-OHT for 2 and 4 h (myrPI3K–ER and myrPKB–ER) or for 2 and 8 h (FOXO3(A3)–ER and FOXO4(A3)–ER) and microarray analyses were performed. Shown is a Venn diagram with the number of genes, which show a differential expression of at least 1.5-fold after 4 h stimulation of myrPI3K–ER and myrPKB–ER and 8 h stimulation of FOXO3(A3)–ER. (**b**) FOXO3 target genes that have a GO-term for the biological process negative regulation of transcription. Data are represented as log2 mean values of one experiment performed in quadruplicate. (**c**) Wild-type Ba/F3 cells and cells expressing FOXO3(A3)–ER or FOXO4(A3)–ER were treated with 100 nM 4-OHT, and RNA was isolated after the indicated time points. Relative mRNA levels of FOXP1 and $p27^{Kip1}$, a known FOXO target gene,[28] were analyzed using qRT-PCR. Data are represented as mean ± S.D. normalized for *GAPDH* ($n = 3$). (**d**) Wild-type DLD1 cells and cells expressing FOXO3(A3)–ER (DL23) were treated with 100 nM 4-OHT, and RNA was isolated after the indicated time points. Relative mRNA levels of FOXP1 and $p27^{Kip1}$ were analyzed using qRT-PCR. Data are represented as mean ± S.D. normalized for *GAPDH* ($n = 3$)

across the genome, which were mostly located outside promoter regions in distal intergenic regions, suggesting FOXP1 regulates gene expression by binding distal enhancers (Supplementary Figure 3 and Supplementary Table 1). In line with our hypothesis, FOXP1 binding was found to be enriched in promoter regions of known FOXO targets, including $p27^{Kip1}$ and *CITED2* (Figure 4a).[21,28] Interestingly, a binding region in the promoter of the *FOXP1* gene itself was among the most enriched binding sites, suggesting it can regulate its own expression (Figure 4a). Multiple detected regions, including binding sites near the *FOXP1*, *CITED2* and *BIK* genes, have previously been identified as FOXP1-binding sites in different cellular systems using ChIP-seq technology,[27,29] validating the specificity of our approach. Importantly, we could independently validate FOXP1 recruitment to these loci in DL23 cells upon 4-OHT treatment by ChIP-qPCR (Figure 4b). *De-novo* motif search verified the presence of a Forkhead-binding motif in a large proportion of the peaks (Figure 4c). The most enriched Forkhead-binding motif (TGTTTAC), which is present in ~30% of the peaks, has recently been shown to be the most optimal binding site for FOXP1 in embryonic stem cells.[27] Notably, this motif has also been described to be to most optimal binding site for FOXO3 in different cell systems, including DL23.[25,30] In addition, we found enrichment for AP-1- and GATA-binding motifs in the FOXP1 peaks, which were also found to be enriched in FOXO3-binding regions,[31] further supporting our hypothesis that these two transcription

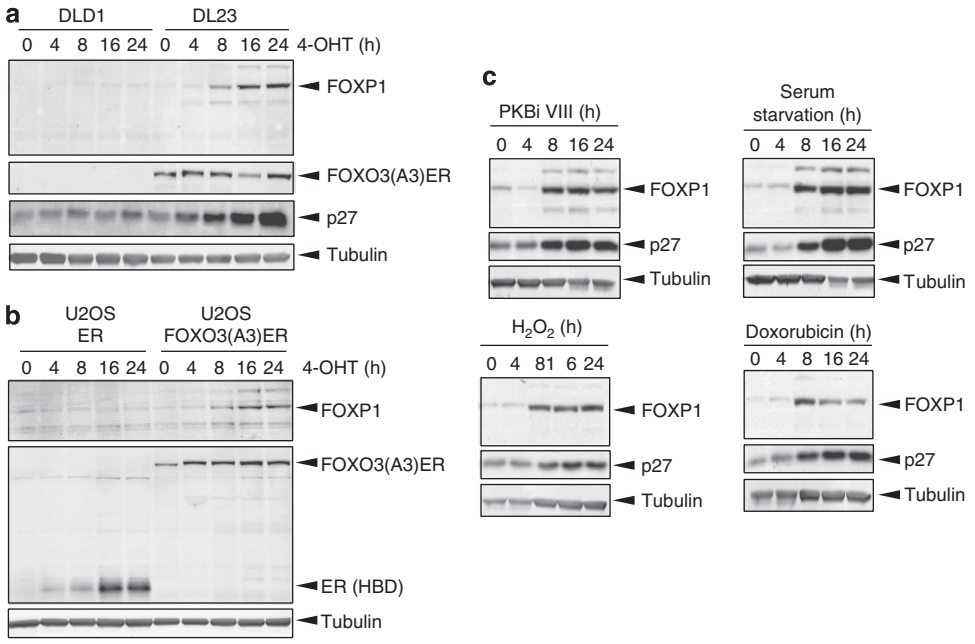

Figure 2 PI3K–PKB–FOXO signaling regulates FOXP1 protein levels. (**a**) DLD1 and DL23 cells were treated with 100 nM 4-OHT for the indicated time points. Cell lysates were analyzed for protein levels of FOXP1, FOXO3(A3)–ER, $p27^{Kip1}$ and tubulin. Shown are representative blots ($n = 3$). (**b**) U2OS cells expressing the HBD of ER or FOXO3(A3)–ER were treated with 100 nM 4-OHT for the indicated time points. Cell lysates were analyzed for protein levels of FOXP1, ER and tubulin. Shown are representative blots ($n = 3$). (**c**) Wild-type DLD1 cells were treated with 10 μM PKB inhibitor VIII, 200 μM H2O2, 0.5 μM doxorubicin or serum starved in DMEM containing 0.2% fatty acid-free BSA for the indicated time points. Cell lysates were analyzed for protein levels of FOXP1, $p27^{Kip1}$ and tubulin

factors bind similar genomic loci. Indeed, when we compared the genome-wide overlap of FOXP1-binding sites with previously determined FOXO3-binding sites in the same cellular system,[25] a considerable overlap was observed (Figure 4d). A similar degree of overlap was observed when we compared the binding sites of FOXP1 and FOXO3(A3)–ER, which was determined by ChIP-seq utilizing an antibody against ER in DL23 cells upon 4-OHT treatment (Supplementary Figure 4).[25] Of note, the duration of PI3K–PKB inhibition used to determine FOXO3 binding (30 min, Figure 4d) was much shorter than the duration of 4-OHT treatment for establishing FOXP1 genomic-binding sites (16 h, Figure 4d), suggesting FOXO3-induced expression of FOXP1 may result in displacement of FOXO3 from DNA-binding sites. To rule out direct interaction of FOXO3 and FOXP1, we performed co-immunopreciptation experiments using overexpressed tagged versions of the two transcription factors, but did not observe any interaction (Supplementary Figure 5). From these data, we conclude that FOXP1 and FOXO3 share a large proportion of genomic-binding sites and that their binding most probably occurs sequentially.

FOXP1 regulates expression of a specific subset of FOXO target genes. To initially investigate the effect of FOXP1 on FOXO3-mediated expression, we performed a FOXO reporter assay.[30] Transfection of FOXO3 with a luciferase reporter construct containing multiple copies of the core FOXO-binding site (TTGTTTAC) resulted in a strong activation of luciferase activity (Figure 4e). Cotransfection of FOXP1 inhibited FOXO3-induced luciferase activity in a dose-dependent manner (Figure 4e), suggesting that FOXP1 can negatively regulate FOXO-induced transcription. To test the effect of FOXP1 on global FOXO3-induced gene expression, we transfected DL23 cells with short interfering RNA (siRNA) targeting *FOXP1*, allowing specific activation of FOXO3(A3)–ER in the presence or absence of FOXP1 knockdown (KD) (Figure 5a). Subsequently, we performed gene expression analysis by next-generation sequencing of transcriptomes (RNA-seq). First, we defined genes regulated by FOXO with expression changes of at least twofold in cells transfected with control siRNA (siSCR) and treated with 4-OHT for 8 h compared with untreated siSCR-transfected cells. Using these criteria, we identified 3014 genes that were differentially expressed after FOXO3(A3)–ER activation, including previously identified FOXO target genes (Figure 5b and Supplementary Table 2). Next, we determined the number of genes that were at least twofold differentially expressed in FOXP1-KD cells treated with 4-OHT compared with siSCR-transfected cells. In total, 1296 differentially expressed genes were identified of which 273 have a FOXP1 ChIP-seq peak at least 10 kb from the TSS (Figure 5b). In line with the 6xDBE reporter assay (Figure 4e), many genes that have a FOXP1 ChIP-seq peak within 10 kb of the TSS were found to be upregulated in FOXP1-depleted cells upon 4-OHT treatment compared with control cells, such as *LGR4* and *ADM* (Figure 5b). This suggests that FOXP1 inhibits FOXO-induced expression of these genes. However, we also observed many FOXO-regulated genes that were unaffected by FOXP1 depletion, but have a FOXP1 ChIP-seq peak within 10 kb of the TSS, such as $p27^{Kip1}$, *CITED2* and *GADD45B* (Figures 4a and 5b). Furthermore, the expression of another subset of genes that have a FOXP1 ChIP-seq peak within 10 kb of the TSS was found to be negatively affected by FOXP1 KD, including

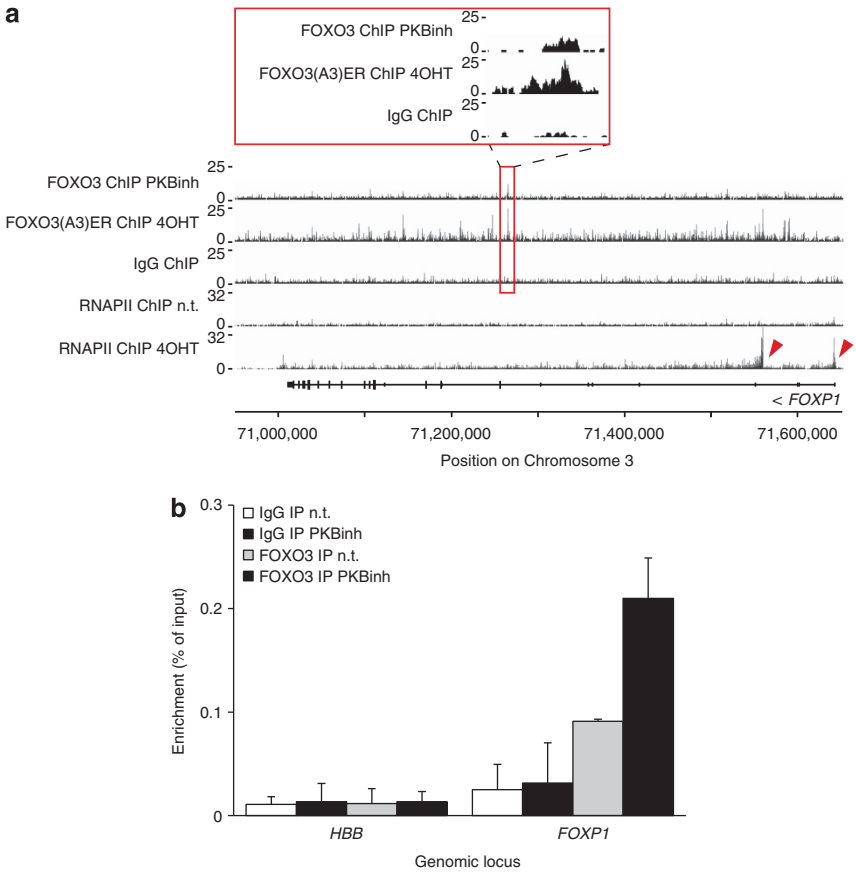

Figure 3 FOXO3 directly regulates FOXP1 expression by binding its gene locus and recruiting RNAPII. (**a**) Browser view of FOXO3, FOXO3(A3)–ER and RNAPII binding at the genetic locus of *FOXP1* in ChIP-seq experiments performed with antibodies against endogenous FOXO3, ER or RNAPII in, respectively, DLD1 or DL23 cells.[25] Red arrow heads indicate location of putative TSS. (**b**) DLD1 cells were treated with 10 μM PKB inhibitor VIII and fixed after 30 min. Subsequently, ChIP-qPCR was performed with an antibody against endogenous FOXO3 or IgG and primers for the locus indicated in (**a**). Data are represented as mean ± S.D. normalized for input ($n = 2$)

ABL1 and *GLUL*, showing that at some loci FOXP1 could function as a positive transcriptional regulator in response to FOXO activation (Figure 5b).

These data suggest that FOXP1 can modify FOXO-induced transcription toward a specific cellular outcome by specifically repressing the expression of a subset of genes while inducing the expression of others. To predict the effect of FOXP1 activity on cellular fate, we performed GO-term analyses evaluating the genes that were differentially expressed in FOXP1-KD cells upon FOXO activation compared with control cells. Interestingly, genes that were upregulated in FOXP1-depleted cells compared with control cells showed enrichment for apoptosis, including genes such as *BIK* and *FAS* (Figures 5c and d). In contrast, the expression of FOXO target genes that were negatively affected by FOXP1 depletion showed enrichment for cell migratory processes, including genes such as *ETS1* and *EFNB2* (Figure 5c). This suggests that on one hand FOXP1 can induce cell survival by inhibiting FOXO-induced expression of pro-apoptotic genes, while on the other hand it may promote cell migration (Figure 5c). Independent evaluation of *BIK* mRNA and protein levels verified that this gene is indeed negatively regulated by FOXP1 activity upon FOXO activation (Figures 5e and f). Importantly, the presence of FOXP1 ChIP-seq peak within the *BIK* gene locus suggests that FOXP1-mediated repression of this gene occurs in a direct fashion (Supplementary Figure 6).

FOXP1 prevents FOXO3-induced cell death. Increased expression of BIK is known to result in apoptosis through mitochondrial-induced cell death.[32] Depending on cell type, FOXO activity can either induce cell death or cell cycle arrest. In DLD1 cells, FOXO-induced transcription results in cell cycle arrest in a $p27^{Kip1}$-dependent manner.[31] Here, we show that FOXP1 negatively affect FOXO-dependent transcription of genes involved in inducing cell death, including *BIK*. Therefore, we questioned whether FOXP1 can indeed promote cellular survival in response to FOXO activation in this system. To test this, we performed FOXP1 KD in DL23 cells, specifically activated FOXO3 by 4-OHT and measured the protein levels of cleaved caspase-3 as a measure of apoptosis. Increased expression of cleaved caspase-3 was only observed upon activation of FOXO3A3–ER in FOXP1-KD cells (Figure 6a), suggesting that FOXP1 expression prevents FOXO3-induced cell death. Indeed, whereas FOXO3 activation in DL23 cells induces an arrest in cell proliferation, we observed significant decreased survival in response to 4-OHT treatment of FOXP1-depleted cells

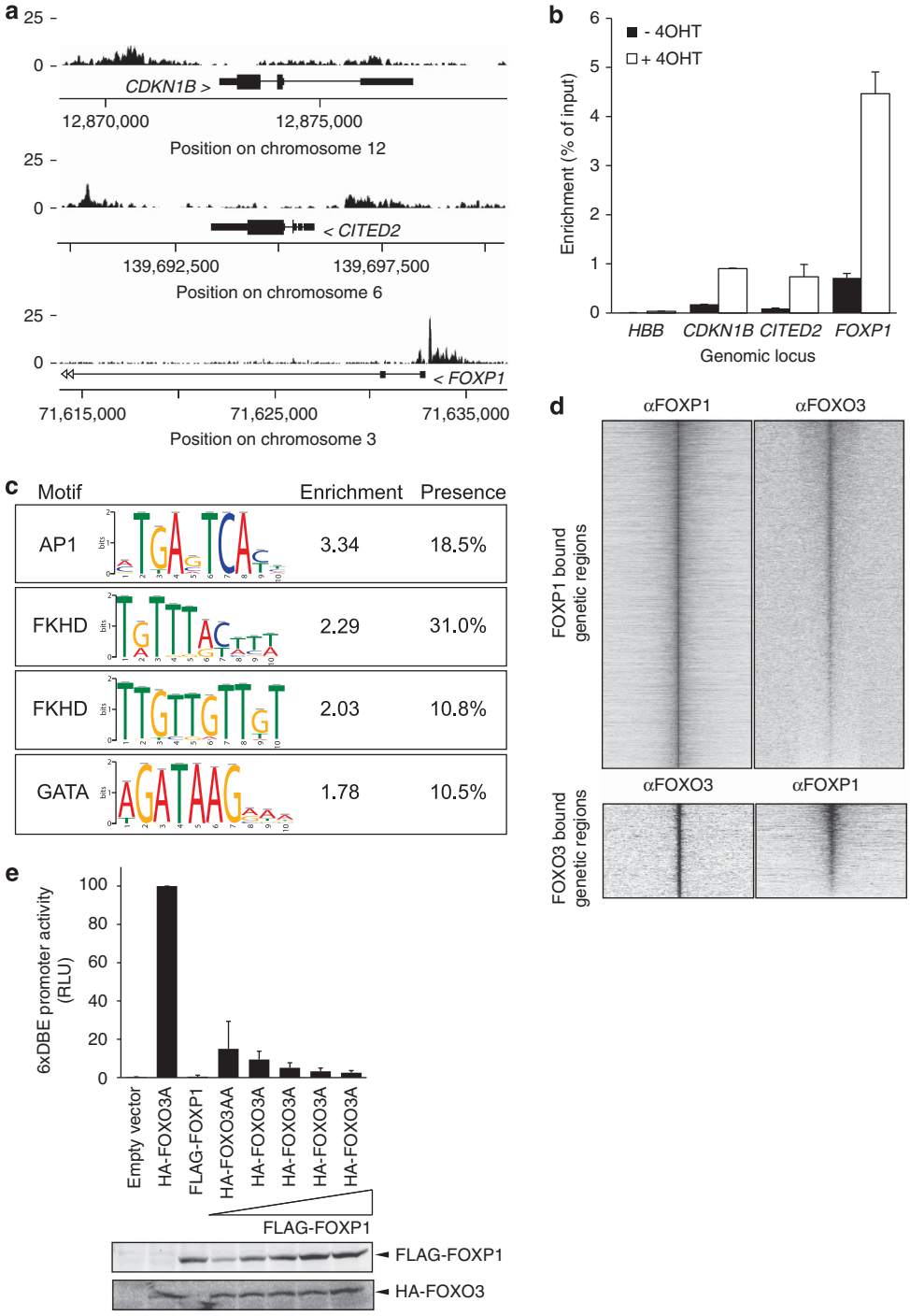

Figure 4 FOXP1 and FOXO3 show a large overlap in DNA-binding sites. (**a**) DL23 cells were treated with 100 nM 4-OHT and fixed after 16 h. Subsequently, ChIP-seq was performed with an antibody against endogenous FOXP1. Shown are representative plots of reads aligned to the *CDKN1B*, *CITED2* and *FOXP1* gene loci. (**b**) DL23 cells were either not treated (− 4-OHT) or treated with 4-OHT for 16 h (+ 4-OHT) followed by ChIP-qPCR using an antibody against FOXP1, and primers for the gene loci shown in (**a**). The gene locus of *HBB* serves as a negative region, which does not bind FOXP1. Data are represented as mean ± S.D. normalized for input ($n = 2$). (**c**) *De-novo* motif analysis reveals the presence of four enriched motifs in the FOXP1-binding regions. Relative enrichment to control regions and percentage of peaks containing the motif are shown. (**d**) Upper panels: heat maps of, respectively, FOXP1 or FOXO3 occupancy at all FOXP1-bound genomic regions. Each row represents the 10 kb region surrounding the FOXP1 peak center. Regions are sorted by descending FOXO3 signal. Signal intensities are normalized for sequencing depth. Heat maps are shown with white indicating no signal and black as high signal. Lower panels: heat maps of, respectively, FOXO3 or FOXP1 occupancy at all FOXO3-bound genomic regions. Regions are sorted by descending FOXP1 signal. (**e**) FOXP1 inhibits FOXO3 activity in a 6xDBE reporter assay. A reporter plasmid carrying six core FOXO-binding sites (TTGTTTAC) in front of a gene encoding luciferase (6xDBE) was transfected in HEK293 together with Renilla, HA-tagged FOXO3 and/or FLAG-tagged FOXP1. Luciferase activity was measured 48 h after transfection. Data are depicted as relative luciferase units compared with FOXO3-activated luciferase, which is set at 100. Shown are mean ± S.D. values ($n = 3$). Lower panels show western blot analysis with antibodies against HA or FLAG of representative samples used in the reporter assay

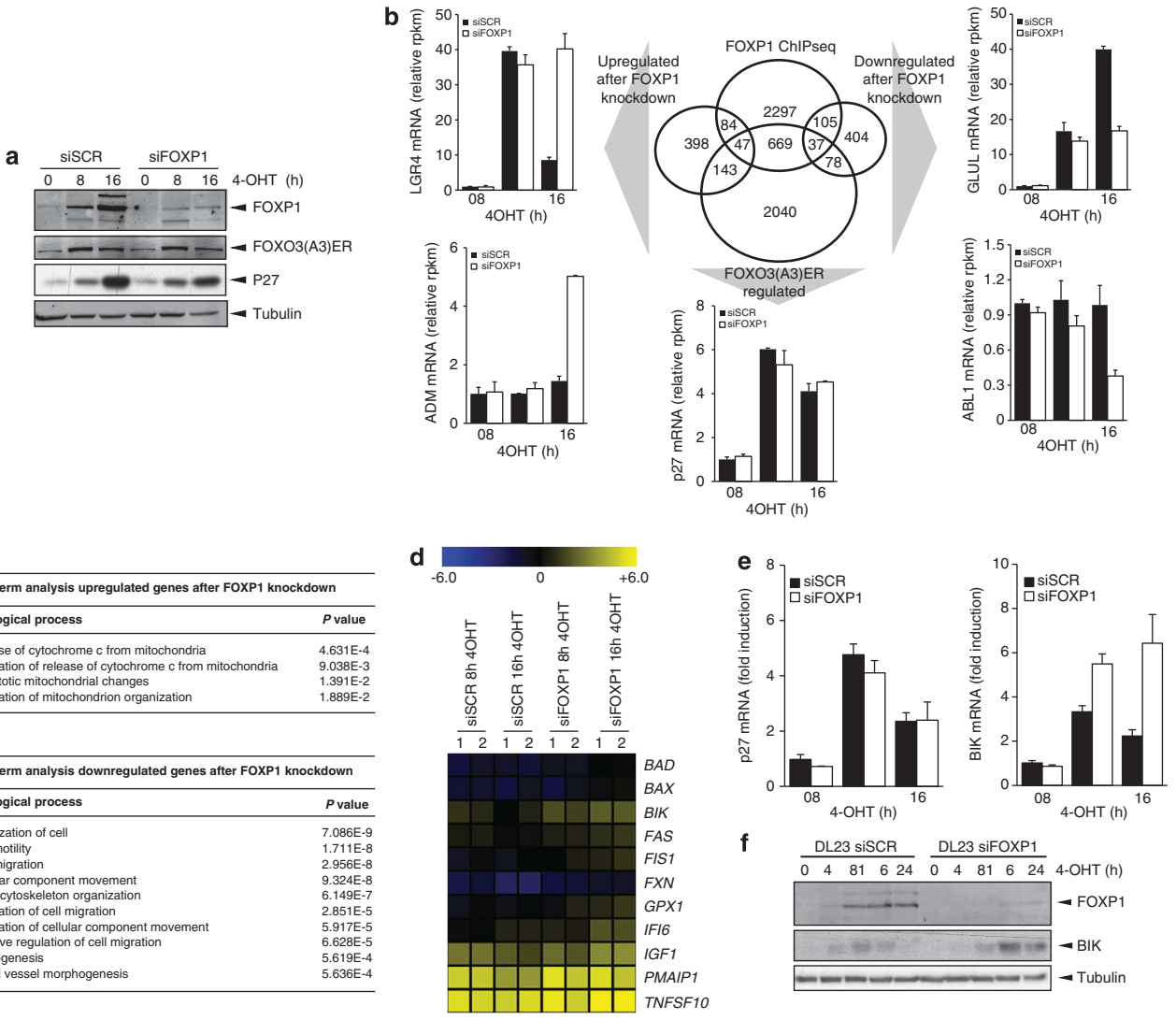

Figure 5 FOXP1 modulates FOXO3-induced transcription. (**a**) DL23 cells were transfected with control siRNA (siSCR) or siRNA against FOXP1 (siFOXP1), and subsequently treated with 100 nM 4-OHT for the indicated time points. Cell lysates were analyzed for protein levels of FOXP1, FOXO3(A3)–ER, $p27^{Kip1}$ and tubulin. Shown are representative blots ($n = 3$). (**b**) FOXP1-depleted DL23 cells or cells transfected with control siRNA were treated with 100 nM 4-OHT for the time point indicated in (**a**). Subsequently, RNA was isolated and analyzed by next-generation sequencing. Shown is a Venn diagram with the number of genes (1) that showed a FOXP1-binding peak at least 10 kb from the TSS as determined by ChIP-seq (FOXP1 ChIP-seq), (2) that showed a differential expression of at least twofold after 8 h stimulation of FOXO3(A3)–ER compared with untreated DL23 cells (FOXO3(A3)–ER regulated), (3) that showed an increased expression of at least twofold in FOXP1-depleted DL23 cells compared with siSCR-transfected cells after 16 h FOXO3(A3)–ER stimulation (upregulated after FOXP1 knockdown) and (4) that showed an decreased expression of at least twofold in FOXP1-depleted DL23 cells compared with siSCR-transfected cells after 16 h FOXO3(A3)–ER stimulation (downregulated after FOXP1 knockdown). In addition, individual examples of genes that have a FOXP1 ChIP-seq peak within 10 kb of the TSS that are differentially expressed by FOXP1 depletion are shown. Stimulation of FOXO3(A3)–ER increases the expression of $p27^{Kip1}$, but this is not affected by FOXP1 depletion (*lower panel*). In contrast, although stimulation of FOXO3(A3)–ER results in an initial increase in *LGR4* expression, prolonged stimulation decreases *LGR4* expression levels, which is dependent on FOXP1 (upper left panel). The expression levels of *ADM* are not affected by FOXO3(A3)–ER stimulation; however, do increase after stimulation of FOXP1-depleted cells (lower left panel). Stimulation of FOXO3(A3)–ER results in increased expression of *GLUL*, which is less profound after FOXP1 depletion (upper right panel). The expression of *ABL1* is not affected by FOXO3(A3)–ER stimulation; however, is decreased after stimulation of FOXP1-depleted cells (lower right panel). Data are represented as mean ± S.D. reads per kilobase per million reads (RPKM) normalized for untreated siSCR-transfected cells ($n = 2$). (**c**) GO-term analysis of genes that show either an increased (upper panel) or decreased (lower panel) expression in FOXP1-depleted DL23 cells compared with control cells after stimulation of FOXO3(A3)–ER. (**d**) Target genes that have GO-terms related to apoptosis as described in (**c**). Data are represented as log2 mean RPKM values ($n = 2$). (**e**) DL23 cells transfected with siSCR or siFOXP1 were treated with 100 nM 4-OHT for the indicated time points. Relative mRNA levels of $p27^{Kip1}$ and BIK were analyzed using qRT-PCR. Data are represented as mean ± S.D. normalized for GAPDH ($n = 3$). (**f**) DL23 cells transfected with siSCR or siFOXP1 were treated with 100 nM 4-OHT for the indicated time points. Cell lysates were analyzed for protein levels of FOXP1, BIK and tubulin. Shown are representative blots ($n = 3$)

(Figures 6b and d). Importantly, FOXP1 KD in untreated DL23 cells does not result in decreased survival compared with control cells (Figures 6b and c). Therefore, FOXP1 specifically prevents FOXO3-induced cell death, and not just an increase in overall survival. Taken together, we show that FOXP1 is an important molecular determinant of FOXO-induced transcriptional outcome by protecting against FOXO3-induced cell death.

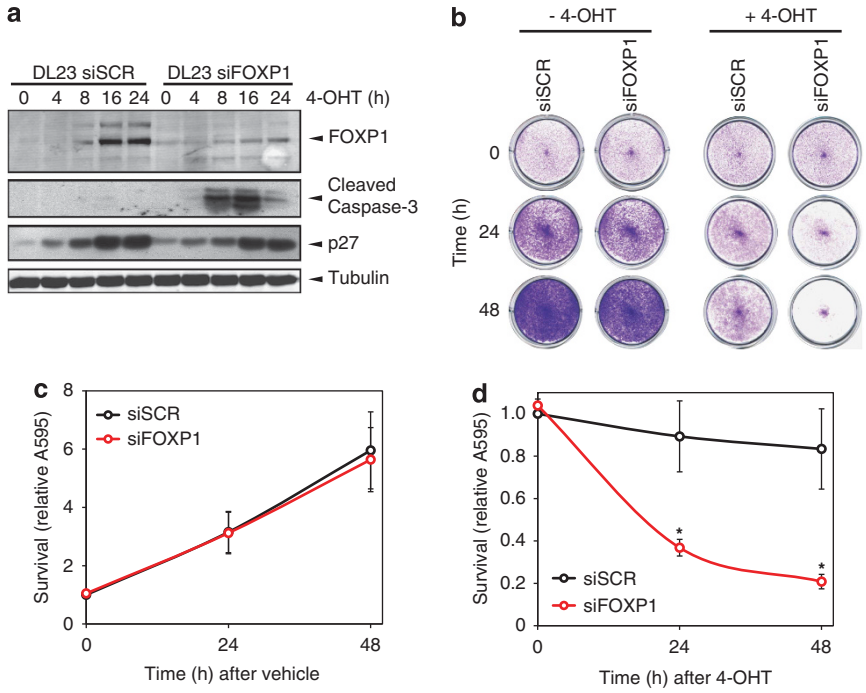

Figure 6 FOXP1 prevents FOXO3-induced apoptosis. (**a**) DL23 cells transfected with control siRNA (siSCR) or siRNA against FOXP1 (siFOXP1) were treated with 100 nM 4-OHT for the indicated time points. Cell lysates were analyzed for protein levels of FOXP1, cleaved Caspase-3, $p27^{Kip1}$ and tubulin. Shown are representative blots ($n=3$). (**b**) DL23 cells transfected with siSCR or siFOXP1 were not treated ($-$ 4-OHT) or treated with 100 nM 4-OHT ($+$ 4-OHT) for the indicated time points, fixed and stained with crystal violet. Shown are representative scans ($n=3$). (**c**) DL23 cells transfected with siSCR or siFOXP1 were fixed at the indicated time points and stained with crystal violet. The crystal violet was subsequently extracted and the dye concentration was determined by measuring absorption at A_{595}. Data are represented as mean ± S.D. normalized for the $t=0$ h value of cells transfected with control siRNA ($n=3$). (**d**) DL23 cells transfected with siSCR or siFOXP1 and treated with 100 nM 4-OHT for the indicated time points, fixed and stained with crystal violet. The crystal violet was subsequently extracted and the dye concentration was determined by measuring absorption at A_{595}. Data are represented as mean ± S.D. normalized for the $t=0$ h value of cells transfected with control siRNA ($n=3$)

Discussion

Activation of FOXO-induced transcription can result in a variety of cellular outcomes, including cell cycle arrest or cell death, which is cell and tissue type dependent.[33] Indeed, whereas FOXO activity induces cell cycle arrest in DLD1 cells, A14 cells, mouse embryonic fibroblasts and 786-0 cells,[31,34] it induces cell death in bone marrow-derived Ba/F3 cells.[35] However, the molecular mechanisms underlying this cell type specificity remains poorly understood. One explanation for cell type-specific outcome of FOXO activation is the availability of functionally interacting proteins, such as other transcription factors or transcriptional cofactors. Here, we demonstrate a novel feedback mechanism in which increased FOXP1 expression prevents FOXO-induced cell death by directly modulating the expression of a specific subset of FOXO target genes, including *BIK* (Figure 5d). Although FOXO proteins also induce the expression of FOXP1 in Ba/F3 cells, these cells still induce apoptosis in response to FOXO activation, in contrast to DLD1 cells where FOXP1 induction prevents FOXO-induced cell death. One explanation for this difference could be that the increase in FOXP1 expression by FOXO3 in Ba/F3 cells is not as prominent as in DLD1 cells (3 to 4-fold increase in Ba/F3 *versus* 15-fold in DLD1, Figures 1c and d). Furthermore, FOXO3-induced FOXP1 mRNA expression appears to be transient in Ba/F3 cells (Figure 1c), whereas this remains high in DLD1 cells (Figure 1d). Another explanation relates to the observations that in Ba/F3 cells FOXO3 induces apoptosis through upregulation of the pro-apoptotic protein BIM.[35] We did not observe upregulation of *BIM* mRNA in FOXP1-KD DL23 cells upon FOXO3 activation, suggesting that BIM is not a FOXP1 target. Indeed, we do not observe a binding peak of FOXP1 in the genomic locus of *BCL2L11*, the gene that encodes BIM. This suggests that apoptosis is induced through a distinct pathway in Ba/F3 cells that is apparently unaffected by FOXP1 activity.

In naïve T cells, it has been shown that FOXP1 antagonizes FOXO1-induced expression of *IL7RA* by directly competing for enhancer-binding site.[36] Our observation that FOXP1 and FOXO3 share a significant number of DNA-binding sites at a genome-wide scale, suggests that antagonizing FOXO-induced transcription may be a general feature of FOXP1. Indeed, we demonstrate that FOXP1 modulates FOXO3-induced gene expression, both in a reporter assay as well as at a global level. However, not all genes that can be bound at their enhancers by both transcription factors are transcriptionally downregulated by FOXP1, arguing against a general antagonistic role in relation to FOXO-induced gene expression. Indeed, the expression of many genes, including prototypic FOXO target genes such as $p27^{Kip1}$, *CITED2* and *GADD45B*, is not affected by binding of FOXP1 to their enhancers. Of note, it is not likely that FOXO3 and FOXP1 interact at these genomic sites, as we could not detect any interaction between the two transcription factors by

co-immunoprecipitation. This suggests that although the expression levels of the target genes are not affected by FOXP1, FOXO3 is likely to be replaced from the enhancers by FOXP1. In addition, the FOXO-induced expression of a subset of genes seems to be enhanced in the presence FOXP1 as depletion of it results in lower FOXO-induced expression levels. These FOXP1-dependent gene expression differences may suggest that the presence or absence of yet other cofactors or transcription factors at specified enhancers can be important to determine if a FOXP1 target is up- or downregulated in response to FOXO activation. In support of this hypothesis, we do find significant enrichment of AP-1- and GATA-binding motifs in FOXP1-bound genomic regions as determined by ChIP-seq, suggesting that these transcription factors might physically or functionally interact. Recently, it was shown that FOXP3 almost exclusively binds enhancer sites, which were pre-accessed by either cofactors or structurally related transcription factors, such as FOXO1.[37] It might well be that FOXP1, similar to FOXP3, exploits pre-existing enhancers in a similar manner by displacing FOXO3 from these sites. This would explain the high degree of DNA-binding site overlap between the two transcription factors. The ultimate result of FOXP1 antagonizing FOXO3 binding of enhancer elements is not overall downregulation of all target genes, but rather a specific transcriptional outcome that protects against FOXO-induced cell death and possibly promotes cell migration.

In agreement with a pro-survival and possibly a pro-migratory function in our system are the reports that indicate an oncogenic role for FOXP1 in cancer. For example, FOXP1 is highly expressed in a subgroup of diffuse large B-cell lymphoma as well as in a mucosal-associated lymphoid tissue type of marginal zone B-cell lymphoma, which in both cancers is strongly associated with decreased patient survival.[38,39] In contrast, loss of FOXP1 expression has been reported in a variety of solid tumors, including colon and breast tumors,[40] indicating a tumor-suppressive role in this context. Indeed, high FOXP1 expression levels in breast cancer were found to be associated with increased patient survival.[41] This latter observation seems to conflict with our finding that FOXP1 protects against cell death while promoting cell migration. However, we observe this FOXP1-induced cell survival phenotype exclusively after activating FOXO3, while FOXP1 KD alone does not affect growth rate. Furthermore, in line with their role in regulating apoptosis, cell cycle arrest and DNA damage response, FOXO proteins are considered to be tumor suppressors. Simultaneous deletion of FOXO1, FOXO3 and FOXO4 results in spontaneous tumor formation in the hematopoietic system (predominantly lymphoblastic thymic lymphoma) of mice.[42] The antagonistic action of FOXP1 against FOXO-induced cell death therefore argues for an oncogenic role of FOXP1 in tumorigenesis. However, deletion of FOXO proteins also results in depletion of multipotent stem cells, including hematopoietic stem cells[43] as well as neural stem cells,[44,45] due to a decrease in self-renewal and an increase in differentiation in different tissues. This latter finding appears at first inconsistent with the tumor-suppressor activity of FOXO proteins as uncontrolled self-renewal is a prerequisite for tumor formation and differentiation is anti-tumorigenic. Furthermore, a recent study demonstrates a pro-oncogenic role for FOXO transcription factors in acute myeloid leukemia.[46] It could be that for FOXP1 a similar cellular context difference might exist; however, this still has to be determined.

Given the relevance of FOXO in inducing cancer cell death in response to anti-cancer treatment,[11] FOXP1 might represent a therapy resistance marker. Indeed, we show that FOXP1 inhibits FOXO3-induced cell death, while promoting the expression of genes that are involved in cell migration and angiogenesis. In line with this, FOXP1 expression is increased in colorectal cancer cells in response to treatment with the chemotherapeutic doxorubicin as well as after specific inhibition of PI3K–PKB signaling. We propose that similar to FOXO-induced HER3 and PIK3CA expression in response to cancer therapy aimed at inhibiting PI3K–PKB signaling,[14,16,47] FOXP1 possibly represents another mode of developing escape from treatment by protecting the cells against FOXO-induced cell death.

Taken together, we have shown that FOXP1 is a direct transcriptional target of FOXO proteins. Importantly, we demonstrate that FOXP1 binds the same genomic enhancer elements as FOXO, potentially in a sequential matter, thereby directing the transcriptional outcome of FOXO proteins toward cellular survival. Therefore, FOXP1 represents an important determinant of FOXO-induced cellular outcome and possibly explains the difference in the response of different cell types to FOXO activation.

Materials and Methods

Cell culture. Ba/F3 cells expressing FOXO3(A3)–ER have been described previously[20] and were cultured in RPMI containing GlutaMAX (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% HyClone serum (Gibco Life Technologies, Carlsbad, CA, USA) and recombinant mouse IL-3 produced in COS cells.[48] DLD1 cells expressing FOXO3(A3)–ER (DL23) have been described previously.[24] For the generation of polyclonal U2OS cells expressing FOXO3(A3)–ER, linearized pcDNA3-HA-FOXO3(A3)–ER or pcDNA3-ER[28] were electroporated into the cells. The cells were subsequently selected and maintained in the presence of 1 mg ml^{-1} G418 (Gibco). DLD1 and U2OS cells were cultured in DMEM containing GlutaMAX supplemented with 10% fetal bovine serum. KD of FOXP1 in DL23 was achieved by transfection using Lipofectamine RNAiMAX reagents (Invitrogen) with 10 nM human *FOXP1* siRNA (Thermo Scientific, ON-TARGET plus SMARTpool, L-004256-01, Waltham, MA, USA) at least 24 h before 4-OHT treatment.

Quantitative real-time PCR. RNA was isolated using a RNeasy kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. cDNA was generated by reverse transcribing 1 μg of total RNA with SuperScript III reverse transcriptase (Invitrogen Life Technologies). Quantitative PCR was subsequently performed using an Biorad Icycler (Bio-Rad, Hercules, CA, USA) with primer pairs for the indicated gene and normalized using primer pairs for GAPDH. Primer sequences can be found in Supplementary Table 3.

Western blotting. Western blot analysis was performed as described.[20] In brief, cells were lysed in Laemmli buffer and protein concentration was determined using a Lowry protein assay. Equal amounts of protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with the appropriate amount of antibody according to the manufacturer's indicated conditions. The antibodies against FOXP1 (2005) and cleaved Caspase-3 (9661) were from Cell Signaling Technology (Danvers, CA, USA), ER (MC-20, sc-542) and BIK (N-19, sc-1710) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), p27^{Kip1} (610241) from BD Biosciences (San Jose, CA, USA), and tubulin (T5168), HA-tag (H3663) and FLAG-tag (F7425) from Sigma Aldrich (Saint Louis, MI, USA). Immunocomplexes were detected using ECL (Amersham Pharmacia Inc. (GE Healthcare), Piscataway, NJ, USA).

Chromatin immunoprecipitation (ChIP) and sequencing. DLD1 or DL23 cells were grown in the absence or presence of 10 μM PKB inhibitor VIII (Santa Cruz Biotechnology) or 100 nM 4-OHT, respectively, for the indicated time points. ChIP was performed as described previously[20] using the following antibodies: 5 μg of anti-FOXO3 (H-144, sc-11351) or anti-ER (MC-20, sc-542) from Santa Cruz Biotechnology, or 5 μg of anti-FOXP1 (ab16645) from Abcam (Cambridge, MA, USA). Chromatin was additionally sheared, end-repaired, sequencing adaptors were ligated and the library was amplified by LMPCR. After LMPCR, the library was purified and checked for the proper size range and for the absence of adaptor dimers on a 2% agarose gel and sequenced on SOLiD/AB sequencer (Applied Biosystems Life Technologies, Carlsbad, CA, USA) to produce 50-bp-long reads. Sequencing reads were mapped against the reference genome (hg19 assembly, NCBI build 37) using the BWA package.[49] Nonuniquely placed reads were discarded. Cisgenome v2.0 software package[50] was used for the identification of binding peaks from the ChIP-seq data and further analysis. Cisgenome 2 was used with settings: − e50, − maxgap 200 and minlen 200. Input sample from the ChIP was sequenced and used as background. *De-novo* motif discovery was done using CisModule function[51] incorporated in Cisgenome sofware, using the following parameters: motif number $K = 15$, mean motif length Lambda = 10, maximal motif length allowed = 18, initial motif length = 10, initial module size $D = 3.0$, module length = 100, order of background Markov chain = 3 and MCM iteration = 500. A combination of Cisgenome functions, custom PERL and R scripts was used for additional data analysis. Similarity search with known transcription factor-binding motifs was performed using TOMTOM motif comparison tool.[52] Primers used for ChIP-qPCR are listed in Supplementary Table 4.

Reporter assay. HEK293 cells were transfected with pGL3-6xDBE-luc[30] together with pcDNA3-HA-FOXO3 and/or pcDNA3-FLAG-FOXP1 and Renilla to normalize for transfection efficiency using polyethylenimine. Cells were lysed in passive lysis buffer 48 h after transfection and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

RNA sequencing. FOXP1 was depleted in DL23 cells as described above. Cells were grown in the absence or presence of 4-OHT for the indicated time point and total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. To isolate mRNA, at least 10 μg of total RNA was purified using Poly(A)Purist MAG Kit (Ambion Life Technologies, Austin, TX, USA) according to manufacturer's instructions. Isolated mRNA was subsequently repurified using mRNA-ONLY Eukaryotic mRNA Isolation Kit (Epicentre (Illumina, Inc.), Madison, WI, USA). Next, sequencing libraries were constructed using SOLiD Total RNA-Seq Kit (Applied Biosystems Life Technologies) according to the standard protocol recommendations for low input and sequenced on SOLiD Wildfire sequencer in a multiplexed way to produce 50-bp-long reads. Sequencing reads were mapped against the reference genome (hg19 assembly) using the BWA package.[49] Only uniquely placed reads were used for further analysis. Cisgenome v2.0[50] software package was used to calculate reads per 1000 base pairs of transcript per million reads sequenced (RPKM) values for all RefSeq annotated genes. RPKMs were quantile normalized throughout all samples and presented as log2(RPKM) after adding small number to RPKM (0.1) to avoid log2 transformation of zero values.

Crystal violet assay. DL23 transfected with siRNA against *FOXP1* or control siRNA were grown in the absence or presence of 100 nM 4-OHT. At the indicated time points, the cells were fixed using 4% paraformaldehyde, washed three times with PBS containing 0.1% Triton-X and an additional two times with PBS. Cells were stained for 30 min at room temperature with 0.1% Crystal violet solution and subsequently rigorously washed. Crystal violet was extracted using a 1% SDS solution and the absorbance was measured at 595 nm. The experiment was performed in triplicates and repeating three times.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Cell Death and Differentiation website (http://www.nature.com/cdd)