FOXO Transcription Factors Control E2F1 Transcriptional Specificity and Apoptotic Function

Igor Shats, Michael L. Gatza, Beiyu Liu, Steven P. Angus, Lingchong You, and Joseph R. Nevins

Abstract

The transcription factor E2F1 is a key regulator of proliferation and apoptosis but the molecular mechanisms that mediate these cell fate decisions remain unclear. Here, we identify FOXO transcription factors as E2F1 target genes that act in a feed-forward regulatory loop to reinforce gene induction of multiple apoptotic genes. We found that E2F1 forms a complex with FOXO1 and FOXO3. RNAi-mediated silencing of FOXO impaired E2F1 binding to the promoters of cooperative target genes. A FOXO3 mutant insensitive to inactivation by survival kinases rescued the inhibitory effect of growth factor signaling on E2F1-mediated transcription and apoptosis. The E2F1/FOXO axis is frequently blocked in cancer, as evidenced by the specific downregulation of the FOXO-dependent E2F1 transcriptional program in multiple cancer types and by the association of a reduced E2F1/FOXO transcriptional program with poor prognosis. HDAC and phosphoinositide 3-kinase (PI3K) inhibitors were identified as specific activators of E2F1/FOXO transcription, acting to enhance E2F1-induced apoptosis in a FOXO3-dependent manner. Notably, combining the histone deacetylase inhibitor vorinostat with a PI3K inhibitor led to enhanced FOXO-dependent apoptosis. Collectively, our results identify E2F1/FOXO cooperation as a regulatory mechanism that places E2F1 apoptotic activity under the control of survival signaling. Therapeutic reactivation of this tumor suppressive mechanism may offer a novel broad-acting therapy for cancer.

Introduction

The role of the retinoblastoma tumor suppressor (Rb) in the control of E2F transcription factors function is now recognized as the key step in the regulation of cell-cycle entry. In response to growth factor signaling, Rb is inhibited by cyclin-dependent kinases (CDK), leading to E2F activation and G0 to G1-S transition. Disruption of various components of this control pathway leads to deregulated proliferation and is central to the development of many forms of human cancer (1).

Previous studies have found that among the E2F family, E2F1 protein is unique in its ability to induce apoptosis in addition to its more conventional role in the control of cellular proliferation (2). For example, E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis (3) and thymocytes derived from E2F1−/− mice are resistant to apoptotic stimuli (4). Following DNA damage, E2F1 is stabilized by ATM and Chk2 phosphorylation, leading to apoptosis induction (5, 6). The E2F1-dependent apoptosis is mediated through transcriptional induction of numerous proapoptotic genes and repression of survival genes (6–10). Collectively, these and other studies suggest that frequently deregulated E2F1 activity in cancer cells represents a potential Achilles heel that might be exploited in cancer therapy. However, to effectively harness this therapeutic potential, we must better understand the mechanisms that inactivate the apoptotic potential of E2F1 in cancer.

Previous studies from our group have shown that the decision to proliferate or undergo a cell death response following E2F1 activation was regulated by PI3K/Akt function, coinciding with a specific repression of only a subset of E2F1 target genes (11, 12). Left unclear from these observations is the mechanism by which phosphoinositide 3-kinase (PI3K) signaling can specifically prevent the induction of apoptotic but not the proliferative E2F1 target genes. Given the evidence for combinatorial mechanisms of transcription control involving other E2F family members, we hypothesized that the outcome of E2F1 activation might also be affected by the status of its different transcriptional partners.

Similar to E2F1, the FOXO family of transcription factors plays an important role in various cellular processes. Activation of FOXO activity can lead to growth arrest, apoptosis, increased stress resistance, differentiation, and metabolic responses in a system-specific manner (13). Phosphorylation of FOXO proteins by kinases such as AKT and SGK downstream of PI3K activation by growth factor signaling, leads to their nuclear exclusion and subsequent degradation (14, 15). FOXO are also regulated by CK1, DYRK1A kinases, and SIRT1...
deacetylase. Different posttranslational modifications not only control FOXO localization but also might affect their transcriptional specificity (16). Thus, FOXO proteins integrate the information on the cell state from multiple signaling pathways and translate it into transcriptional responses. Here, we identify FOXO family of transcription factors as E2F1 transcriptional partners that control E2F1 transcriptional specificity and apoptosis providing a mechanistic link between PI3K signaling and E2F1.

Materials and Methods

Detailed Materials and Methods are available in Supplementary Information. Catalog numbers and oligonucleotide sequences used in this study can be found in Supplementary Table S7.

Cell culture and drugs

U2OS human osteosarcoma cells stably expressing ER-HA-E2F1 were obtained from Dr. Rotter (Weizmann Institute of Science, Israel). IMR90, 293T, and U2OS cells were grown in Dulbecco’s Modified Eagle Medium with 10% fetal calf serum (FCS). Cell line identity was authenticated by DNA STR profiling assay. 4-Hydroxytamoxifen (OHT), LY294002, and G418 were from Sigma. Vorinostat (SAHA) was from ChemieTek.

Microarray analysis

For microarray analysis of U2OS ER-E2F1 cells, RNA was prepared using RNeasy Kit (Qiagen) and analyzed on Affymetrix U133A 2.0 microarrays. Microarray expression data are available in the Gene Expression Omnibus database under the accession number GSE39136.

Cell viability and apoptosis assays

Relative cell numbers were quantified using MTS or CellTiter-Glo assays (Promega). Caspase-3/7 activity assays were conducted using Caspase-GLO 3/7 luminogenic substrate (Promega).

Adenovirus, lentivirus infection, and siRNA

Adenoviruses expressing FOXO3 AAA and LacZ (Vector Biolabs) were used at multiplicity of infection (MOI) 10. For construction of pLEX-FOXO3, HindIII XRxbal fragment from pCDNA3-FLAG-FOXOs (Addgene) was cloned into BamHI site of pLEX-puro (Open Biosystems).

Reporter assays

APAF1 promoter reporter construct (−396/+208) was kindly provided by Dr. Helin (University of Copenhagen, Denmark) and described in (17). FOXO binding site was mutated using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Reporter activities were measured using Dual-Glo Luciferase Assay System (Promega).

Chromatin immunoprecipitation

Chromatin immunoprecipitations of ER-HA-E2F1 were conducted using EZ-ChIP Kit (Millipore) according to manufacturer’s instructions.

Coimmunoprecipitation and Western blotting

FLAG-FOXO1 or FLAG-FOXO3 (Addgene) were precipitated from 293 cell lysates with anti-FLAG (M2) beads (Sigma) and cotransfected E2F1 was detected by Western blotting.

The following antibodies were used: anti-OctA-probe (Flag, sc-807), anti-E2F1 (sc-193), anti-β-tubulin (sc-8035) from Santa Cruz Biotechnology; anti-APAF1 (ALX-804-348) from Enzo; anti-cleaved PARP (9546), anti-FOXO1 (2880) from Cell Signaling; and anti-FOXO3 (07–702) from Millipore.

Statistical analysis

All pooled results are presented as mean and SEM of triplicate experiments. Kaplan–Meier survival curves and their significance levels were generated using GraphPad software and compared using and the log-rank test. P values for the significance of non-zero linear regression trend in the drug sensitivity analysis was generated using GraphPad software.

Results

A feed-forward gene regulatory circuit involving E2F1 and FOXO transcription factors

Previous studies have suggested that specificity and consequently the outcome of transcription factor activation might be determined by the status of its transcription factor partners (18–20). Accordingly, considering the possible mechanisms that underlie the specificity of E2F1 function, we have focused on the identification of transcription factors that might function together with E2F1. In this context, we made use of findings from the systems biology field showing that feed-forward regulation is the most highly overrepresented network motif in transcriptional networks (21). In this motif, one transcription factor activates a second transcription factor that then cooperates with the initial inducer to regulate target genes (Fig. 1A). Using the feed-forward mode of regulation as a working hypothesis, we decided to look for potential E2F1 cooperating transcription factors among the genes that are induced by E2F1. To identify transcription factors that are induced by E2F1, we conducted expression microarray experiment in an inducible system of U2OS cells that express an ER-E2F1 fusion protein. Addition of OHT leads to nuclear translocation of the chimeric protein and activation of E2F1-mediated transcription. This system was characterized previously and microarray analysis showed the lack of OHT effect on transcription in the parental U2OS cells (22).

Among 1,098 genes activated more than two-fold in response to E2F1, 113 genes encoded transcriptional regulators (Supplementary Table S1), suggesting that regulation of other transcription factors represents an important part of E2F1 transcriptional program. Upregulation of two members of the FOXO family of transcription factors, FOXO1 and FOXO3, was of particular interest to us given their established role in apoptosis (23). In addition, the well established inactivation of FOXO by PI3K signaling coupled with our previous findings on the role of this survival signaling pathway in the specific repression of the apoptotic E2F1 transcriptional program suggested that FOXO transcription factors might function as E2F1 apoptotic transcriptional cofactors (11, 14, 15). Notably, the induction of FOXO1 and FOXO3 mRNA by E2F1 is direct as...
it occurred in the presence of protein synthesis inhibitor, and E2F1 binding to FOXO1 and FOXO3 promoters was shown by chromatin immunoprecipitation experiments (24).

To explore a role of the FOXO proteins in E2F1-mediated transcriptional activity, we knocked down their expression and conducted a microarray analysis of gene expression before and after induction of E2F1 activity by OHT. Given previous work that has suggested overlapping and potentially compensatory function for FOXO1 and FOXO3 (25), at this stage we have not sought to distinguish individual roles for these proteins in the function of E2F1 and used two different short hairpin RNA (shRNA) constructs (Foxo33 and Foxo707) targeting both FOXO1 and FOXO3. Real-time PCR analysis validated shFOXO effects on ER-E2F1 levels or nuclear translocation. The majority of E2F1 target genes rules out the possibility of shFOXO effects on ER-E2F1 levels or nuclear translocation.

Examination of the list of FOXO-dependent E2F1 targets identified several genes that had been previously linked with apoptosis, including EGR genes (26–29), APAF1 (30), PTCH1 (31), EPIC (32), NLRP3 (33), and CTSB (34). Notably, the list also contained NR4A3, identified as a serum-repressed apoptotic E2F1 target in a previous study (12). In addition, a CDK inhibitor CDKN1C (p57), was also induced by E2F1 in a FOXO-dependent manner. Finally, we also note the E2F1-mediated induction of numerous developmental genes, including several components of the Wnt pathway that were attenuated by FOXO knockdown.

To further explore the role of E2F1 and FOXO in the control of these genes, reverse transcription (RT)-PCR analysis was conducted. As shown in Fig. 1D, the E2F1-mediated induction of the EGR1, EGR2, and EGR4 genes was abrogated following FOXO knockdown. In addition to the EGR genes, E2F1-mediated induction of APAF1, a key component of apoptosis pathway was also attenuated by FOXO knockdown (30). RT-PCR analysis also confirmed a role for FOXO in E2F1-mediated
induction of additional genes, including CDKN1C (Fig. 1D) and several components of Wnt pathway (Supplementary Fig. S1). In contrast, the induction of a cell-cycle-promoting gene encoding ribonucleotide reductase subunit M2 (RRM2) was unaffected by FOXO knockdown (Fig. 1D).

To further establish a role for FOXO in the E2F1-mediated induction of selected genes, we used a complementary approach in which FOXO3 was ectopically expressed in the U2OS ER-E2F1 cells. RT-PCR analysis following induction of E2F1 activity by OHT showed that E2F1-mediated activation of the EGR4, APAF1, and CDKN1C genes by E2F1 was enhanced by FOXO3. This cooperation was gene-specific as RRM2 was also induced by E2F1 but this induction was unaffected by FOXO3 (Supplementary Fig. S2).

**E2F1 and FOXO transcriptional cooperation**

Considering the mechanism by which E2F1 and FOXO proteins might synergize in the activation of target genes, we found that E2F1 physically interacts with both FOXO1 and FOXO3 in communoprecipitation experiments using tagged proteins (Fig. 2A). Similar experiments using endogenous E2F1 and FOXO proteins were inconclusive, likely due to technical limitations of the antibodies used. To address a role for FOXO in the function of E2F1, we carried out chromatin immunoprecipitation assays to measure the interaction of E2F1 with target promoters. As shown in Fig. 2B, the induction of E2F1 with 4-OHT led to the recruitment of E2F1 to the APAF1 promoter. Importantly, this binding was dependent on FOXO because combined knockdown of FOXO1 and FOXO3 eliminated the binding of E2F1 to the APAF1 promoter. A similar result was seen for E2F1 recruitment to the EGR1 promoter. In contrast, binding of E2F1 to the promoter of a proliferative gene MCM4 was only marginally affected by FOXO knockdown showing promoter specificity of FOXO effect.

To test whether direct binding of FOXO proteins to the APAF1 promoter is necessary for modulation of the E2F1-mediated APAF1 induction, we used a reporter gene under the control of APAF1 promoter sequence (Fig. 2C). The activity of the reporter was induced by cotransfected FOXO3 (Fig. 2D). We identified a potential FOXO recognition sequence located just upstream of an E2F binding site (Fig. 2C). Mutation of this site abolished the activation of the reporter by FOXO3 showing that this site is, indeed, a functional FOXO site responsible for the induction of APAF1 by FOXO (Fig. 2D). Importantly, the E2F1-mediated induction of APAF1 promoter reporter was also attenuated by the mutation of the FOXO site (Fig. 2E). Taken together, these results establish APAF1 as novel direct FOXO target, cooperatively regulated by E2F1 and FOXO.

**Survival signaling regulates the capacity of E2F1 to activate transcription and apoptosis through FOXO**

A role for FOXO in the activation of a subset of E2F1 target genes, coupled with previous work showing that FOXO activity is inhibited by growth factor-induced survival signaling, suggest a mechanism by which the apoptotic function of E2F1 could be controlled independent of the role of E2F1 in proliferation. To determine the extent to which FOXO-dependent E2F1 transcription is regulated by growth factor signaling, we examined the induction of two representative cooperative target genes, APAF1 and CDKN1C. As shown in Fig. 3A, the induction of these genes by E2F1 was inhibited in the presence of serum (FCS). To assess the contribution of FOXO inactivation to the inhibition of E2F1-mediated transcription by serum, we made use of a FOXO3 mutant that is immune to phosphorylation by survival kinases AKT and SGK (FOXO3-AAA; refs. 14, 15). As shown in Fig. 3A, this constitutively active FOXO3 mutant rescued the induction of APAF1 and CDKN1C by E2F1 in the presence of serum. In contrast, induction of a FOXO-independent proliferative E2F1 target, RRM2, was unaffected by serum and inhibited by FOXO3 AAA.

To extend the observation of cooperation between E2F1 and FOXO to normal cells and endogenous E2F1, we evaluated the effects of FOXO3 AAA on serum-induced transcription in IMR90 fibroblasts. While E2F1 was robustly upregulated following addition of serum to starved cells infected with a control LacZ virus, APAF1 was not induced. Infection of cells with FOXO3 AAA adenovirus resulted in significant induction of APAF1 mRNA in starved cells and enabled its further upregulation by serum (Fig. 3B).

Previous work has shown that E2F1-induced apoptosis can be suppressed by survival signals provided by the addition of serum (12). This result is recapitulated by the experiment in Fig. 3C, showing that the induction of E2F1 activity in U2OS ER-E2F1 cells following OHT addition resulted in a dose-dependent cell death response that was inhibited by serum (FCS). This apoptosis was E2F1-dependent because similar treatment of parental U2OS cells with OHT did not affect their growth (Supplementary Fig. S3). Making use of low OHT concentrations where apoptosis is inhibited by serum, we have further explored the role of FOXO and APAF1 in E2F1-mediated apoptosis.

As shown by the Western blot analysis in Fig. 3D, activation of E2F1 by OHT led to a moderate induction of APAF1 in the presence of serum (lane 1, 2) as did overexpression of a phosphorylation-resistant FOXO3-AAA mutant (compare lanes 1 and 3). The combined action of E2F1 and FOXO led to a further increase in APAF1 levels (lane 4). Coincident with the induction of APAF1 was the cleavage of PARP as seen by the reduction in full-length PARP and the appearance of a cleaved form of the protein. Importantly, knockdown of APAF1 using two independent siRNAs strongly attenuated this apoptotic response (lane 5, 6) establishing a central role for APAF1 in the E2F1/FOXO apoptotic axis.

**The E2F1/FOXO transcriptional program is reduced in cancer**

Among the various changes accompanying the development of an oncogenic phenotype is the loss of capacity to initiate cell death (35). Given the apparent role of FOXO transcription factors in defining the transcriptional specificity of E2F1 to include the induction of apoptosis, we evaluated the pattern of expression of FOXO-dependent E2F1 target genes as cells transition from a normal state to an oncogenic state. To develop a measure of the E2F1/FOXO transcription program, we first identified those probe sets on the microarray that were induced at least two fold by E2F1 in the presence of FOXO...
activity. Then, we identified the subset from this group whose induction by E2F1 was reduced at least 1.5 fold by FOXO knockdown. This yielded a total of 52 probe sets representing 51 genes (Supplementary Table S3). We then compared the average expression of this group of genes in normal and tumor samples making use of several publicly available expression datasets.

We started our analysis with a sarcoma dataset, given the fact that our initial microarray experiments were carried out in the osteosarcoma cell line U2OS. As seen in Fig. 4A, the expression of the FOXO-dependent E2F1 transcriptional signature was significantly reduced in sarcomas as compared with normal fat tissue. To evaluate the extent to which this distinction between normal and tumor samples was a reflection of the
FOXO-dependent component of the E2F1 transcriptional program, we generated a second signature that included genes whose induction by E2F1 was not affected by FOXO in our microarray analysis (Supplementary Table S4). As seen in Fig. 4A, this signature failed to exhibit a significant distinction between tumor and normal samples.

To extend our observations to epithelial cancer types, we analyzed five additional datasets representing breast, colorectal, and liver cancers. The characteristics of all datasets and summary of the results are shown in Supplementary Table S5. In all datasets, we found a significant reduction of the FOXO-dependent E2F1 transcriptional signature in tumor samples (Fig. 4; Supplementary Table S5). In contrast, the expression of the FOXO-independent E2F1 transcriptional signature did not significantly differ between tumor and normal samples in four datasets (Fig. 4; Supplementary Table S5) and was downregulated to a smaller extent than the FOXO-dependent E2F1 program in two datasets (Supplementary Table S5). The difference in the extent of downregulation in tumors between FOXO-dependent and FOXO-independent E2F1 signatures.

Figure 3. Survival signaling regulates the capacity of E2F1 to activate transcription and apoptosis through FOXO. A, U2OS ER-E2F1 cells were either serum-starved (no FCS) or infected in the presence of serum with adenovirus encoding FOXO 3 AAA constitutively active mutant (FCS FOXO3 AAA) or with control LacZ adenovirus (FCS LacZ). Twenty-four hours later, medium was replaced with (OHT) or without (con) 30 nmol/L OHT for 5 hours. Expression levels of the indicated genes were determined by real-time RT-PCR. B, IMR90 was infected with adenovirus encoding FOXO 3 AAA constitutively active mutant (FOXO3 AAA) or with control LacZ adenovirus (LacZ) in serum-free medium. After 40 hours, cells’ medium was replaced with either serum-free medium (starv) or medium containing 10% serum (FCS) for 20 hours. Expression levels of the indicated genes were determined by real-time RT-PCR. C, dose-response curves in U2OS cells treated with a range of OHT concentrations in the presence (10% FCS) or the absence (0% FCS) of serum. Relative cell numbers were determined using MTS assay 48 hours after OHT addition. See also Supplementary Fig. S3. Measurements were done in triplicate wells with SDs smaller than the symbols representing the averages. D, U2OS ER-E2F1 cells were transfected with the indicated siRNA duplexes in the presence of 10% FCS. Twenty-four hours later, cells were infected with adenoviruses encoding FOXO 3 AAA constitutively active mutant (FOXO3) or with control LacZ adenovirus. Twenty-four hours later, medium was replaced with (+) or without (−) 20 nmol/L OHT for 48 hours. Expression of APAF1 and PARP was analyzed by Western blotting. Tubulin served as a loading control.
Unsupervised clustering of samples resulted in identity (Table S3) from a large breast cancer dataset (36). We extracted the expression values of the 52 FOXO-dependent probes described above (Supplementary Dataset). We performed survival analysis studies in breast and lung cancer. To this end, we made use of the Connectivity Map, a reference collection of gene-expression profiles from three human cancer cell lines treated with bioactive small molecules that were frequently targeted in cancer. Survival analysis showed that patients whose tumors were characterized by a high expression of this program had significantly better prognosis than those from the low expression cluster. Similar results were obtained in an analysis of large lung adenocarcinoma dataset (Fig. 5B; ref. 37).

In summary, lower expression of the FOXO-dependent arm of E2F1 transcriptional program associates with poor prognosis in breast and lung cancer. Taken together with our results on a widespread downregulation of this program in multiple cancer types, and the experimental results on the cooperation between E2F1 and FOXO3 in apoptosis induction, these results suggest that FOXO-dependent part of E2F1 transcriptional program is an important novel tumor suppressive mechanism, frequently targeted in cancer.

**A reduced FOXO-dependent E2F1 signature is associated with poor prognosis in breast and lung cancer**

To evaluate the potential role of the FOXO-dependent E2F1 transcriptional program in cancer outcomes, we conducted survival analysis studies in breast and lung cancer. We extracted the expression values of the 52 FOXO-dependent E2F1 target probes sets described above (Supplementary Table S3) from a large breast cancer dataset (36). Unsupervised clustering of samples resulted in identification of two main sample clusters, stratifying the tumors into high and low expressors of the FOXO-dependent E2F1 transcriptional program (Fig. 5A). Survival analysis showed that patients whose tumors were characterized by a high expression of this program had significantly better prognosis than those from the low expression cluster. Similar results were
knockdown, suggesting important role of FOXO3 in mediating this apoptotic caspase activation was attenuated by FOXO3 enhanced the apoptotic activity of E2F1 (Fig. 6B). Furthermore, alone resulted in only mild apoptotic response, it potently or with their combination. While treatment of cells with SAHA we treated U2OS ER-E2F1 cells with SAHA alone, OHT alone, (Supplementary Table S6; refs. 14, 15).

E2F1 signature by HDACi was highly specific to this signature as shown by low specificity values. In stark contrast to the activation of the FOXO-dependent E2F1 signature by HDACi, the signature consisting of FOXO-dependent E2F1 targets identified multiple histone deacetylase (HDAC) inhibitors (HDACi) including trichostatin A (TSA), vorinostat (SAHA), and scriptaid as the top ranking inducers of the E2F1/FOXO transcriptional program. Notably, highly significant upregulation of E2F1/FOXO signature was observed in all three cell lines used for the Connectivity Map analysis representing prostate, breast, and blood cancers (Fig. 6A; Supplementary Table S6). The induction of the FOXO-dependent E2F1 signature by HDACi was highly specific to this signature as shown by low specificity values.

In stark contrast to the activation of the FOXO-dependent E2F1 signature by HDACi, the signature consisting of FOXO-independent E2F1 targets identified multiple histone deacetylase (HDAC) inhibitors (HDACi) including trichostatin A (TSA), vorinostat (SAHA), and scriptaid as the top ranking inducers of the E2F1/FOXO transcriptional program. Notably, highly significant upregulation of E2F1/FOXO signature was observed in all three cell lines used for the Connectivity Map analysis representing prostate, breast, and blood cancers (Fig. 6A; Supplementary Table S6). The induction of the FOXO-dependent E2F1 signature by HDACi was highly specific to this signature as shown by low specificity values.

To examine the role of HDACi in E2F1-induced apoptosis, we treated U2OS ER-E2F1 cells with SAHA alone, OHT alone, or with their combination. While treatment of cells with SAHA alone resulted in only mild apoptotic response, it potently enhanced the apoptotic activity of E2F1 (Fig. 6B). Furthermore, this apoptotic caspase activation was attenuated by FOXO3 knockdown, suggesting important role of FOXO3 in mediating the apoptotic synergy between E2F1 and SAHA. We then studied the effect of individual and combined knockdown of FOXO1 and FOXO3 on cell viability following separate and combined treatment of cells with SAHA and OHT (Fig. 6C). Western blot analysis confirmed efficient knockdown of FOXO1 and FOXO3 by corresponding siRNAs. While the low concentration of OHT did not affect cell proliferation in the presence of serum, SAHA strongly synergized with OHT in decreasing cell numbers. Importantly, single knockdowns of FOXO1 or FOXO3 significantly attenuated this toxicity with combined knockdown, resulting in the greatest degree of protection.

Next, we combined SAHA with a PI3K inhibitor (LY294002) to further activate FOXO activity. As shown in Fig. 6D, treatment of U2OS with this combination led to a synergistic apoptosis induction, which was again attenuated by FOXO3 knockdown. Thus, the synergy between HDACi and PI3K inhibitors during apoptosis induction is mediated at least in part through FOXO-dependent pathway.

Our results so far suggested that HDACi and PI3K inhibitors specifically activate FOXO-dependent E2F1 transcription and that activation of this transcriptional program has tumor suppressive properties. Accordingly, we would expect that these drugs will be more active in tumors where this program is inactivated, reflected by a lower basal E2F1/FOXO signature. To test this hypothesis, we made use of publicly available data from a recent study that profiled 50 breast cancer cell lines for drug sensitivity and basal gene expression (39). As shown in Fig. 6E, we indeed found a statistically significant inverse correlation between the
average expression of FOXO-dependent E2F1 signature and sensitivity to an HDACi, trichostatin A. Similar result was observed for a PI3K inhibitor GSK2126458.

In summary, we identify HDACi and PI3K inhibitors as specific activators of FOXO-dependent E2F1 transcriptional program and show FOXO-dependent apoptotic synergy of
HDAC inhibitor with E2F1 and with PI3K inhibitor. Growth inhibition by PI3K and HDAC1 correlates with the extent of E2F1/FOXO signature downregulation, suggesting potential use of this signature as a mechanism-based biomarker for patient selection in future clinical trials for these drugs.

Discussion

The role of the Rb/E2F pathway in the control of critical cell fate decisions including proliferation, quiescence, development, differentiation, metabolism, and cell death, has been well established in previous work (40). Particularly intriguing is the apparent dual role of the E2F1 transcription factor in the activation of gene expression programs leading to proliferation and apoptosis (41). The identification of FOXO as component of the E2F1-induced apoptotic program, coupled with the established role of survival signaling events regulating FOXO activity, provides a molecular basis by which survival signals can control the outcome of E2F1 activation and block the death component (Fig. 7).

The role for E2F1 and FOXO acting in combination highlights an example of the concept of combinatorial transcription control by which individual transcription factors mediate their function through cooperative interactions. The significance of combinatorial transcription control is at least twofold. First, a mechanism whereby two transcription factors act in combination generates a much greater specificity of promoter recognition than can be achieved with either factor alone as it is the recognition of the combined target sequences that defines function. Second, the opportunity for the individual transcription factors to form multiple combinations provides the potential for a much greater repertoire of specificity than what could be achieved through the action of single proteins. Furthermore, the coherent feed-forward loop established by E2F1 and FOXO may enhance the cell’s ability to distinguish normal and abnormal E2F1 signals by generating a delay in the activation of targets such as APAF1 due to the time required to accumulate FOXO. Upon E2F1 removal, however, there is no delay in shutting off APAF1 even though FOXO remains present. Such an asymmetric delay device can thus work as a noise filter, which responds only to sustained activity of the upstream activator and rejects response to transient input.

We also believe the spectrum of control involving the combination of E2F1 and FOXO goes beyond the activation of an apoptotic program, given the identification of multiple genes as FOXO-dependent E2F1 targets. For instance, the identification of multiple developmental genes, including several Wnt signaling pathway components, as cooperative targets suggests a potential role for E2F1/FOXO crosstalk in development and differentiation. In addition, we identify CDKN1C (p57), a negative regulator of the cell cycle through CDK inhibition, as FOXO-dependent E2F1 target (Fig. 1C). Thus, the feed-forward loop between E2F1 and FOXO family might contribute not only to apoptosis but also to growth arrest. The repression of proliferative E2F1 targets such as RRMI by FOXO3 AAA (Fig. 3A) is consistent with this notion and might be either direct (as described previously for cyclin D) or indirect through the induction of CDK inhibitors (42). Although additional studies will be required to better understand the role of E2F1/FOXO cooperation in different cellular contexts, the overall effect seems to be tumor suppressive based on the observation that the E2F1/FOXO transcriptional program is reduced as normal cells transition to an oncogenic state. The implication of this finding is that while E2F1 likely participates in various cellular processes, it is the FOXO-dependent subset of E2F1 targets that identifies a tumor suppressing transcriptional program. Given the frequent functional inactivation of FOXO in human tumors (43), this underscores the significance of recognizing the complexity of gene control mediated by individual transcription factors and the importance of identifying the combinations that define unique transcriptional regulatory programs.

Intriguingly, in addition to joint regulation by E2F1 and FOXO, APAF1, as well as other apoptotic genes, are regulated by a tumor suppressor, p53. As p53 itself is activated by E2F1, this suggest an even more complex apoptotic scheme where activities of multiple checkpoint pathways interconnected through an array of feed-forward loops and controlled by upstream signaling pathway need to be activated to trigger apoptosis (44).

Finally, the identification of a regulatory system involving E2F1 and FOXO, and the importance of control of this pathway in determining oncogenic outcomes, provides an opportunity to develop new and novel therapeutic strategies that could reactivate the E2F1/FOXO program. The identification of HDAC and PI3K as targets, and the potential of a SAHA/PI3K...
inhibitor combination to have therapeutic benefit in tumors lacking the E2F1/FOXO3A program, represents one such opportunity, SAHA is already approved for the treatment of cutaneous T-cell lymphoma and currently is tested in clinical trials for additional cancer types. Likewise, many PI3K inhibitors are currently in early-phase clinical studies. The strong apoptotic synergy between SAHA and a PI3K inhibitor suggests that a combination of these two drugs, coupled with the selective use in a population of patients exhibiting reduced E2F1/FOXO3A program, could represent a novel therapeutic strategy to test in future clinical studies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

Conception and design: I. Shats, J.R. Nevins
Development of methodology: I. Shats
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Shats, M.L. Gatza, S.P. Angus
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): I. Shats, M.L. Gatza, B. Liu, J.R. Nevins
Writing, review, and/or revision of the manuscript: I. Shats, M.L. Gatza, S.P. Angus, L. You, J.R. Nevins
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Liu, J.R. Nevins
Study supervision: J.R. Nevins

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