Cell Reports
Article

FOXO3 Selectively Amplifies Enhancer Activity to Establish Target Gene Regulation

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http://dx.doi.org/10.1016/j.celrep.2013.11.031

SUMMARY

Forkhead box O (FOXO) transcription factors regulate diverse cellular processes, affecting tumorigenesis, metabolism, stem cell maintenance, and lifespan. We show that FOXO3 transcription regulation mainly proceeds through the most active subset of enhancers. In addition to the general distinction between "open" and "closed" chromatin, we show that the level of activity marks (H3K27ac, RNAPII, enhancer RNAs) of these open chromatin regions prior to FOXO3 activation largely determines FOXO3 DNA binding. Consequently, FOXO3 amplifies the levels of these activity marks and their absolute rather than relative changes associate best with FOXO3 target gene regulation. The importance of preexisting chromatin state in directing FOXO3 gene regulation, as shown here, provides a mechanism whereby FOXO3 can regulate cell-specific homeostasis. Genetic variation is reported to affect these chromatin signatures in a quantitative manner, and, in agreement, we observe a correlation between cancer-associated genetic variations and the amplitude of FOXO3 enhancer binding.

INTRODUCTION

The Forkhead box O (FOXO) family of transcription factors comprises FOXO1, FOXO3, FOXO4, and FOXO6. Multiple signaling pathways converge on FOXOs, regulating their activity mainly through posttranslational modifications and altering cellular localization. Two conserved pathways function at the core of FOXO regulation. In the presence of growth factors and insulin, through PI3K/PKB signaling, FOXOs are inactivated by cytoplasmic retention. In contrast, FOXOs are activated in the presence of oxidative stress (for a recent review, see Hedrick et al., 2012). All FOXOs bind the same consensus sequence (5′-TGTTTAC-3′) and FOXO activity regulates a plethora of target genes (reviewed in van der Vos and Coffer, 2011). Genetic studies in *C. elegans*, *D. melanogaster*, and mice have shed light on the versatility of FOXO functions in vivo. FOXOs were shown to act as redundant tumor suppressors, to control glucose homeostasis, and to be involved in immune cell regulation and (cancer) stem cell maintenance (reviewed in Eijkelenboom and Burgering, 2013). In model organisms, FOXO function also greatly influences lifespan and variations in human *FOXO3* are associated with human longevity (reviewed in Kenyon, 2010). At present, the cell-type-specific nature of FOXO transcriptional output is attributed to the activity of specific signaling pathways, resulting in different combinations of posttranslational modifications (Calnan and Brunet, 2008), and to functional and physical interactions with other transcription factors (reviewed in van der Vos and Coffer, 2008, 2011).

Enhancers are DNA elements driving cell-type-specific gene expression and can be located at great distances from the genes they regulate. They are bound by transcription factors, transcriptional coactivators, and chromatin regulators and are considered to modulate transcription from promoters through delivery of factors that directly regulate transcription (reviewed in Ong and Corces, 2011). Although genome-wide approaches have greatly progressed the identification of enhancers and transcription factor binding events (for example, the ENCODE project; Bernstein et al., 2012), the relationships among transcription factors, regulatory regions, and their contribution to gene expression are far from completely understood. For example, transcription factor binding does not necessarily result in regulation of gene expression and the majority of transcription factor binding events might not actually be functional (reviewed in Spitz and Furlong, 2012). Previously, we observed that the majority of FOXO3 peaks occupy regions located distal from promoters. Given this large number of FOXO3-bound regions, we aimed to study the role of enhancers in FOXO3-mediated gene regulation and identify features that classify the subset of binding events that actually contribute to target gene regulation. We therefore generated genome-wide profiles of chromatin modifications H3K4me1, H3K4me3, and H3K27ac (histone H3 lysine 4 mono- and trimethylation and lysine 27 acetylation), identified "open" chromatin through formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq; Giresi et al., 2007), followed by massive parallel sequencing, and analyzed transcriptional changes by RNA sequencing (RNA-seq). Combined with previously generated FOXO3 and RNAPII genome-wide binding profiles, these data sets provide a comprehensive overview of chromatin status prior to and upon FOXO3 activation, combined with FOXO3 DNA

binding and transcriptional output. We show that the amplitude of FOXO3 DNA binding to an enhancer determines FOXO3 output on the same enhancer, which is the local increase in enhancer activity marks like H3K27Ac and RNAPII. In addition, FOXO3 transcriptional output affecting target gene expression also depends on the amplitude of FOXO3 binding. FOXO3 enhancer binding in turn is determined by a combination of sequence content and chromatin context. Chromatin context we define here as a quantitative feature, not merely a distinction between open and "closed" chromatin but rather the levels of activity marks at these open chromatin regions. We show that it is not only the presence of Forkhead motifs and DNA accessibility but also the levels of initial enhancer activity that affects FOXO3 binding. As a result, FOXO3 preferentially binds to a subset of enhancers that already display high activity marks prior to FOXO3 induction, with binding levels reflecting the initial levels of these marks and preexisting accessibility. Consequently, FOXO3 amplifies the levels of initial enhancer activity marks, with the greatest absolute changes on enhancers displaying the highest initial levels. We show absolute rather than relative change in enhancer activity marks has a better correlation with target gene regulation. FOXO3-induced changes in enhancer activity marks bridge FOXO3 enhancer binding with gene regulation, since we show that only the "responsive" subset of FOXO3 enhancer binding events (with changes in enhancer activity marks) is associated with target gene induction. Together, this suggests that the result of individual FOXO3 binding events is dictated by preexisting enhancer activity. Our integrative analysis of chromatin state and gene expression profiles both before and after FOXO3 activation provides insight into the complex relationship among transcription factor binding, the quantitative features of chromatin state, and their influence on gene expression. Furthermore, it provides a perspective on FOXO function that may explain how FOXOs, and FOXO3 in particular, can contribute to cellular homeostasis and thereby affect lifespan and disease.

RESULTS

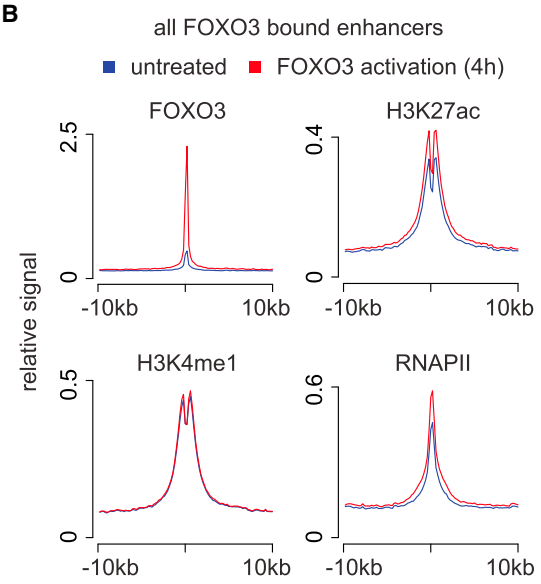
FOXO3 Binds Enhancers and Induces an Active Chromatin Profile

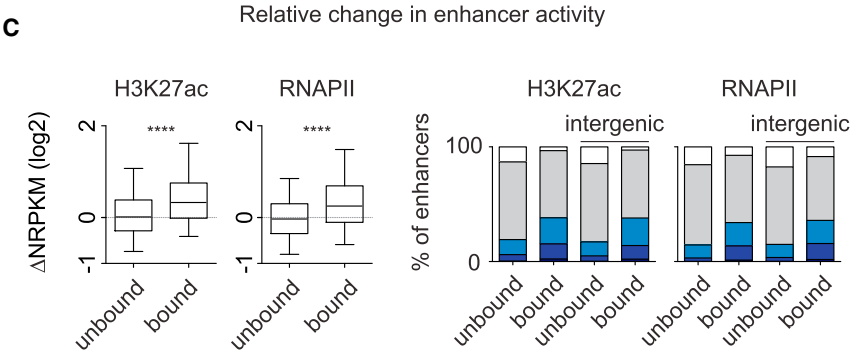
To study the transcriptional output of FOXO3 activation, we required a system to rapidly and specifically activate FOXO3. Physiological stimuli, which can activate FOXO (e.g., inhibition of the PI3K/PKB axis), also influence parallel pathways, possibly confounding the global analysis of FOXO3 transcriptional output. We and others have previously used DLD1-F3 cells (also called DL23 cells) to study FOXO3 function. These colon carcinoma cells contain a fusion of a constitutively active mutant (FOXO3A3), lacking inhibitory PKB phosphorylation sites, fused with the ligand binding domain of the estrogen receptor (ER) (Kops et al., 2002), allowing rapid and specific induction by 4-OH tamoxifen (4OHT) (Littlewood et al., 1995). The ER domain is transcriptionally inactive and the FOXO3-ER fusion protein faithfully mimics endogenous FOXO3 (Eijkelenboom et al., 2013). Therefore, the FOXO3-ER system provides a robust system to specifically study the transcriptional output of the isolated activation of a single transcription factor.

We have previously shown that the majority of FOXO3 peaks are located beyond the immediate vicinity (>5 kb) of any annotated transcription start site (TSS) (Eijkelenboom et al., 2013). Given that initial analysis suggested binding of FOXO3 to enhancers and regulation of enhancer activity by FOXO3, we analyzed enhancer regulation by FOXO3 in a systematic manner. As enhancers can be discriminated from promoter regions by high levels of H3K4me1 and low H3K4me3 (Heintzman et al., 2007, 2009) and active enhancers are marked by H3K27ac (Creyghton et al., 2010), we performed chromatin immunoprecipitation with antibodies against H3K4me1, H3K4me3, and H3K27ac followed by massive parallel sequencing (ChIP-seq) on DLD1-F3 cells, both before and after FOXO3 activation. Combined with previously generated FOXO3 and RNAPII genome-wide binding profiles (Eijkelenboom et al., 2013), this allows for an integrative genome-wide analysis of FOXO3 DNA binding and transcriptional output with chromatin context (an overview of our approach is depicted in Figure S1A). First, we explored FOXO3 enhancer binding and induced changes in enhancer activity. Based on H3K4 methylation status, we identified 37,051 enhancers, of which 5,731 overlap with previously identified FOXO3-bound regions (Figure 1A). A total of 65% of all FOXO3 peaks (6,489 out of 9,932 peaks) overlap with identified enhancers. FOXO3 binding increases enhancer activity marks, as can be observed by local increases in H3K27ac and RNAPII (Figure 1B), which is consistent with previous observations on increased RNAPII signal at intergenic FOXO3-bound regions (Eijkelenboom et al., 2013) and a role as a transcriptional activator. Although FOXO has been reported to act as a repressor in a limited number of studies, previous genome-wide analyses by us and others in a variety of systems have all shown FOXO generally acts as a transcriptional activator, without significant evidence for a direct role in repression (Alic et al., 2011; Eijkelenboom et al., 2013; Riedel et al., 2013; Schuster et al., 2010; Webb et al., 2013). This suggests that gene repression generally occurs through DNA-binding-independent or secondary mechanisms. The relative depletion of both H3K4me1 and H3K27ac at the FOXO3 peak center prior to FOXO3 activation suggests that FOXO3 binds nucleosome-free regions. This was confirmed by ChIP-seq on total histone H3, as well as FAIRE-seq (Figure S1B). Changes in ChIP-seq signals were determined for all enhancers and comparisons between FOXO3-bound and FOXO3-unbound enhancers confirmed the activating output of FOXO3 binding (Figures 1C and 1D). Both the H3K27ac and RNAPII occupancy levels provide measures for enhancer activity, which allows us to study local consequences of FOXO3 binding with two independent parameters. To discriminate the RNAPII signal from enhancers and transcribed genes, all subsequent analyses involving RNAPII occupancy changes are performed on intergenic enhancers. These enhancers show very similar dynamics (Figures 1C and 1D) and distribution profiles (Figure S1C) compared with all enhancers. The outcome of FOXO3 binding on H3K27ac and RNAPII levels varies, with no detectable changes at a substantial proportion of regions. FOXO3-bound and responsive enhancers are more conserved relative to the FOXO-bound and unresponsive enhancers, indicating their biological relevance (Figure S1D).

A

	all	intergenic
all enhancers	37051	9809
FOXO3 bound	5731	1576
unbound	31320	8233





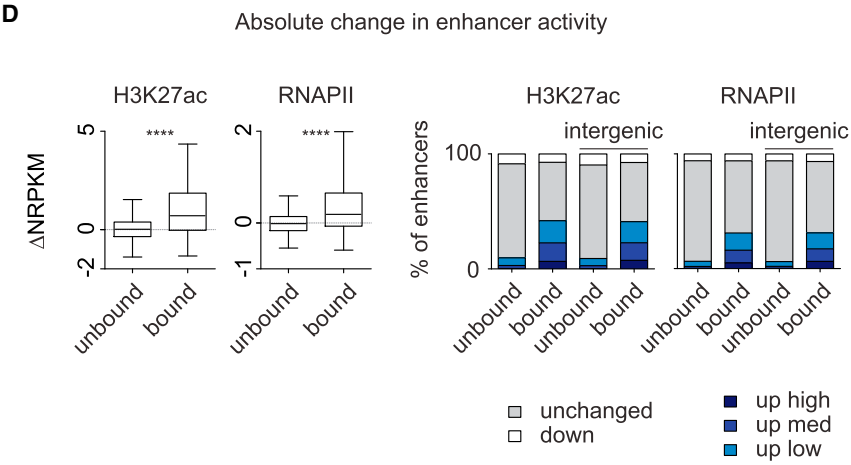


Figure 1. FOXO3 Binds Enhancers and Increases Enhancer Activity Marks

(A) Overview of enhancers identified in DLD1-F3 cells and the overlap with FOXO3 bound genomic locations. Intergenic enhancers do not overlap with annotated transcripts.

(B) Average signals of FOXO3, H3K4me1, H3K27ac, and RNAPII ChIP-seq profiles at FOXO3-bound enhancers in DLD1-F3 cells both before and after FOXO3 activation (4 hr 4OHT). Enhancers are centered on the FOXO3 peak center. Untreated signal in the FOXO3 graph represents background signal from DLD1 cells.

(C and D) Normalized read density was determined for H3K27ac and RNAPII (NRPKM) on all enhancers and changes upon FOXO3 activation were calculated. Relative (log2, C) and absolute (linear, D) changes in H3K27ac and RNAPII are shown for FOXO3-unbound and FOXO3-bound enhancers in boxplots with 5%–95% whiskers (p values are from a two-tailed Mann-Whitney *U* test; ****$p < 2.2 \times 10^{-16}$). Enhancers were categorized based on FOXO3 binding and location relative to annotated transcripts and changes in H3K27ac and RNAPII levels were determined (down, < -0.5; up low, 0.5 to 1.0; up med, 1.0 to 2.0; up high, > 2.0 for all except absolute changes in H3K27ac in which values are < -1.0, 1.0 to 2.0, 2.0 to 4.0, and > 4.0, respectively; these cutoffs are used throughout this study).

See also Figure S1.

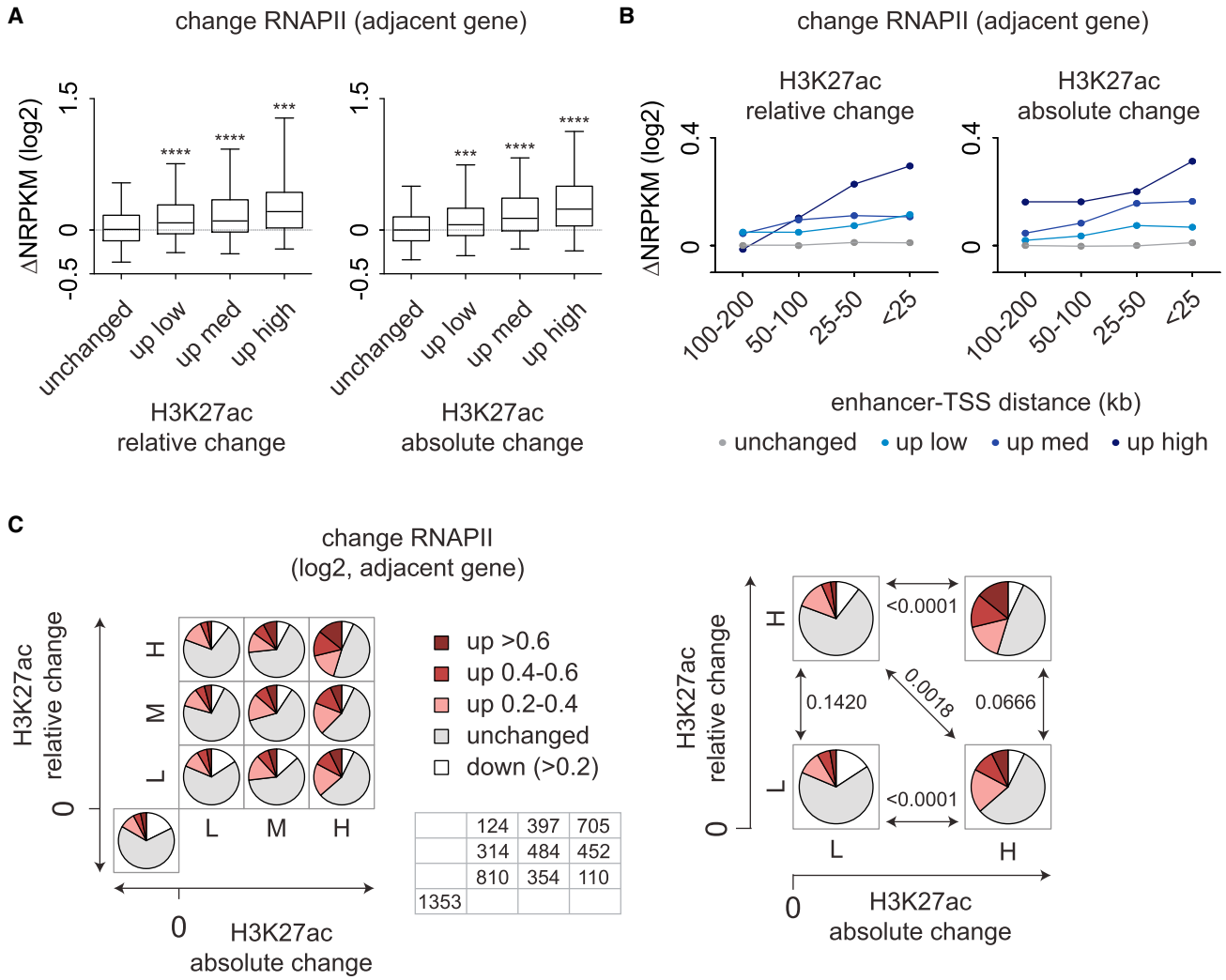


Figure 2. Absolute Changes in Enhancer Activity Marks Associate Most with FOXO3-Mediated Target Gene Regulation

(A and B) FOXO3-bound enhancers were categorized by H3K27ac changes (as in Figures 1C and 1D) and the changes in RNAPII occupancy of the proximate gene, including all annotated transcripts, were calculated. Boxplot with 5%–95% whiskers are shown for all enhancers with a maximum enhancer-TSS distance of 100 kb (A); p values from a two-tailed Mann-Whitney *U* test relative to unchanged (***$p < 10^{-10}$, ****$p < 10^{-15}$). Median values are shown for various enhancer-TSS distance windows (B), with colors representing categories of H3K27ac.

(C) FOXO3-bound enhancers were divided in three groups, sorted for increasing changes in H3K27ac (L = low, M = medium, H = high). Formation of three groups was separately performed for absolute changes (left to right) and relative changes (bottom to top), of which the combination results in nine categories. Relative changes in RNAPII levels at adjacent genes are determined (≤200 kb enhancer-TSS; table represents number of enhancers within each category). Categories with most extreme values are shown to the right, with values between categories representing p values from a two-tailed Mann-Whitney *U* test.

See also Figure S2.

The FOXO3-Mediated Accumulation of Enhancer Activity Marks Associates with Target Gene Regulation

Enhancers can associate with genes through looping and influence gene transcription (reviewed in Splinter and de Laat, 2011), and this also holds for some selected FOXO3-bound enhancers and target genes (Eijkelenboom et al., 2013). To determine if changes in enhancer activity marks are associated with target gene regulation, we assigned all enhancers to the most proximate gene, judged by enhancer-TSS distance, because the proximity of regulatory elements is a major determinant of selectivity (Splinter and de Laat, 2011) and is commonly used (e.g., Creyghton et al., 2010; Lovén et al., 2013). Using this assumption, we will miss a proportion of bona fide enhancer-gene interactions. In addition, we will score false-positive enhancer-gene pairs. The most responsive FOXO3-bound enhancers, as judged by increased activity marks, are associated with the most prominent changes in gene transcription (calculated through changes in RNAPII occupancy at the gene body) (Figures 2A and S2A). These associations exist up to 200 kb enhancer-TSS maximum distances but are more evident within smaller distances (Figure 2B), probably due to more accurate enhancer-gene pairing. These results show that even though

FOXO3 binds thousands of enhancers, only the subset of FOXO3 binding events with a concomitant increase in enhancer activity marks—the subset of responsive enhancers—is relevant to target gene regulation.

Changes in enhancer activity marks can be expressed in relative and absolute values. We reasoned that the most informative groups of enhancers to distinguish between the effect of relative and absolute changes are enhancers with low relative and high absolute changes and vice versa. Interestingly, enhancers with high absolute but low relative changes are more associated with gene activation than enhancers with high relative but low absolute changes. In addition, the subset of enhancers with the highest absolute change also associate the most with changes in gene activation, irrespective of the relative amount of change. We observe this phenomenon for any combination of enhancer activity marks (H3K27ac or RNAPII changes) with measures for gene transcription and expression (RNAPII occupancy and RNA-seq) (Figures 2C and S2B–S2D; data not shown). In an alternative approach, we assigned the closest FOXO3-bound enhancer to all genes (not just the closest one), with several windows up to 200 kb. The mere presence or absence of a FOXO3-bound enhancer poorly associates with changes in gene transcription, but the association increases greatly when changes in enhancer activity marks are taken into account (Figure S2E). In addition, we could confirm that absolute changes are best associated with gene activation (Figures S2F and S2G). Possibly, the observed changes in enhancer activity marks represent the delivery of activating factors to target gene promoters. Absolute numbers might be most relevant in this context, as they represent the actual amount of activating factors.

FOXO3 Output Is Determined by the Amount of Bound FOXO3

To investigate what features distinguish FOXO3-induced from nonresponding bound enhancers, we visually inspected FOXO3-bound enhancers and noticed that highly responsive enhancers contain more prominent FOXO3 peaks. To test if local FOXO3 levels are associated with the outcome of FOXO3 activation, we determined FOXO3 ChIP-seq signal on all bound enhancers. Indeed, the amount of FOXO3 binding progressively increases with increased enhancer induction (Figures 3A and S3A). Endogenous FOXO3 levels show a similar progressive increase (Figure S3B). The amount of FOXO3 binding also positively correlates with changes in activity marks without using arbitrary thresholds (Figures 3B, S3C, and S3D). As supported by inspection of individual tracks and genome-wide analysis, unchanged FOXO3-bound enhancers do show a local high H3K4me1 signal (Figure S3E) and a clear FOXO3 signal in two independent FOXO3-ER and endogenous FOXO3 profiles (Figures 3A and S3F), thus excluding the presence of a significant number of false positives in FOXO3 binding status or enhancer identification that would account for the lack of response. Because an induction in enhancer activity marks is associated with target gene regulation, we categorized FOXO3-bound enhancers according to increasing FOXO3 levels. The most highly bound enhancers were also associated with the most changes in adjacent gene transcription (Figure 3C). Several examples of FOXO3 responsive enhancers near known target genes are shown (Figures 3D and S3G). The paradigm that more bound FOXO3 results in more target gene regulation also extends to the total amount of bound FOXO3 in the vicinity of a gene (Figure S3H). Although peak size may reflect a combination of properties, including affinity and residence time, these results show that the amount of bound FOXO3 determines the local output of FOXO3 activity at enhancers and FOXO3 target gene regulation.

FOXO3 Binding Is Determined by Sequence Content and Chromatin Context

Because the amount of FOXO3 DNA binding is relevant for enhancer and target gene activation, we sought to investigate what parameters determine FOXO3 binding. The presence of multiple Forkhead motifs within one enhancer could result in more FOXO3 binding, providing a simple explanation for the differences in FOXO3 binding levels. To test this hypothesis, we determined the frequency of previously identified Forkhead motifs enriched within FOXO3 peaks (Eijkelenboom et al., 2013) and noticed an increasing frequency of Forkhead motif presence corresponds with greater changes in activity marks (Figures 3E and S3I), suggesting sequence content directs the outcome of FOXO3 activation. However, Forkhead motif presence is not the single parameter determining FOXO3 binding, because mere classification of enhancers by the amount of Forkhead motifs does not correlate with differences in FOXO3 binding (Figure S3J; compare with Figures 3A and S3A). Previously, we have observed that FOXO3-bound intergenic regions are marked by RNAPII peaks prior to FOXO3 induction (Eijkelenboom et al., 2013), suggesting that the preexisting chromatin context influences motif accessibility and subsequent binding. To verify this suggestion, we show that FOXO3 preferentially binds the most open and active enhancers based on FAIRE, H3K27ac values (Figures 4A and 4B), and RNAPII occupancy (Figure S4A) prior to FOXO3 activation. Endogenous FOXO3 and an independent FOXO3-ER ChIP-seq confirm this preference (Figure S4B). The chromatin state not only determines whether FOXO3 is bound but also influences the binding intensity, as the amount of FOXO3 binding shows a gradual and continuous increase with increasing initial enhancer activity marks and accessibility (Figures 4C and S4C). Similar results were obtained for endogenous FOXO3 binding (Figures S4D–S4F). Initial levels of enhancer activity marks are independent of Forkhead motif presence (Figure S4G) and, when corrected for motif presence, FOXO3 preferentially binds the subset of Forkhead motifs contained in enhancers with the highest activity marks and accessibility (Figure 4D). The combination of both parameters confirms FOXO3 DNA binding affinity is determined by both motif presence and accessibility (Figures 4E and S4H). We could confirm this preference in other genome-wide binding profiles for FOXO3 (breast cancer cells; Ruben van Boxtel and Paul Coffer, personal communication) and FoxO1 (mouse liver and T cells; Ouyang et al., 2012; Shin et al., 2012) (Figure S4I). Although both FOXO family members preferentially bind the Forkhead motif, these data also suggest that DNA binding by FOXOs is promiscuous, meaning FOXOs are also able to bind less specific sequences. This is in line with previous observations regarding the identification of Forkhead motifs in only

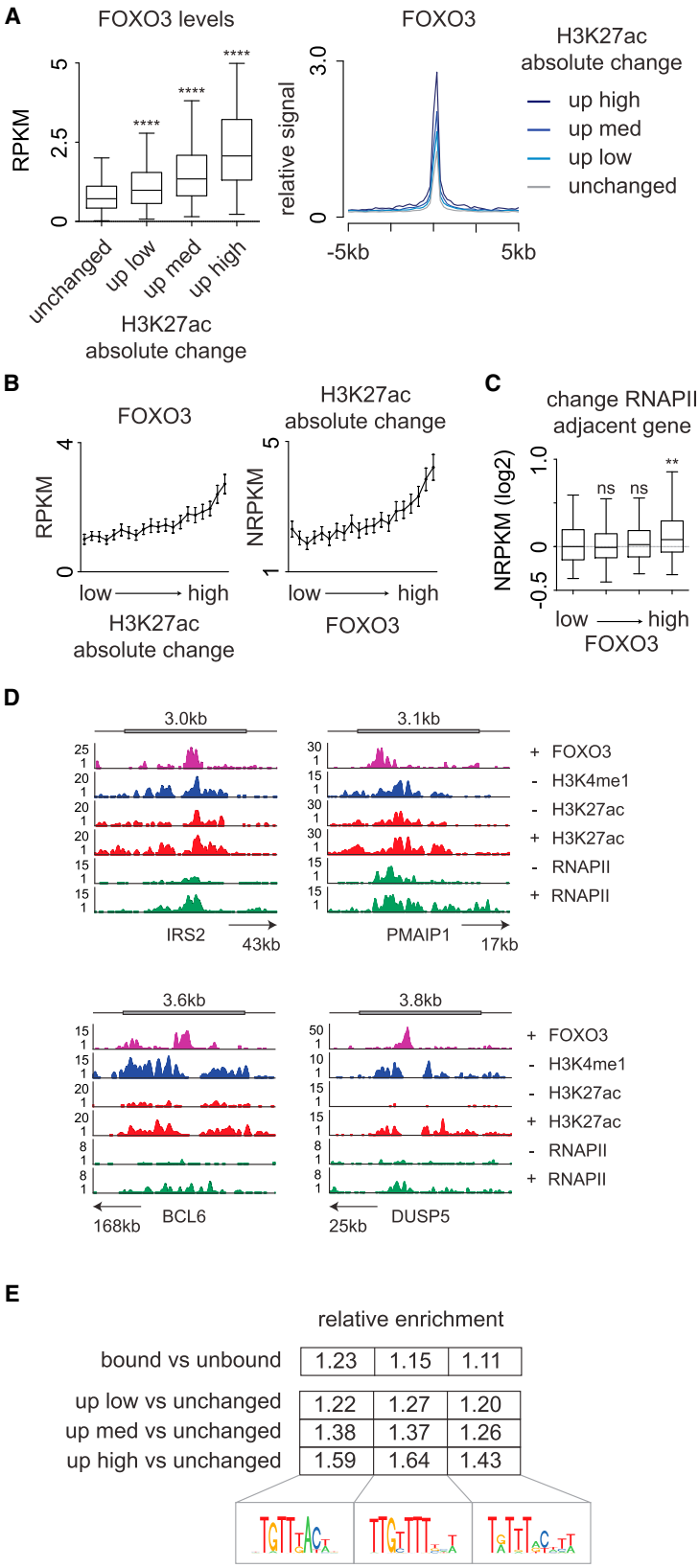


Figure 3. FOXO3 Output Is Determined by FOXO3 Binding Levels

(A) FOXO3 ChIP-seq signal at all FOXO3 bound enhancers, corrected for background levels. Boxplots with 5%–95% whiskers show FOXO3 levels on enhancers categorized by changes in H3K27ac (p values from a two-tailed Mann-Whitney *U* test relative to unchanged; ****$p < 10^{-15}$). Average FOXO3 ChIP-seq signals at FOXO3 bound enhancers for same categories are shown. Enhancers are centered on the FOXO3 peak center.

(B) Responsive FOXO3 bound enhancers were identified by absolute changes in H3K27ac (>1) and sorted in 20 equal sized bins for H3K27ac changes (left) or FOXO3 occupancy (right). Mean and 95% confidence intervals of FOXO3 levels (left) and H3K27ac absolute changes (right) are shown.

(C) FOXO3 bound enhancers were categorized in four equal-sized groups with increasing FOXO3 levels and changes in RNAPII occupancy of the proximate gene were calculated. Boxplot with 5%–95% whiskers are shown for all enhancers (≤200 kb enhancer-TSS; p values from a two-tailed Mann-Whitney *U* test, relative to lowest; ns = $p > 0.05$, **$p < 10^{-4}$).

(D) Example tracks of FOXO3, H3K4me1, H3K27ac, and RNAPII ChIP-seq occupancy in untreated (−) or FOXO3 activated (+) conditions. Four enhancers (gray boxes) are shown. Proximate genes with enhancer-TSS distance are indicated below tracks.

(E) The frequency of Forkhead motif occurrence as identified in FOXO3-bound regions (Eijkelenboom et al., 2013) was determined for all FOXO3-bound and FOXO3-unbound enhancers, and bound enhancers were categorized by absolute changes in H3K27ac.

See also Figure S3.

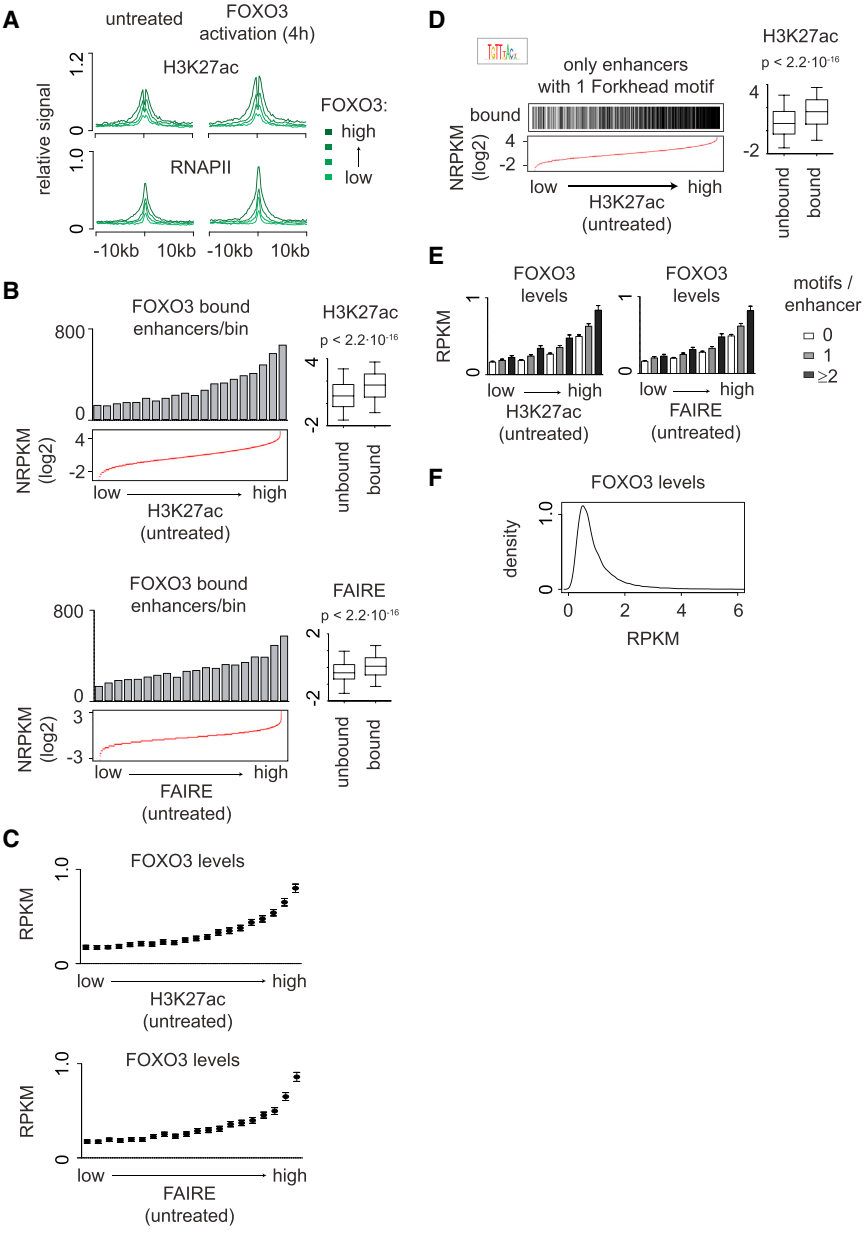


Figure 4. FOXO3 Preferentially Binds Accessible Enhancers with an Active Chromatin Profile

(A) FOXO3-bound intergenic enhancers were categorized in four equal-sized groups with increasing FOXO3 levels. Average signals at FOXO3-bound enhancers of H3K27ac and RNAPII ChIP-seq profiles for the same categories are shown before and after FOXO3 activation (4 hr 4OHT).

(B) Enhancers were sorted in 20 equal-sized bins according to increasing initial H3K27ac (top) or FAIRE (bottom) signals and FOXO3 binding status was determined. The number of FOXO3-bound enhancers within each bin is shown. Boxplots with 5%–95% whiskers show corresponding levels of activity marks (p values from a two-tailed Mann-Whitney *U* test).

(C) FOXO3 levels were determined on all enhancers (regardless of FOXO3 binding status) and corrected for background. Enhancers were sorted in 20 equal bins for increasing initial H3K27ac (top) or FAIRE (bottom) signals. Mean and 95% confidence intervals of FOXO3 levels are shown for each bin.

(D) Enhancers with one canonical Forkhead motif were selected and FOXO3 binding within this subset was determined. Sorted H3K27ac values are shown, with black lines indicating FOXO3 binding. Boxplots with 5%–95% whiskers show corresponding H3K27ac levels (p value from a two-tailed Mann-Whitney *U* test).

(E) Enhancers were divided in four equal groups for increasing initial H3K27ac and FAIRE levels and categorized by the number of canonical Forkhead motifs. Mean and 95% confidence intervals of FOXO3 levels are shown for each category.

(F) Distribution of FOXO3 levels on all enhancers.

See also Figure S4.

45% percent of FOXO3-bound regions (Eijkelenboom et al., 2013). In addition, the distribution of FOXO3 binding shows a continuous rather than a binary profile (Figures 4F and S4J). A binary profile is expected when enhancers display either background or specific binding, dictated by, for example, motif presence. The continuous distribution is more consistent with a gradual increase in binding affinity and is analogous to distributions of promoter-bound c-Myc, for which binding was shown to mirror promoter activity (Lin et al., 2012; Nie et al., 2012). Although promoter binding is much less frequent than enhancer binding (8.8% and 65% of all peaks, respectively), FOXO3 does bind promoter regions (877 peaks are within 2.5 kb from annotated gene TSSs). As expected, FOXO3 promoter binding is similar to enhancer binding, with a preference for active promoters judged by initial H3K27ac values (Figure S4K) and a concomitant increase in these H3K27ac levels (Figure S4L). These results argue against FOXO3 generally operating as a pioneer factor, which has been suggested by in vitro studies for Foxo1 (Hatta and Cirillo, 2007; Hatta et al., 2009), but actually support the opposite: upon activation, FOXO3 occupies a preexisting network of enhancers displaying high activity marks and FOXO3 binding levels mirror initial levels of these marks and preexisting accessibility.

Pre-existing Levels of Enhancer Activity Marks Direct the FOXO3 Transcriptional Response

As the output of FOXO3 enhancer binding depends on FOXO3 levels and FOXO3 preferentially binds to enhancers with the highest level of activity marks, this suggests FOXO3 will induce highly active enhancers the most. To test this hypothesis, FOXO3-bound enhancers were categorized by absolute changes in H3K27ac. We focused on absolute changes, because we already showed that these are more relevant to target gene

regulation than relative changes (Figure 2). Enhancers with the highest absolute change in enhancer activity marks initially also display the highest level of these marks (Figures 5A and 5B). We could confirm this with independent H3K27ac and RNAPII ChIP-seq experiments and upon endogenous FOXO3 activation (Figure S5A) and exclude the influence of arbitrary cutoffs (Figures 5C and S5B). Inspection of individual tracks supports FOXO3-induced amplification of activity marks; Figure 5D shows an example of a genomic locus containing multiple enhancers with variable initial levels and concomitant FOXO3-induced changes.

Initially, we sought to determine what features distinguish responsive from nonresponsive enhancers and influence FOXO3-mediated target gene regulation. We showed that the combination of Forkhead motif presence and high levels of enhancer activity marks predicts FOXO3 binding levels. To determine if these parameters also predict the outcome of FOXO3 activation, we combined the two parameters on all enhancers bound by FOXO3. Bound enhancers with multiple Forkhead motifs and high initial activity marks are most likely to be induced with associated activation of adjacent genes (Figures 5E, 5F, and S5C). Within the subset of induced FOXO3-bound enhancers, the enhancers with the highest initial activity marks are most associated with target gene induction (Figures 5G and S5D–S5F). Also irrespective of FOXO3 binding status, genes associated with enhancers with multiple Forkhead motifs and the highest initial activity marks are most prone to induction by FOXO3 (Figure S5G). Together, these results are in agreement with the concept that FOXO3 target gene regulation is dictated by quantitative features of the preexisting chromatin profile. H3K27ac levels at enhancers are correlated with higher gene expression levels of adjacent genes (Creyghton et al., 2010). Given this initial high levels are associated with FOXO3 binding and response, we questioned if FOXO3-regulated genes adjacent to these enhancers are also initially more highly expressed. As judged by RNAPII occupancy and expression data from RNA-seq, genes with nearby FOXO3-bound enhancers are enriched for expressed genes (Figure S5H). Although the most responsive enhancers initially show the highest levels of activity marks, the expression level of the associated genes is only marginally higher before FOXO3 activation (Figures S5I and S5J). This suggests that if actual transcriptional activity of a gene is the sum of all regulatory impulses active within the cell, then the preexisting level of activity marks of an individual enhancer might be less correlated with ongoing transcription and more relevant for transcriptional changes.

FOXO3 Activation Potentiates Production of Enhancer RNAs

RNAPII enhancer occupancy was recently shown to produce transcripts termed enhancer RNAs (eRNAs), for which the presence correlates with the mRNA levels of adjacent protein-coding genes. In addition, target gene induction was shown to be reduced upon eRNA depletion, suggestion a requirement for these transcripts in gene regulation (reviewed in Pennacchio et al., 2013). To determine if FOXO3-responsive enhancers also produce eRNAs, we performed RNA-seq on total RNA depleted for ribosomal RNA (Ribominus, or rRNA−) and poly(A)-enriched [poly(A)+] RNA. We calculated changes in RNA-seq reads at all intergenic enhancers. Responsive enhancers show increased levels of transcription (Figures 6A, S6A, and S6B). Enhancer transcription provides a third readout for enhancer activity and confirms the results obtained for H3K27ac and RNAPII. First, the highest FOXO3-bound enhancers show the most prominent increase in eRNA transcription (Figures 6B and S6C). Second, FOXO3 preferentially binds to more transcribed (and thus active) enhancers preceding FOXO3 activation (Figures 6C and S6D). Third, FOXO3-induced eRNA transcription associates with nearby gene activation (Figures 6D, S6E, and S6F). In addition, we determined the presence of, and changes in, transcription of intergenic enhancers categorized by increasing initial H3K27ac levels and Forkhead motif presence (Figures 6E and S6G). This also shows that the proportion of responsive enhancers is highest in the subset of enhancers with multiple Forkhead motifs and the highest initial activity marks. For a proportion of bound enhancers, no transcription is detected. This proportion decreases both with higher initial H3K27ac levels and upon FOXO3 activation (indicated in the pie charts in Figures 6E and S6G). Similar results were obtained using categorization by RNAPII levels (not shown). Therefore, eRNA production serves an alternative transcriptional readout for the local response at bound enhancers, underlining the role of the preexisting chromatin state in directing the FOXO3 transcriptional program.

Compared with RNAPII ChIP-seq, strand-specific RNA-seq can provide additional information on the transcription of enhancers. Figure 6F shows two examples of FOXO3-bound and FOXO3-transcribed enhancers near regulated target genes, which are also bound and regulated by endogenous FOXO3 activated through PKB inhibition (Figure S6H). Transcription of enhancers was described to be either nonpolyadenylated or polyadenylated (Pennacchio et al., 2013). In these two examples, transcription is evident both from rRNA− and poly(A)+ RNA-seq. Generally, poly(A)+ RNA sequencing yields similar results as rRNA− sequencing, suggesting at least some proportion of enhancer transcripts are polyadenylated. Transcription of the two examples is clearly unidirectional, while generally transcription from FOXO3-responsive enhancers becomes more unidirectional upon FOXO3 activation (Figure S6I). This suggests that eRNAs required for gene activation are produced from a specific DNA strand and indicates that functional transcription from enhancers is unidirectional.

Possible Disruption of FOXO3 Activity through Genetic Alterations in Enhancers

The PI3K/PKB axis is frequently mutated in a wide variety of cancer types. Specifically, loss-of-function mutations in PTEN and activating mutations in PI3K subunits and (less frequently) PKB have been identified, resulting in increased signaling (Hennessy et al., 2005). FOXOs make an important contribution to the transcriptional output of PI3K/PKB signaling and the above mutations generally result in inactivation of FOXOs through constitutive cytoplasmic retention, as occurs in the DLD1 cells used in this study (Samuels et al., 2005). Alteration of regulatory regions bound by FOXO could provide an alternative mechanism to disrupt FOXO tumor suppressive function. Mutations in the

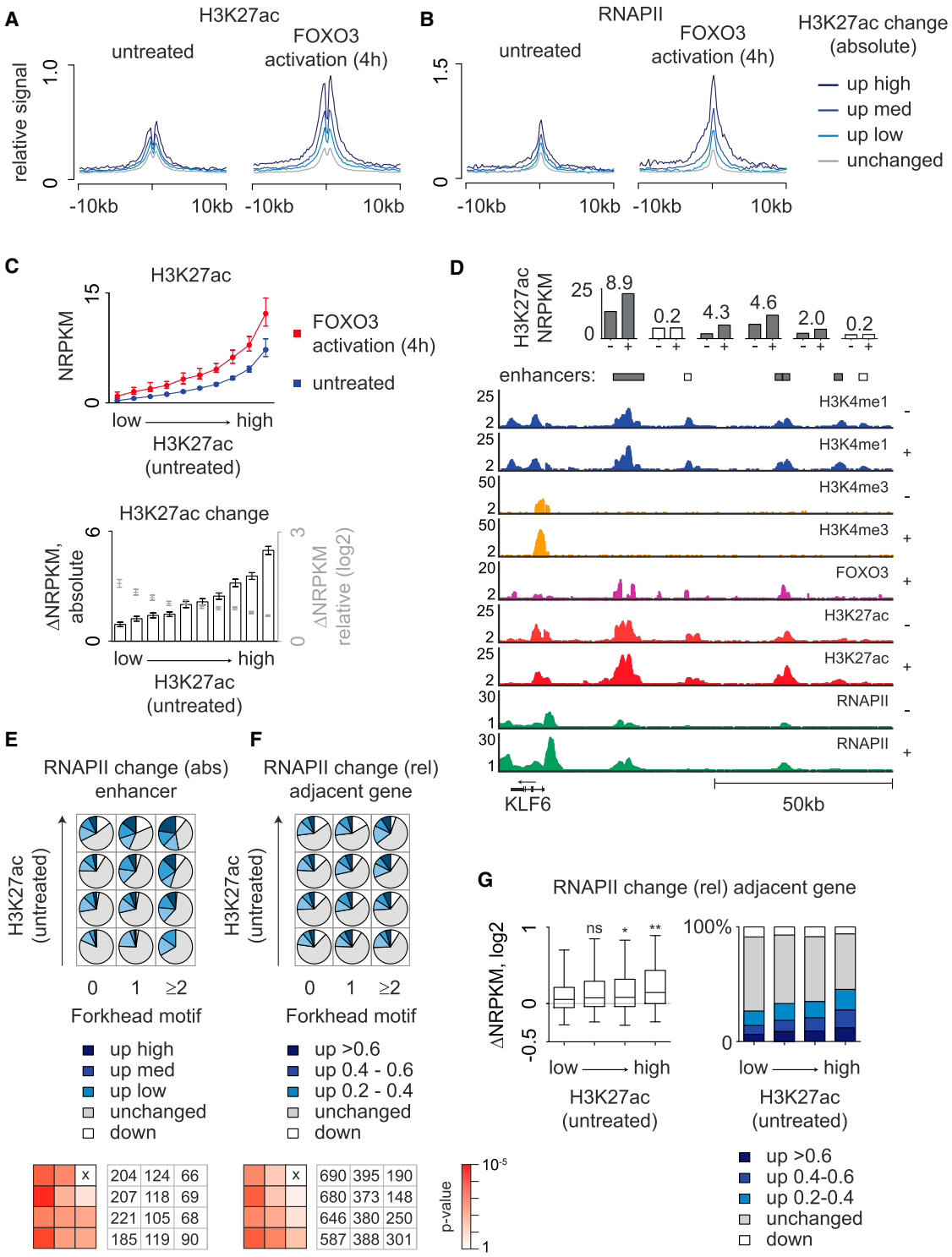


Figure 5. FOXO3 Transcriptional Output Is Predetermined by Initial Levels of Enhancer Activity Marks

(A and B) Average signals at FOXO3 bound enhancers of H3K27ac (A) and RNAPII (B, intergenic only) ChIP-seq profiles prior to and after FOXO3 activation (4 hr 4OHT) categorized by absolute changes in H3K27ac. Enhancers are centered on the FOXO3 peak.

(C) Responsive FOXO3-bound enhancers were identified by changes in H3K27ac (>0.5 relative change, log2) and sorted in ten equal-sized bins for initial H3K27ac levels. Mean and 95% confidence intervals of H3K27ac levels before and after FOXO3 activation are shown for each bin (top), with absolute and relative changes in H3K27ac levels (below).

(legend continued on next page)

Forkhead motif could disable FOXO binding but are unlikely to occur frequently. Our results, however, suggest that alterations in enhancer activity could also greatly influence FOXO3 DNA binding and subsequently affect the transcriptional program. One potential factor influencing enhancer activity is genetic variation (Pennacchio et al., 2013). We determined FOXO3 binding levels on enhancers containing variants associated with colorectal cancer (CRC) (Akhtar-Zaidi et al., 2012). Besides the overlap between CRC SNPs and regulatory elements in DLD1 cells, which is much larger than expected from randomly selected SNPs (Figure 7A), we also observed relatively high FOXO3 levels on these enhancers (Figure 7B). In contrast, FOXO3 levels on enhancers with variants associated with inflammatory bowel disease (Jostins et al., 2012) display normal occupancy values relative to all enhancers. Although the numbers of genetic variants are small and it is unclear if they represent differences in enhancer activity, these results indicate that in addition to the mutations found in components of the PI3K signaling pathway, a functional relationship between alterations in enhancer activity and FOXO3 function might also be biologically relevant in the context of cancer (a model is depicted in Figure S7).

DISCUSSION

This study establishes the role for chromatin context in the regulation of FOXO3-induced target gene regulation. Whereas we previously noted FOXO3 binding to distal regions and initial analysis suggested enhancer binding and FOXO3-induced changes in enhancer activity marks, we now provide a genome-wide and systematic analysis while showing the underlying mechanistic insights and consequences of this phenomenon. We have made use of the H3K4me1 signal to identify enhancers independently of three activity marks (RNAPII, H3K27ac, and eRNAs), which we subsequently related to both changes of these enhancer activity marks at FOXO3-bound enhancers and FOXO3-induced changes in adjacent gene expression. First, our integrative genomic approach shows that FOXO3 enhancer binding is in part determined by the presence of one or multiple canonical Forkhead motifs within an enhancer. Clustered binding sites of the same transcription factor are not uncommon but rather enriched in human regulatory regions (Gotea et al., 2010) and might provide a common mechanism to obtain high local transcription factor concentrations. Second, we observed targeting of FOXO3 specifically to a subset of binding elements within regions of open chromatin that exhibit high levels of activity marks. This has been observed for other transcription factors, including HSF, glucocorticoid receptor, and FOXP3 (Guertin and Lis, 2010; John et al., 2011; Samstein et al., 2012). The chromatin landscape can be directive in transcription factor binding, as we show for FOXO3 binding, and can actually be used to predict transcription factor binding amplitudes (Guertin et al., 2012; Pique-Regi et al., 2011). Although these studies indicate the importance of chromatin context in directing transcription factor binding, they did not relate chromatin context with the output of transcription factor activity. Here, we relate the quantitative features of preexisting chromatin state to the consequences of FOXO3 induction. The genome-wide analysis of enhancer-associated activity marks and gene expression both before and after FOXO3 activation allows a systematic analysis to further understand the interplay between the preexisting chromatin state and the consequences of transcription factor activation. We show that the H3K27ac and RNAPII enhancer occupancy levels that are predictive for FOXO3 binding also increase upon FOXO3 activation. The relevance of this amplification for target gene regulation is highlighted by the greater significance of absolute changes in enhancer activity marks, compared to relative changes, in transcriptional activation of associated genes. In line with this, bound regulatory regions with the highest preexisting activity marks are most associated with gene activation. We not only show the importance of the preexisting chromatin state for directing FOXO3 DNA-binding but also provide evidence for its importance in target gene regulation. Our results indicate that the FOXO3 transcriptional response is both dictated by, and limited to, the cell's specific epigenetic program that is active at the moment of FOXO3 induction. This is in line with observations on short-term transcriptional changes upon signaling cues, which are generally limited to genes with active (or poised) enhancers (Ghisletti et al., 2010; Heintzman et al., 2009). Taken together, and from a mechanist point of view, our data strongly suggest that the number of FOXO3 binding events, determined by a combination of Forkhead DNA elements and the chromatin context, sets a threshold for productive FOXO3 target gene regulation.

The FOXO Transcriptional Program

The notion that FOXO function connects lifespan with two major age-related diseases, i.e., cancer and diabetes, has resulted in an intense search for FOXO-regulated genes. The underlying premise is that a FOXO-specific gene program drives optimal lifespan with a concomitant reduction in disease onset. To date, numerous studies have reported FOXO-induced gene expression changes in various organisms and cellular settings.

(D) Profiles of FOXO3, H3K4me1, H3K4me3, H3K27ac, and RNAPII ChIP-seq occupancy in untreated (−) or FOXO3 activated (+) conditions at the *KLF6* gene locus. Identified enhancers (boxes, gray = FOXO3 bound, white = unbound) are indicated above, with H3K27ac values before and after FOXO3 activation (bars, − and +) and absolute changes (numbers above bars).
(E) All intergenic FOXO3-bound enhancers were sorted into four groups with increasing initial H3K27ac levels and categorized by the number of canonical Forkhead motifs. Absolute changes in RNAPII occupancy are shown (numbers of enhancers within each category are indicated below with p values from a two-tailed Mann-Whitney *U* test on all categories relative to "x").
(F) Enhancers were categorized as in (E), but for all FOXO3-bound enhancers. Changes in RNAPII occupancy of the proximate gene were calculated for each group (≤200 kb enhancer-TSS).
(G) FOXO3 bound responsive enhancers (H3K27ac > 0.5 relative induction, log2) were categorized in four equally sized groups with increasing initial H3K27ac levels and RNAPII occupancy changes of the adjacent gene were calculated (≤200 kb enhancer-TSS). Boxplots and bars show changes in RNAPII gene occupancy (p values from a two-tailed Mann-Whitney *U* test, relative to lowest; ns = $p > 0.05$, *$p < 0.05$, **$p < 10^{-7}$).
See also Figure S5.

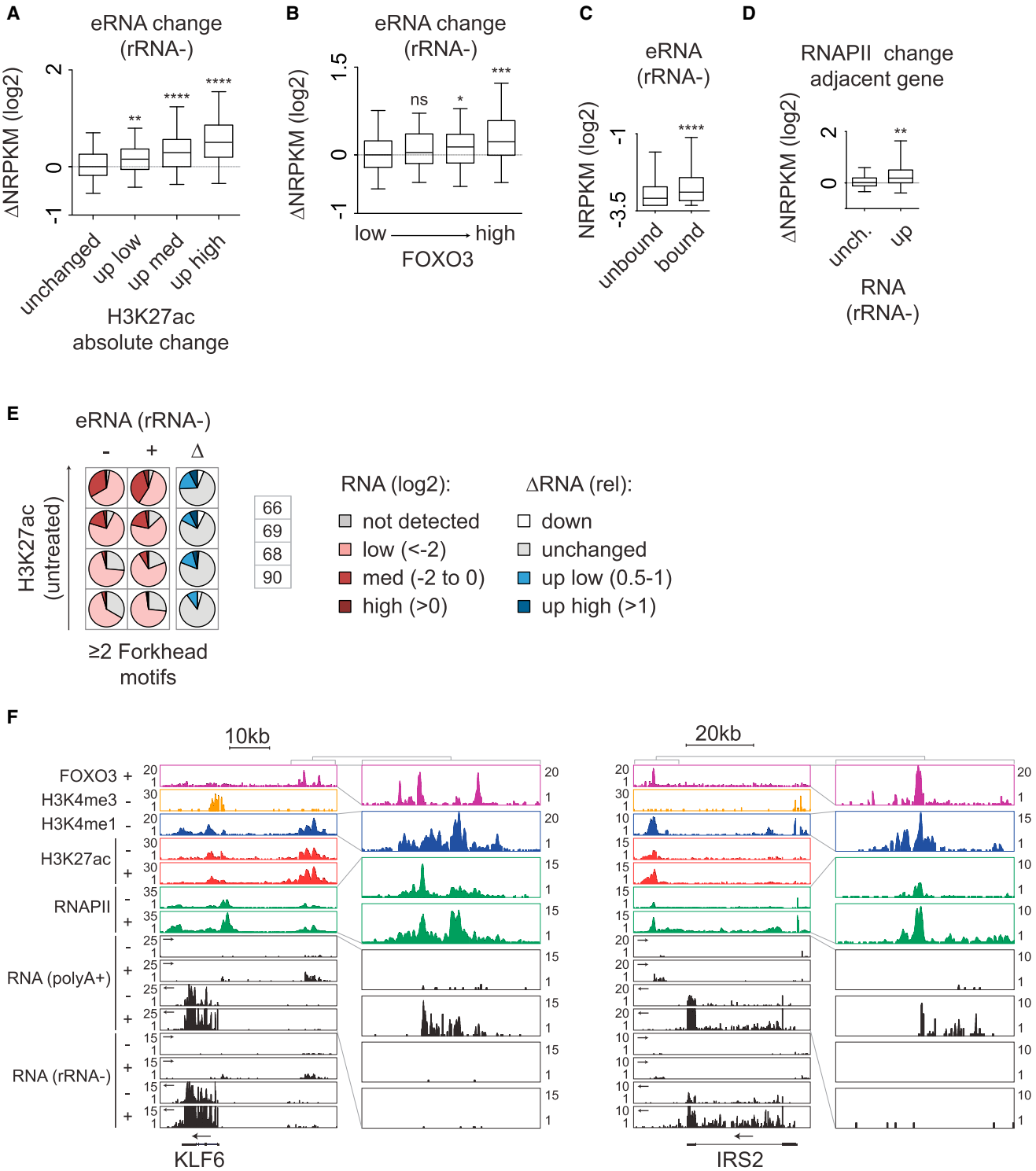


Figure 6. FOXO3 Potentiates Transcription of Enhancers

(A) RNA-seq signal (rRNA depleted) at all intergenic FOXO3-bound enhancers categorized by absolute changes in H3K27ac.

(B) Same as (A), but categorized by FOXO3 levels.

(C) FOXO3 binding status was determined for all intergenic enhancers and corresponding levels of initial transcription determined by RNA-seq are shown.

(D) FOXO3 bound intergenic enhancers were categorized by changes in RNA levels (up is > 1 change, log2) and the changes in RNAPII occupancy of the proximate gene were calculated. Boxplot are shown for all enhancers with a maximum enhancer-TSS distance of 200kb.

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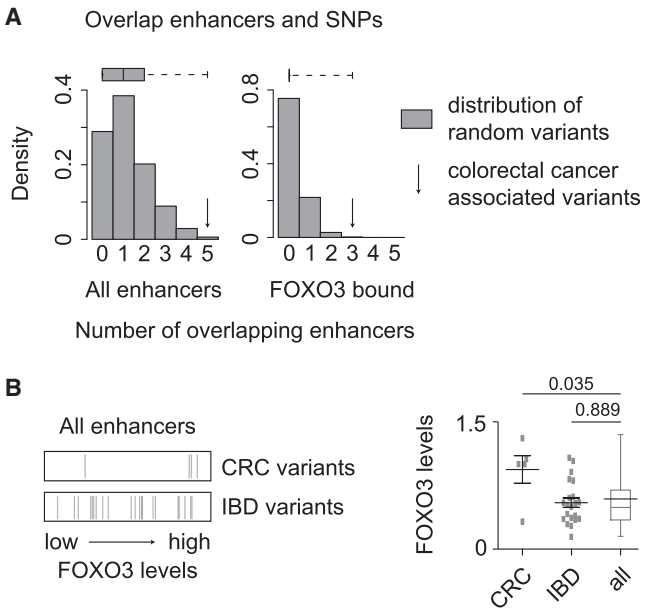

Figure 7. Genetic Variations Could Be Relevant for FOXO3 Binding

(A) The overlap between sequence variants associated with increased colorectal cancer risk and enhancers identified in this study compared to random variants (gray bars and boxplot).

(B) Enhancers were ranked for increasing FOXO3 levels and enhancers overlapping with risk variants for colorectal cancer (CRC) and inflammatory bowel disease (IBD) are indicated by black lines. FOXO3 levels on CRC or IBD variants containing enhancers relative to all enhancers are indicated to the right, with mean and SEM for all categories. The distribution of FOXO3 levels on all enhancers is indicated with a boxplot with 5–95 percentile values. p values are from a two-tailed Mann-Whitney *U* test.

See also Figure S7.

Comparison of these results has not yet resulted in the definition of a comprehensive set of genes that may indeed represent a "FOXO signature" that can mediate a healthy trade-off between lifespan and disease. On the contrary, extensive comparison has yet to result in a single conserved gene regulated by FOXO throughout all organisms and/or cell lines. Thus, the question arises whether a FOXO signature actually exists. Possibly, similarities can only be found when extending the analysis to biological processes regulated by FOXOs, instead of focusing on single genes (Linda Partridge and Irene Papatheodorou, personal communication). Considering the flexible and cell-type-specific nature of regulatory regions and their activity (Heintzman et al., 2009), our observations provide a mechanistic explanation for the diversity in FOXO transcriptional output and lack of a clear FOXO signature. Recently, mainly through studies in model organisms, it has become evident that the biological role of FOXO transcription factors primarily involves the response to stress conditions. In this context, others and we have argued that FOXOs mainly function to maintain cellular and organismal homeostasis over time (Eijkelenboom and Burgering, 2013; Salih and Brunet, 2008). Our results provide an elegant rationale on how homeostasis can be achieved. The use of a preexisting gene program directed by chromatin context of regulatory regions (as illustrated in Figure S7) could allow for a rapid and temporary adjustment in gene transcription, sufficient to re-establish homeostasis, while preventing elaborate time- and energy-consuming permanent alterations in the cell's specific gene expression program.

In many respects, FOXO and c-Myc are functional antagonists, and FOXOs can actively repress c-Myc function in several ways (reviewed in Peck et al., 2013). This is in agreement with c-Myc being an oncogene and FOXOs being bona fide tumor suppressors. However, the aforementioned lack of a FOXO gene expression signature is highly reminiscent of what has been observed for c-Myc (Lin et al., 2012; Nie et al., 2012). The major distinction between FOXO3- and c-Myc-mediated gene regulation is that FOXO3 appears to select regulatory regions, guided by sequence content and cellular chromatin architecture, to restrict gene expression to a limited set of genes that are already controlled by active enhancers, whereas c-Myc acts as a global amplifier of transcription of a large number of active genes. Whether these distinctions between FOXO and c-Myc regarding transcription control contribute to their opposing role with respect to cancer remains to be investigated.

FOXOs as Pioneer Factors?

FOXA, like FOXO, is a member of the larger Forkhead family and has been shown to act as a pioneer factor in establishing chromatin opening at regulatory regions to enable subsequent recruitment of transcription regulators (reviewed in Lalmansingh et al., 2012). In vitro studies have shown mouse Foxo1 binding to disrupt histone-DNA contacts, resulting in chromatin opening (Hatta and Cirillo, 2007; Hatta et al., 2009). Also, binding of the chromatin-remodeling complex Swi/Snf to DAF-16/FOXO bound *C. elegans* genomic locations has been taken to suggest that FOXO may act as a pioneer factor (Riedel et al., 2013). Our results are not compatible with FOXO3 generally acting as a pioneer factor, but they also do not exclude that this occurs in a small subset of bound locations, which could imply a dual role as both a classic transcriptional activator and a pioneer factor (as suggested by Lalmansingh et al., 2012). In line with this, it has been proposed that the mechanism and function of DNA binding of pioneer factors within the chromatin landscape is not substantially different from nonpioneer transcription factors, with preferential binding of both categories to regions marked

(A–D) p values are from a two-tailed Mann-Whitney *U* test relative to the first category (ns = $p > 0.05$, *$p < 0.05$, **$p < 10^{-3}$, ***$p < 10^{-10}$, ****$p < 10^{-15}$). Boxplots are with 5%–95% whiskers.

(E) Same as Figure 5E, but with the presence of and changes in enhancer transcription. Pie charts show RNA levels prior to (−) and after FOXO3 activation (+) as well as FOXO3-induced changes (Δ). Only pie charts for enhancers with multiple canonical Forkhead motifs are shown (all 12 categories in S6G, numbers of enhancers within each category in table).

(F) Tracks of FOXO3, H3K4me1, H3K27ac, RNAPII, RNA poly(A)+, and rRNA− in untreated (−) or FOXO3-activated (+) conditions. Transcribed enhancers from the left panel are displayed in the right panels in more detail. See also Figure S6.

by active histone marks. Thus, many transcription factors might actually have the potential to function as a pioneer in a subset of regions (Guertin and Lis, 2013). The timing of FOXO activation is possibly relevant in this context, as we focus on short-term effects of FOXO3 activation and the transcriptional consequences of the actions of a pioneer factor might be more evident upon prolonged activation. However, FOXO3-induced changes in RNAPII occupancy upon short (4 hr) and long (24 hr) activation are similar (Eijkelenboom et al., 2013), arguing against this. Measurements on nuclear mobility provide an alternative method to distinguish pioneers from nonpioneers. Nuclear mobility of pioneers FoxA1 and FoxA2 was much lower compared with other transcription factors, indicative of tight chromatin binding (Sekiya et al., 2009). Similar measurements of FOXO4 show high nuclear mobility (Tobias Dansen, personal communication), also arguing against a general function as pioneer factors.

Relevance of Genetic Variation for FOXO Function

The role of epigenetics in aging and disease has recently attracted great interest. Epigenetic changes caused by environmental changes or genetic variation within regulatory regions are thought to be involved in the pathogenesis of, and predisposition to, aging and disease (Oberdoerffer and Sinclair, 2007; Rando and Chang, 2012; Sakabe et al., 2012). Several studies, especially in the aging field, have searched for genetic variation within the FOXO genes to link genetic variation to human lifespan (Kenyon, 2010). Our results suggest a conceptual mechanism, indicating that genetic variation that affects levels of enhancer activity, but without affecting the Forkhead motif sequence, could also influence FOXO3 DNA binding and subsequently affect the FOXO3-mediated regulation of target genes. This indicates that genetic variation, especially in enhancer regions near FOXO target genes, may prove to be a relevant determinant to modulate FOXO function and transcriptional output. In agreement with this, we observe a specific correlation between FOXO binding and enhancers with CRC-associated genetic variants. This possibility is further underscored by recent reports showing that quantitative changes in histone marks and RNAPII levels at regulatory regions are associated with genetic variation (Kasowski et al., 2013; Kilpinen et al., 2013; McVicker et al., 2013). As shown here for FOXO3, these quantitative changes could affect DNA binding and transcriptional output of other transcription factors as well, without affecting their recognition sequence, providing an additional explanation for the functional consequences of these quantitative effects.

Deregulation of PI3K/PKB signaling is one of the most frequent events in cancer in general, as well as in CRC, and FOXOs are themselves regulated by PI3K/PKB signaling. Our findings therefore add another possible mode of deregulation of this pathway: genetic disruption of regulatory regions required for FOXO-mediated gene regulation. Analogously, it will be of interest to analyze if a similar correlation may exist between FOXO-bound enhancers in relevant tissues and genetic variants associated with other diseases involving FOXO deregulation, such as diabetes, or even human lifespan. Clearly, further understanding the influence of chromatin state on directing and possibly disrupting the FOXO transcriptional program might prove necessary to fully understand FOXO function in aging and disease.

EXPERIMENTAL PROCEDURES

ChIP-Seq

Cells were grown in the absence or 4 hr presence of 4OHT or PKB inhibitor. ChIPs were performed as described in detail elsewhere (Eijkelenboom et al., 2013). Immunoprecipitations were performed with antibodies recognizing H3K4me1, H3K4me3, H3K27ac, total H3 (ab8895, ab8580, ab4729, and ab1791; Abcam) or FOXO3 (H144; Santa Cruz) for the FOXO3-ER (2) ChIP-seq.

RNA-Seq

Cells were grown in the absence or 8 hr presence of 4OHT. RNA was isolated from two independent biological replicates, generated in parallel with two out of three batches of chromatin for ChIP-seq. Sequencing libraries were constructed using SOLiD Total RNA-Seq Kit (Life Technologies) according to the standard protocol recommendations for low input.

FAIRE-Seq

The FAIRE sample was generated from 10×10^6 DLD1-F3 cells as described in detail previously (Simon et al., 2012). Sequencing library preparation, sequencing, and mapping were performed on isolated DNA as for the ChIP-seq.

Data Analysis

FOXO3 Peak Identification

The Cisgenome software package (Ji et al., 2008) was used for the identification of binding peaks from the ChIP-seq data and further analysis.

Enhancer Identification

Peaks were identified in H3K4me1 (untreated and +4 hr 4OHT) and H3K4me3 (untreated and +4 hr 4OHT) data sets, and H3 was used as background control. Enhancers consist of all identified H3K4me1 peaks excluding peaks overlapping with H3K4me3 peaks.

Quantification of Gene Expression

To set gene expression from RNAPII ChIP-seq (untreated and +4 hr 4OHT) and RNA[poly(A)]-seq (untreated and +8 hr 4OHT, performed in duplicate) data, we counted the number of the sequencing tags aligned to annotated transcript coordinates (Mokry et al., 2012).

Motif Presence

Motif frequency was determined within the 2 kb surrounding the enhancer centers on the subsets of enhancers described in the text. Motif mapping was performed using Cisgenome v2.0 utilities (threshold: −r 500).

SNP Overlap

To determine overlap with variants associated with colorectal cancer and inflammatory bowel disease, a SNP falling within enhancer coordinates was considered as an overlapping SNP. Random SNP sets were generated from variants present on Human Omni1S genotyping chip (Illumina).

Statistical Analysis

Analysis were performed in R, Excel, or Graph Pad. The details of tests used are given in figure captions.

Extended experimental procedures can be found in the Supplemental Information.

ACCESSION NUMBERS

ChIP-seq, RNA-seq, and FAIRE-seq raw data are available from the Gene Expression Omnibus with accession number GSE50243. An overview of all data sets used in this study can be found in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.11.031.

AUTHOR CONTRIBUTIONS

A.E., M.M., B.B., and E.N. designed experiments. A.E. and L.S. performed experiments. M.M. and A.E. performed data analysis. A.E., M.M., and B.B. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Edwin Cuppen, Ewart de Bruijn, Nico Lansu, and Wensi Hao for deep sequencing and Sander Boymans for mapping of the sequencing data. We thank Ruben van Boxtel, Paul Coffer, Linda Partridge, Irene Papatheodorou, and Tobias Dansen for sharing unpublished results. We thank Paul Coffer and Eoghan O'Duibhir for careful reading of the manuscript and suggestions. We thank Frank Holstege, Wouter de Laat, Arne Smits, and all Burgering group members for discussion and suggestions. We thank Maaike van den Berg for initiating FAIRE sequencing. We thank Miranda van Triest for technical support. Research was funded in part by the Centre for Biomedical Genetics, Cancer Genetics Centre, and Centre for Translational Molecular Medicine and the WKZ research fund.

Received: July 5, 2013
Revised: August 29, 2013
Accepted: November 15, 2013
Published: December 19, 2013

REFERENCES

Akhtar-Zaidi, B., Cowper-Sal-lari, R., Corradin, O., Saiakhova, A., Bartels, C.F., Balasubramanian, D., Myeroff, L., Lutterbaugh, J., Jarrar, A., Kalady, M.F., et al. (2012). Epigenomic enhancer profiling defines a signature of colon cancer. Science *336*, 736–739.

Alic, N., Andrews, T.D., Giannakou, M.E., Papatheodorou, I., Slack, C., Hoddinott, M.P., Cochemé, H.M., Schuster, E.F., Thornton, J.M., and Partridge, L. (2011). Genome-wide dFOXO targets and topology of the transcriptomic response to stress and insulin signalling. Mol. Syst. Biol. *7*, 502.

Bernstein, B.E., Birney, E., Dunham, I., Green, E.D., Gunter, C., Snyder, M., Doyle, F., Epstein, C.B., Frietze, S., Harrow, J., et al.; ENCODE Project Consortium (2012). An integrated encyclopedia of DNA elements in the human genome. Nature *489*, 57–74.

Calnan, D.R., and Brunet, A. (2008). The FoxO code. Oncogene *27*, 2276–2288.

Creyghton, M.P., Cheng, A.W., Welstead, G.G., Kooistra, T., Carey, B.W., Steine, E.J., Hanna, J., Lodato, M.A., Frampton, G.M., Sharp, P.A., et al. (2010). Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proc. Natl. Acad. Sci. USA *107*, 21931–21936.

Eijkelenboom, A., and Burgering, B.M. (2013). FOXOs: signalling integrators for homeostasis maintenance. Nat. Rev. Mol. Cell Biol. *14*, 83–97.

Eijkelenboom, A., Mokry, M., de Wit, E., Smits, L.M., Polderman, P.E., van Triest, M.H., van Boxtel, R., Schulze, A., de Laat, W., Cuppen, E., and Burgering, B.M. (2013). Genome-wide analysis of FOXO3 mediated transcription regulation through RNA polymerase II profiling. Mol. Syst. Biol. *9*, 638.

Ghisletti, S., Barozzi, I., Mietton, F., Polletti, S., De Santa, F., Venturini, E., Gregory, L., Lonie, L., Chew, A., Wei, C.L., et al. (2010). Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. Immunity *32*, 317–328.

Giresi, P.G., Kim, J., McDaniell, R.M., Iyer, V.R., and Lieb, J.D. (2007). FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. Genome Res. *17*, 877–885.

Gotea, V., Visel, A., Westlund, J.M., Nobrega, M.A., Pennacchio, L.A., and Ovcharenko, I. (2010). Homotypic clusters of transcription factor binding sites are a key component of human promoters and enhancers. Genome Res. *20*, 565–577.

Guertin, M.J., and Lis, J.T. (2010). Chromatin landscape dictates HSF binding to target DNA elements. PLoS Genet. *6*, e1001114.

Guertin, M.J., and Lis, J.T. (2013). Mechanisms by which transcription factors gain access to target sequence elements in chromatin. Curr. Opin. Genet. Dev. *23*, 116–123.

Guertin, M.J., Martins, A.L., Siepel, A., and Lis, J.T. (2012). Accurate prediction of inducible transcription factor binding intensities in vivo. PLoS Genet. *8*, e1002610.

Hatta, M., and Cirillo, L.A. (2007). Chromatin opening and stable perturbation of core histone:DNA contacts by FoxO1. J. Biol. Chem. *282*, 35583–35593.

Hatta, M., Liu, F., and Cirillo, L.A. (2009). Acetylation curtails nucleosome binding, not stable nucleosome remodeling, by FoxO1. Biochem. Biophys. Res. Commun. *379*, 1005–1008.

Hedrick, S.M., Hess Michelini, R., Doedens, A.L., Goldrath, A.W., and Stone, E.L. (2012). FOXO transcription factors throughout T cell biology. Nat. Rev. Immunol. *12*, 649–661.

Heintzman, N.D., Stuart, R.K., Hon, G., Fu, Y., Ching, C.W., Hawkins, R.D., Barrera, L.O., Van Calcar, S., Qu, C., Ching, K.A., et al. (2007). Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat. Genet. *39*, 311–318.

Heintzman, N.D., Hon, G.C., Hawkins, R.D., Kheradpour, P., Stark, A., Harp, L.F., Ye, Z., Lee, L.K., Stuart, R.K., Ching, C.W., et al. (2009). Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature *459*, 108–112.

Hennessy, B.T., Smith, D.L., Ram, P.T., Lu, Y., and Mills, G.B. (2005). Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat. Rev. Drug Discov. *4*, 988–1004.

Ji, H., Jiang, H., Ma, W., Johnson, D.S., Myers, R.M., and Wong, W.H. (2008). An integrated software system for analyzing ChIP-chip and ChIP-seq data. Nat. Biotechnol. *26*, 1293–1300.

John, S., Sabo, P.J., Thurman, R.E., Sung, M.H., Biddie, S.C., Johnson, T.A., Hager, G.L., and Stamatoyannopoulos, J.A. (2011). Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. Nat. Genet. *43*, 264–268.

Jostins, L., Ripke, S., Weersma, R.K., Duerr, R.H., McGovern, D.P., Hui, K.Y., Lee, J.C., Schumm, L.P., Sharma, Y., Anderson, C.A., et al.; International IBD Genetics Consortium (IIBDGC) (2012). Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature *491*, 119–124.

Kasowski, M., Kyriazopoulou-Panagiotopoulou, S., Grubert, F., Zaugg, J.B., Kundaje, A., Liu, Y., Boyle, A.P., Zhang, Q.C., Zakharia, F., Spacek, D.V., et al. (2013). Extensive variation in chromatin states across humans. Science *342*, 750–752.

Kenyon, C.J. (2010). The genetics of ageing. Nature *464*, 504–512.

Kilpinen, H., Waszak, S.M., Gschwind, A.R., Raghav, S.K., Witwicki, R.M., Orioli, A., Migliavacca, E., Wiederkehr, M., Gutierrez-Arcelus, M., Panousis, N.I., et al. (2013). Coordinated effects of sequence variation on DNA binding, chromatin structure, and transcription. Science *342*, 744–747.

Kops, G.J., Medema, R.H., Glassford, J., Essers, M.A., Dijkers, P.F., Coffer, P.J., Lam, E.W., and Burgering, B.M. (2002). Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol. Cell. Biol. *22*, 2025–2036.

Lalmansingh, A.S., Karmakar, S., Jin, Y., and Nagaich, A.K. (2012). Multiple modes of chromatin remodeling by Forkhead box proteins. Biochim. Biophys. Acta *1819*, 707–715.

Lin, C.Y., Lovén, J., Rahl, P.B., Paranal, R.M., Burge, C.B., Bradner, J.E., Lee, T.I., and Young, R.A. (2012). Transcriptional amplification in tumor cells with elevated c-Myc. Cell *151*, 56–67.

Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G., and Evan, G.I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res. *23*, 1686–1690.

Lovén, J., Hoke, H.A., Lin, C.Y., Lau, A., Orlando, D.A., Vakoc, C.R., Bradner, J.E., Lee, T.I., and Young, R.A. (2013). Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell *153*, 320–334.

McVicker, G., van de Geijn, B., Degner, J.F., Cain, C.E., Banovich, N.E., Raj, A., Lewellen, N., Myrthil, M., Gilad, Y., and Pritchard, J.K. (2013). Identification of genetic variants that affect histone modifications in human cells. Science *342*, 747–749.

Mokry, M., Hatzis, P., Schuijers, J., Lansu, N., Ruzius, F.P., Clevers, H., and Cuppen, E. (2012). Integrated genome-wide analysis of transcription factor occupancy, RNA polymerase II binding and steady-state RNA levels identify differentially regulated functional gene classes. Nucleic Acids Res. *40*, 148–158.

Nie, Z., Hu, G., Wei, G., Cui, K., Yamane, A., Resch, W., Wang, R., Green, D.R., Tessarollo, L., Casellas, R., et al. (2012). c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. Cell *151*, 68–79.

Oberdoerffer, P., and Sinclair, D.A. (2007). The role of nuclear architecture in genomic instability and ageing. Nat. Rev. Mol. Cell Biol. *8*, 692–702.

Ong, C.T., and Corces, V.G. (2011). Enhancer function: new insights into the regulation of tissue-specific gene expression. Nat. Rev. Genet. *12*, 283–293.

Ouyang, W., Liao, W., Luo, C.T., Yin, N., Huse, M., Kim, M.V., Peng, M., Chan, P., Ma, Q., Mo, Y., et al. (2012). Novel Foxo1-dependent transcriptional programs control T(reg) cell function. Nature *491*, 554–559.

Peck, B., Ferber, E.C., and Schulze, A. (2013). Antagonism between FOXO and MYC Regulates Cellular Powerhouse. Front Oncol *3*, 96.

Pennacchio, L.A., Bickmore, W., Dean, A., Nobrega, M.A., and Bejerano, G. (2013). Enhancers: five essential questions. Nat. Rev. Genet. *14*, 288–295.

Pique-Regi, R., Degner, J.F., Pai, A.A., Gaffney, D.J., Gilad, Y., and Pritchard, J.K. (2011). Accurate inference of transcription factor binding from DNA sequence and chromatin accessibility data. Genome Res. *21*, 447–455.

Rando, T.A., and Chang, H.Y. (2012). Aging, rejuvenation, and epigenetic reprogramming: resetting the aging clock. Cell *148*, 46–57.

Riedel, C.G., Dowen, R.H., Lourenco, G.F., Kirienko, N.V., Heimbucher, T., West, J.A., Bowman, S.K., Kingston, R.E., Dillin, A., Asara, J.M., and Ruvkun, G. (2013). DAF-16 employs the chromatin remodeller SWI/SNF to promote stress resistance and longevity. Nat. Cell Biol. *15*, 491–501.

Sakabe, N.J., Savic, D., and Nobrega, M.A. (2012). Transcriptional enhancers in development and disease. Genome Biol. *13*, 238.

Salih, D.A., and Brunet, A. (2008). FoxO transcription factors in the maintenance of cellular homeostasis during aging. Curr. Opin. Cell Biol. *20*, 126–136.

Samstein, R.M., Arvey, A., Josefowicz, S.Z., Peng, X., Reynolds, A., Sandstrom, R., Neph, S., Sabo, P., Kim, J.M., Liao, W., et al. (2012). Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. Cell *151*, 153–166.

Samuels, Y., Diaz, L.A., Jr., Schmidt-Kittler, O., Cummins, J.M., Delong, L., Cheong, I., Rago, C., Huso, D.L., Lengauer, C., Kinzler, K.W., et al. (2005). Mutant PIK3CA promotes cell growth and invasion of human cancer cells. Cancer Cell *7*, 561–573.

Schuster, E., McElwee, J.J., Tullet, J.M., Doonan, R., Matthijssens, F., Reece-Hoyes, J.S., Hope, I.A., Vanfleteren, J.R., Thornton, J.M., and Gems, D. (2010). DamID in C. elegans reveals longevity-associated targets of DAF-16/FoxO. Mol. Syst. Biol. *6*, 399.

Sekiya, T., Muthurajan, U.M., Luger, K., Tulin, A.V., and Zaret, K.S. (2009). Nucleosome-binding affinity as a primary determinant of the nuclear mobility of the pioneer transcription factor FoxA. Genes Dev. *23*, 804–809.

Shin, D.J., Joshi, P., Hong, S.H., Mosure, K., Shin, D.G., and Osborne, T.F. (2012). Genome-wide analysis of FoxO1 binding in hepatic chromatin: potential involvement of FoxO1 in linking retinoid signaling to hepatic gluconeogenesis. Nucleic Acids Res. *40*, 11499–11509.

Simon, J.M., Giresi, P.G., Davis, I.J., and Lieb, J.D. (2012). Using formaldehyde-assisted isolation of regulatory elements (FAIRE) to isolate active regulatory DNA. Nat. Protoc. *7*, 256–267.

Spitz, F., and Furlong, E.E. (2012). Transcription factors: from enhancer binding to developmental control. Nat. Rev. Genet. *13*, 613–626.

Splinter, E., and de Laat, W. (2011). The complex transcription regulatory landscape of our genome: control in three dimensions. EMBO J. *30*, 4345–4355.

van der Vos, K.E., and Coffer, P.J. (2008). FOXO-binding partners: it takes two to tango. Oncogene *27*, 2289–2299.

van der Vos, K.E., and Coffer, P.J. (2011). The extending network of FOXO transcriptional target genes. Antioxid. Redox Signal. *14*, 579–592.

Webb, A.E., Pollina, E.A., Vierbuchen, T., Urbán, N., Ucar, D., Leeman, D.S., Martynoga, B., Sewak, M., Rando, T.A., Guillemot, F., et al. (2013). FOXO3 shares common targets with ASCL1 genome-wide and inhibits ASCL1-dependent neurogenesis. Cell Rep *4*, 477–491.