Published OnlineFirst July 27, 2010; DOI: 10.1158/0008-5472.CAN-10-0582

Cancer Research

Genome-Wide Identification of PAX3-FKHR Binding Sites in Rhabdomyosarcoma Reveals Candidate Target Genes Important for Development and Cancer

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Abstract

The PAX3-FKHR fusion protein is present in a majority of alveolar rhabdomyosarcomas associated with increased aggressiveness and poor prognosis. To better understand the molecular pathogenesis of PAX3-FKHR, we carried out the first, unbiased genome-wide identification of PAX3-FKHR binding sites and associated target genes in alveolar rhabdomyosarcoma. The data shows that PAX3-FKHR binds to the same sites as PAX3 at both *MYF5* and *MYOD* enhancers. The genome-wide analysis reveals that the PAX3-FKHR sites are (*a*) mostly distal to transcription start sites, (*b*) conserved, (*c*) enriched for PAX3 motifs, and (*d*) strongly associated with genes overexpressed in PAX3-FKHR–positive rhabdomyosarcoma cells and tumors. There is little evidence in our data set for PAX3-FKHR binding at the promoter sequences. The genome-wide analysis further illustrates a strong association between PAX3 and E-box motifs in these binding sites, suggestive of a common coregulation for many target genes. We also provide the first direct evidence that *FGFR4* and *IGF1R* are the targets for PAX3-FKHR. The map of PAX3-FKHR binding sites provides a framework for understanding the pathogenic roles of PAX3-FKHR, as well as its molecular targets to allow a systematic evaluation of agents against this aggressive rhabdomyosarcoma. *Cancer Res; 70(16); 6497–508. ©2010 AACR.*

Introduction

The paired box transcription factor (PAX) family consists of nine highly related members with critical roles in organogenesis and tissue specification during development, including skeletal muscle, pancreas, central nervous system, kidney, and immune system (1). In various cell model systems, *PAX* genes have been implicated in the maintenance of the multipotent state, the initiation of differentiation programs, cell migration, proliferation, and survival. Mutations and gene rearrangement of *PAX* genes are frequently associated with human diseases and cancers (2–4). In particular, *PAX3* and *PAX7* are required during early embryogenesis for the development of the neural crest and skeletal muscle (2, 5), and gene rearrangements with *FKHR* are frequently implicated with rhabdomyosarcoma (6).

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-10-0582

Rhabdomyosarcoma is the most common soft tissue sarcoma in children (7), and consists of alveolar (ARMS) and embryonal subtypes. The majority of ARMS are associated with specific chromosome translocations that result in PAX3-FKHR (FOXO1A) or PAX7-FKHR fusion protein, consisting of the NH_2-terminal PAX3/PAX7 DNA binding domain and the COOH-terminal FKHR transactivation domain (8, 9). The fusion protein was suggested as a more potent transactivator than PAX3 *in vitro* (10, 11) and *in vivo* (12). Conditional activation of PAX3-FKHR was shown to collaborate with the inactivation of *Ink4a/Arf* or *p53* in facilitating the development of murine ARMS (13). The *PAX3-FKHR* translocation is not only the most common translocation in ARMS but is also associated with increased treatment failure and mortality rate (14). Thus, it is important to identify the direct transcription targets of PAX3-FKHR. Nearly all published studies on PAX3-FKHR targets and their cis-acting sequences focused on promoter elements immediately adjacent to the transcription start sites, even though the role of PAX3-FKHR binding sites as a promoter has not been verified. In fact, for the best-studied targets of PAX3, the myogenic determination genes, *MYF5* and *MYOD*, the cis-acting sequences responsible for PAX3 regulation are distal enhancers (15–18). As the identification of the direct effectors of PAX3-FKHR might have pivotal roles in delineating its molecular pathogenic mechanism and in identifying new targets for therapy, a genome-wide analysis of the direct targets of PAX3-FKHR is the next necessary step.

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To elucidate the targets of PAX3-FKHR, chromatin immunoprecipitation and DNA sequencing (ChIP-seq) were performed with a novel antibody specific for PAX3-FKHR fusion proteins. Our results show that the majority of binding sites are conserved distal regulatory elements, enriched for PAX3 and E-box/MYF motifs, and are strongly associated with genes upregulated by PAX3-FKHR. The data set of PAX3-FKHR binding sites has a high degree of accuracy, as shown in the examples of *MYF5* and *MYOD* enhancers, with a resolution sufficient for rapid identification and confirmation of specific recognition sequences. The identification of the PAX3-FKHR binding sites and associated genes enables the rapid identification and validation of its targets, which would enhance our understanding of myogenic development, the molecular pathogenesis of rhabdomyosarcoma, and the evaluation of targeted agents for this cancer.

Materials and Methods

Cell lines and reagents

All human rhabdomyosarcoma cell lines, along with an Rh1 Ewing's sarcoma cell line, were maintained in RPMI 1640 with 10% fetal bovine serum. *PAX3-FKHR*-HA and *PAX3* and *PAX3-FKHR* expression plasmids were kindly provided by Drs. Michael Anderson and Fred Barr. Small hairpin RNA (shRNA) plasmids targeting FKHR were obtained from Open Biosystems with the target sequences: s2, GCTTAGACTGTGACATGGAAT; s4, CAGGACAATAAGTCGAGTTAT. Lentiviruses for shRNA vectors were generated. For RNAi experiments, cells were infected with the lentiviruses for 3 days with at least 50% of the cells infected.

Quantitative PCR analysis

Total and immunoprecipitated DNA were used for quantitative PCR (qPCR) on glyceraldehyde-3-phosphate dehydrogenase promoter and MYF5 enhancer using SYBR Green PCR kit (Qiagen); the primers used are listed in Supplementary Table S7. For quantitative expression analysis, we used cells (3 days after infection with lentiviruses) carrying scramble or shRNA vectors against the FKHR portion of PAX-FKHR. Total RNA was isolated with Trizol (Invitrogen) and cDNA was generated using SuperScript III (Invitrogen). qPCR analysis was performed in triplicate with SYBR Green PCR kit, with primers listed in Supplementary Table S7 to determine the relative levels of specific transcripts, normalized against that of glyceraldehyde-3-phosphate dehydrogenase. All qPCR assays were reproduced at least three times.

Antibodies and immunoblots

Cell lysate preparations and immunoblots were done as previously described (19). A monoclonal antibody against PAX3-FKHR (PFM2) was generated against a fusion region peptide (Supplementary Methods). Ascites fluid was obtained for PFM2 and the IgG fraction was isolated. Antibodies against PAX3, FKHR, MYCN, MET, ERK, and AKT, as well as actin, were from Cell Signaling Technology; the antibody against IGF1R-β (C20) was from Santa Cruz Biotechnology.

Luciferase reporter assay for PAX3-FKHR enhancer

PAX3-FKHR binding sites were amplified from human genomic DNA using the primers listed in Supplementary Table S7. They were cloned into the *Sal*I and *Bam*HI sites of the pGL3-promoter vector (Promega), which contains an SV40 promoter upstream of the firefly luciferase reporter gene. Mutant enhancers were generated with a site-directed mutagenesis kit (Stratagene) with primers listed in Supplementary Table S7. All mutations were confirmed by DNA sequencing. Enhancer assays were performed with a Dual Luciferase Assay System (Promega) to adjust for differences in transfection efficiency. Reporter cotransfection experiments were done with reporter to activator plasmid ratios of 1:1. All reporter assays were reproduced at least three times.

Statistical analyses

All quantitative experiments, including qPCR for immunoprecipitated DNA, RT-qPCR for specific gene expression, and luciferase analyses were performed in triplicate. Data analyses were performed with Prism (GraphPad) and shown in charts as mean ± SEM. Its effect on gene expression patterns was tested based on the null hypothesis that the number of genes associated with binding sites within the set of differentially expressed genes was not larger than expected by chance when compared with the set of all annotated genes. *P* values were calculated using the hypergeometric distribution.

Results

Genome-wide mapping of PAX3-FKHR binding sites in rhabdomyosarcoma

To identify the targets of PAX3-FKHR, a novel monoclonal antibody PFM2 was generated using a peptide corresponding to the fusion region. The antibody is specific to PAX3-FKHR (Fig. 1A). The precipitation with PFM2 in PAX3-FKHR$^+$Rh4 cells resulted in a 20- to 40-fold enrichment of the *MYF5* enhancer over the input DNA, the best known target of PAX3 (Fig. 1B). In contrast, parallel ChIP with fusion-negative RD cells gave no enhancer enrichment (Fig. 1B). Both the ChIP and input DNA were sequenced to obtain 3.96 and 4.06 million aligned sequence tags, respectively, with similar chromosome distributions (Supplementary Fig. S1, Supplementary Table S1). Although the input DNA tags were evenly distributed, distinct sequence tag clusters were identified with PFM2 ChIP DNA, as shown in chromosome 11 (Fig. 1C). Altogether, a total of 1,463 statistically significant putative PAX3-FKHR binding sites were identified in the human genome with an average size of 186 bp, at approximately one site per 2 million base pairs (GEO no. GSE19063, see Supplementary Methods for a detailed description of selection criteria and data access). The chromosome distribution of the binding sites is closely correlated with the length of the chromosome (Supplementary Fig. S1, r^2= 0.83), similar to that of estrogen receptor and p53 (Supplementary Fig. S1, Supplementary Table S1; refs. 20, 21). The ChIP-seq result was highly reproducible with 80% (1,166 sites) identified in a repeat experiment (data not shown). The specificity of the binding

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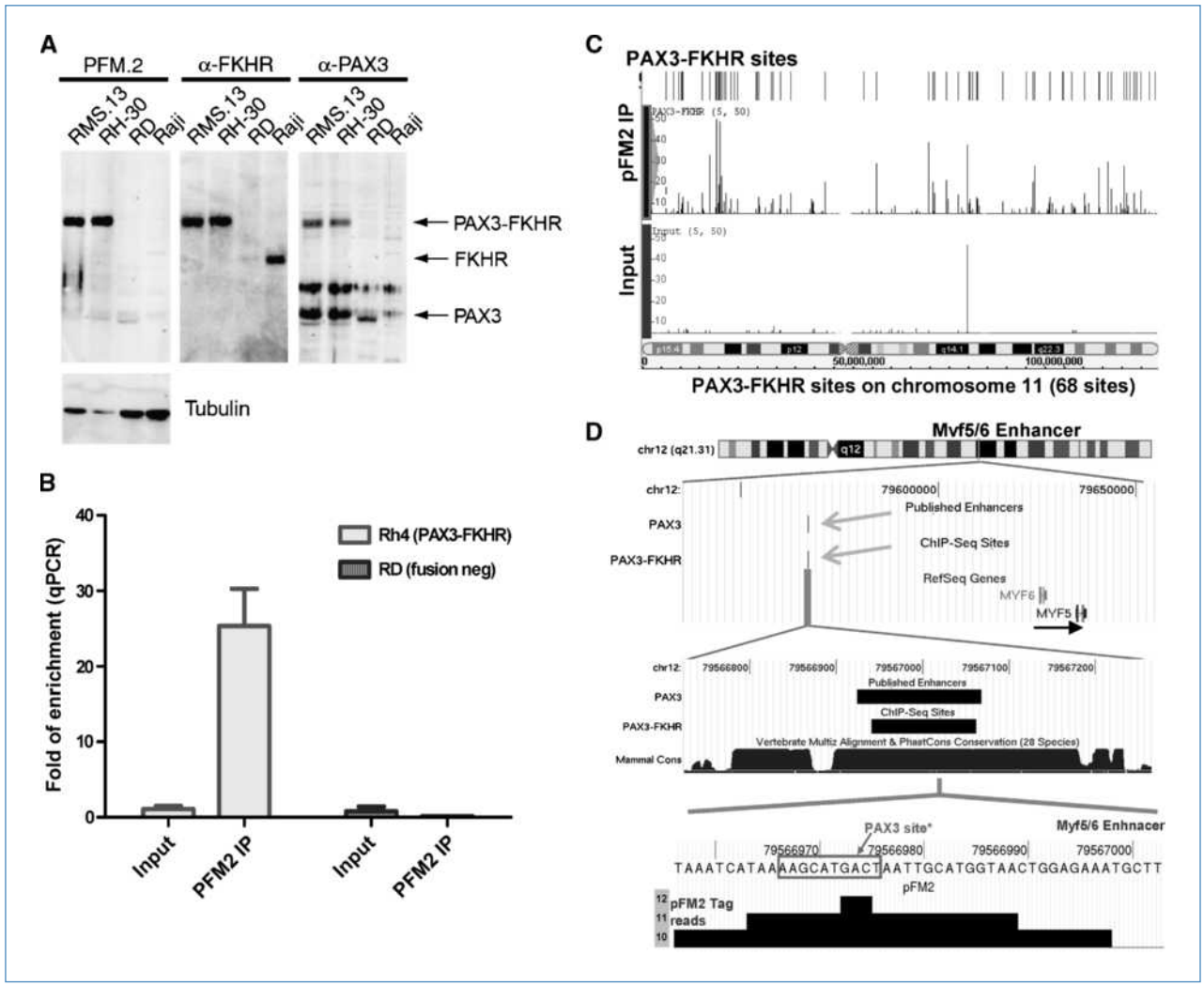

Figure 1. ChIP-seq analysis of PAX3-FKHR binding sites in rhabdomyosarcoma cells. A, immunoblots show that PFM2 recognizes PAX3-FKHR proteins in rhabdomyosarcoma cell lines positive for the translocation (RMS13 and Rh30). B, qPCR analysis shows that PFM2 selectively immunoprecipitates the *MYF5* enhancer from PAX3-FKHR$^+$ Rh4 cells, not from the negative RD cells. C, distribution of PAX3-FKHR binding sites in chromosome 11. Peak height represents the number of sequence tags. D, a PAX3-FKHR site mapped within the *MYF5* enhancer. The maximum signal aligns with a known PAX3 recognition site at this PAX3-dependent enhancer.

sites was very good, as there was no overlap between the 1,463 sites in Rh4 and the 223 sites obtained from fusion-negative RD cells via ChIP-seq, which also gave 4 million aligned sequences (data not shown).

The specificity of the binding sites was verified with the best characterized PAX3-dependent enhancer located at −58/−56 kb distal to *MYF5* (Fig. 1D). The binding site identified by ChIP-seq is defined in a 108 bp region, within the previously identified 145 bp minimal PAX3-responsive *MYF5* enhancer (17), demonstrating that PAX3-FKHR binds to identical sites in rhabdomyosarcoma cells as PAX3 does during development. The maximum of the tag density was located at the boxed PAX3 binding sequence AAGCATGACT, at the enhancer previously shown to be critical for PAX3-dependent activation (17, 22). This result shows that the data set might have sufficient resolution for identifying specific recognition sequences.

PAX3-FKHR binding at conserved distal regulatory elements

Analysis of the binding sites showed that the vast majority of the sites were either more than 4 kb from the transcription start sites outside of the genes (60.2%), or in the introns (32.5%). Only 0.4% of the binding sites were located within 1 kb upstream of the initiation sites (Fig. 2A). In addition, there was no binding site enrichment within either a 1 or 4 kb window upstream of the start sites (Fig. 2A). The data suggests that PAX3-FKHR binds primarily to distal sites. The enrichment analysis further revealed reduced binding sites in the exons (Fig. 2A), consistent with distinctive functions for

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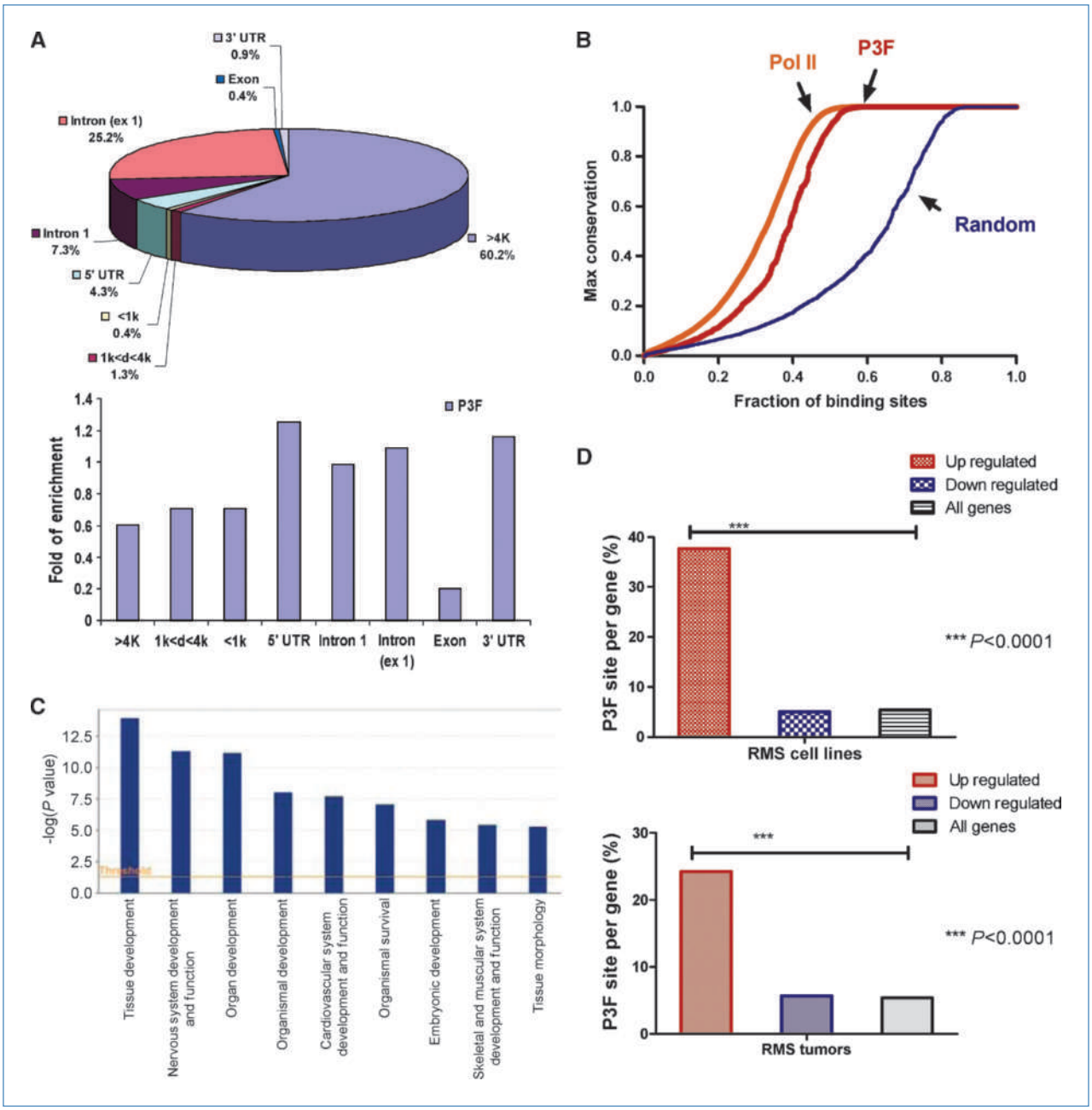

Figure 2. PAX3-FKHR binding sites as putative enhancers. A, distribution of PAX3-FKHR sites relating to the transcription start sites, exons, and introns. Enrichment or suppression PAX3-FKHR sites. Unit of 1 represents the average number of sites across the genome. B, degree of conservation of PAX3-FKHR sites compared with that of Pol-II ChIP-seq sites and randomly generated sequence tags. C, gene ontology analysis of genes associated with PAX3-FKHR sites with Ingenuity IPA. D, the association of PAX3-FKHR sites with genes upregulated by PAX3-FKHR in RD cells (Supplementary Table S3), and genes upregulated in a panel of PAX3/7-FKHR–positive rhabdomyosarcoma tumors (Supplementary Table S4).

the two. Maximal conservation analysis shows that there is a significant conservation among these PAX3-FKHR binding sites in vertebrates, in contrast to that of randomly selected sequences. The degree of conservation is similar to that of RNA Pol-II binding sites (Fig. 2B), again indicating the functionality of these PAX3-FKHR sites. The analysis of the 1,463 binding sites identifies 1,072 genes either overlapping or located proximal to them (Supplementary Table S2). Gene ontology analysis shows a bias towards developmental processes, including those of the nervous, skeletal, and muscular systems (Fig. 2C), consistent with the known roles of PAX3 in early development and its expression in those systems. Thus, the binding sites are conserved and identify potential targets of PAX3 during development.

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Inspection of the PAX3-FKHR sites provides a direct link between PAX3/PAX3-FKHR and the genes implicated in skeletal development, muscular development, and rhabdomyosarcoma (Table 1). These genes are involved in growth, survival and oncogenesis, cell migration and tumor metastasis, as well as being important for myogenic differentiation. All their binding sites were re-confirmed in the repeat experiment. Despite their roles in muscle development and cancer, many were not previously known to be direct targets of either PAX3 or PAX3-FKHR, and in most cases, the PAX3-FKHR–dependent regulatory elements were not defined.

PAX3-FKHR sites associated with genes upregulated by PAX3-FKHR in rhabdomyosarcoma

To establish the association between the binding sites and genes regulated by PAX3-FKHR, correlative analysis was performed with the genes upregulated and downregulated by *PAX3-FKHR* when introduced to the RD cells via retroviral transduction (23). There is a 7-fold enrichment of PAX3-FKHR binding sites in the genes upregulated by PAX3-FKHR at 37.7%; whereas no enrichment of PAX3-FKHR sites was seen for genes downregulated by PAX3-FKHR at 5% (Fig. 2D, Supplementary Table S3). Hypergeometric distribution analysis shows that the association between genes with PAX3-FKHR sites and those upregulated by PAX3-FKHR is statistically significant ($P < 0.0001$). In addition to the association with syngeneic rhabdomyosarcoma cells with exogenously introduced PAX3-FKHR, there is a strong correlation between the binding sites and the upregulated genes in a panel of 160 rhabdomyosarcoma tumors (24). Of the 363 genes with increased expression in PAX3/7-FKHR–positive rhabdomyosarcoma, 24.2% have the binding sites (Fig. 2D, Supplementary Table S4). In contrast, of the 122 genes with decreased expression, only 5.7% have the binding sites. Again, hypergeometric distribution analysis shows that such an association is significant ($P < 0.0001$). Thus, PAX3-FKHR sites are strongly associated with genes upregulated by PAX3-FKHR *in vitro* in rhabdomyosarcoma cells and in rhabdomyosarcoma tumors with PAX3/7-FKHR translocations.

PAX3-FKHR binds *MYOD* core enhancer at a newly defined PAX3 recognition sequence and regulates other limb development genes

MYOD is the master controller of skeletal muscle development (25). PAX3 regulation of *MYOD* is mediated via the *MYOD* core enhancer (15, 26). Our genome-wide analysis of PAX3-FKHR sites shows a binding site in a region of 161 bp, located within the previously mapped 258-bp *MYOD* core enhancer (Fig. 3A). Interestingly, sequence examination at the binding signal peak identified a novel PAX3 recognition sequence, AACCCGTGAC (box). To confirm the role of this PAX3 recognition sequence, an enhancer reporter was constructed with this MYOD enhancer and a mutant was then derived by site-directed mutagenesis to inactivate the PAX3 recognition sequence. Enhancer analysis showed that this particular PAX3 recognition sequence was responsible for PAX3- and PAX3-FKHR–dependent activation of the core enhancer (Fig. 3A). Thus, our data enabled the identification of a novel recognition sequence necessary for PAX3- and PAX3-HKFR–mediated activation of *MYOD* core enhancer. In addition to *MYF5* and *MYOD*, we identified novel PAX3-FKHR binding sites in several additional transcription factors important for muscle and limb development, and potentially for ARMS, including homeobox genes *MEOX1, MEOX2*, and *PRRX1* (Table 1). Selected sites were validated by enhancer assays, site-directed mutagenesis, and shRNA depletion of PAX3-FKHR (Supplementary Figs. S2 and S3).

Genome-wide coenrichment of PAX3 and E-box motifs within the PAX3-FKHR site

There is limited knowledge on how PAX3 functions in regulating gene expression. It is a weak transactivator and might require other elements in regulating gene expression. Genetic hierarchy analysis of skeletal muscle differentiation reveals that *MYOD* expression requires both *PAX3* and *MYF5* (27). Analysis of the *MYOD* core enhancer shows several E-box motifs that are required for its expression in the skeletal muscle lineage (26). We asked if the presence of both PAX3 and E-box motifs was a more common property of PAX3-FKHR binding sites. Transcription factor motif analysis of PAX3-FKHR sites showed that PAX3_B was the most enriched motif at 21.7-fold, together with several related paired box motifs (Supplementary Table S5; Fig. 3B). Interestingly, several other motifs were also enriched: AP4 by 12.9-fold and LBP1 by 11.1-fold, sharing a common CAGCTG E-box motif. Thus, motif analysis of PAX3-FKHR sites reveals a genome-wide enrichment of PAX3 and E-box motifs. To confirm the results, *de novo* motif analysis of the ChIP-Seq data was performed with Discriminating Motif Enumerator (DME) (28). Of the top 10 output motifs, at least 5 were PAX3 or highly related motifs, and 4 others were E-box–related motifs using the TRANSFAC database (Fig. 3C; Supplementary Table S6). Thus, motif and *de novo* analysis of PAX3-FKHR sites revealed a genome-wide selective enrichment of PAX3 and E-box motifs.

We asked whether the enrichment was due to the colocalization of these two motifs adjacent to each other. When PAX3_B was used as the anchor, both LBP1 and AP4 motifs (E-box) were enriched by 7- to 8-fold within 100 bp of the anchor (Fig. 3D). Neither the PAX3_B site nor controls (CREBP1 or PPAR-α) were enriched adjacent to the PAX3_B anchor. Similarly, when LBP1 was used as the anchor, only the PAX3_B motif was enriched by 14-fold. Thus, the genome-wide analysis of PAX3 and E-box motifs shows a strong colocalization at PAX3-FKHR binding sites. These observations suggest a broad role for PAX3 and MYF/E-box proteins in regulating the expression of many targets in addition to *MYOD*.

FGFR4 is a direct target of PAX3-FKHR

The fibroblast growth factor (FGF) signaling pathway has been implicated *in vivo* in promoting cell proliferation and differentiation during myogenesis (29). FGFR4 is expressed during myogenic differentiation under the control of PAX3 via a downstream enhancer (30). The importance of this pathway has recently been emphasized by the identification of activating mutations of FGFR4 in 7% of rhabdomyosarcomas (31), which promotes metastasis in the xenograft mouse.

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Table 1. Fold of induction was normalized against that of the vector reporter without an activator

(A) Selected genes with PAX3-FKHR binding sites

ID	Entrez gene name	Location	Type	Drugs
Self-renewal, growth, survival, oncogenesis				
ALK	Anaplastic lymphoma receptor tyrosine kinase	Plasma membrane	Receptor kinase	PF-02341066
FGFR2	Fibroblast growth factor receptor 2	Plasma membrane	Receptor kinase	TKI-258, AZD2171, brivanib, PHA-739358
FGFR4	Fibroblast growth factor receptor 4	Plasma membrane	Receptor kinase	
IGF1R	Insulin-like growth factor 1 receptor	Plasma membrane	Receptor kinase	CP-751871, AMG479, R1507, MK-0646, AVE1642
KDR	Kinase insert domain receptor, VEGFR2	Plasma membrane	Receptor kinase	Bevacizumab, sunitinib, vatalanib, sorafenib, vandetanib
MEIS1	Meis homeobox 1	Nucleus	Transcription factor	
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma-derived	Nucleus	Transcription factor	
SPRY1	Sprouty homologue 1, antagonist of FGF signaling (Drosophila)	Plasma membrane	FGFR4 ligand	
Migration, metastasis				
CXCR4	Chemokine (C-X-C motif) receptor 4	Plasma membrane	GPCR	JM 3100
MET	met proto-oncogene (hepatocyte growth factor receptor)	Plasma membrane	Receptor kinase	PF-02341066
Myogenic differentiation				
EYA2	Eyes absent homologue 2 (Drosophila)	Nucleus	Phosphatase	
EYA4	Eyes absent homologue 4 (Drosophila)	Cytoplasm	Phosphatase	
MEOX1	Mesenchyme homeobox 1	Nucleus	Transcription factor	
MEOX2	Mesenchyme homeobox 2	Nucleus	Transcription factor	
MYF5/6	Myogenic factor 6 (herculin)	Nucleus	Transcription factor	
MYOD1	Myogenic differentiation 1	Nucleus	Transcription factor	
PRRX1	Paired related homeobox 1	Nucleus	Transcription factor	
Other potential targets				
ABAT	4-Aminobutyrate aminotransferase	Cytoplasm	Enzyme	Valproic acid
ADRA1D	Adrenergic, α-1D-, receptor	Plasma membrane	GPCR	Paliperidone, risperidone, antazoline, acetaminophen, epinephrine, acetaminophen
ADRA2A	Adrenergic, α-2A-, receptor	Plasma membrane		
ADRA2C	Adrenergic, α-2C-, receptor	Plasma membrane		
DHFR	Dihydrofolate reductase	Unknown	Enzyme	Pyrimethamine, trimethoprim, iclaprim, methotrexate

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Published OnlineFirst July 27, 2010; DOI: 10.1158/0008-5472.CAN-10-0582

Table 1. (Cont'd)

(B) Verification of PAX3-FKHR binding site via enhancer assay

Gene	Enhancer no.	Binding intensity	Fold induction by activators			Rel. Location	Dist. Tx Start bp	Dist. Tx End bp
			None	PAX3	PAX3-FKHR			
Vector	None	NA	1.0	1.0	0.9	NA	NA	NA
ALK	1	High	2.6	10.8	19.5	3rd Intron	–262,835	464,601
FGFR4	1	Low	1.5	1.9	3.7	3′ downstream	–8,913	–839
FGFR4	2	High	1.9	5.7	8.3	3′ downstream	–16,273	–8,199
IGF1R	1	Med	1.8	4.0	4.8	2nd Intron	–136,375	171,486
MET	1	Low	3.9	10.2	7.4	3rd Intron	–35,329	61,711
MET	2	High	1.3	2.5	3.8	18th Intron	–83,277	13,763
MYCN	1	Med	0.9	3.8	5.4	3′ downstream	–96,819	–92,786
MEOX1	1	Med	1.2	2.4	5.4	5′ upstream	994	20,274
MEOX2	1	High	0.8	0.8	3.8	1 intron	–798	73,218
PRRX1	1	Med	0.6	2.1	18.2	1st intron	–4,226	67,742

NOTE: The locations of the binding sites are the distances to transcription start and end shown as upstream (+) or downstream (-).

The expression level of FGFR4 was associated with ARMS and poor survival (31). Our results revealed two binding sites downstream of *FGFR4* (Fig. 4A). Both putative *FGFR4* enhancer sequences were cloned into the enhancer reporter and used for enhancer analysis. The results showed that the enhancer activities were detected in PAX3-FKHR$^+$Rh4 and Rh30 cells, but not in the negative Rh18 cells (Fig. 4B). The distal FGFR4.E2 gave significantly greater activity than that of FGFR4.E1. In cotransfection experiments into RD cells, *PAX3-FKHR* was able to induce both E1 and E2 activity (Fig. 4B), of which E2 is a stronger enhancer.

The examination of FGFR4.E2 reveals PAX3 and E-box recognition sequences that are both conserved in vertebrates (Fig. 4A). A mutant in the putative PAX3 recognition site resulted in the complete inactivation of PAX3-FKHR–dependent induction (Fig. 4C). The data show that the PAX3-FKHR binding site downstream of *FGFR4* is a PAX3-FKHR–dependent enhancer.

To determine if the expression of *FGFR4* in rhabdomyosarcoma cells is dependent on PAX3-FKHR, Rh4 cells were infected with a lentivirus containing a shRNA (s2) targeting PAX3-FKHR. RNA was isolated and analyzed for PAX3-FKHR and FGFR4 mRNA levels. The results showed that both PAX3-FKHR and FGFR4 mRNA levels were reduced with this shRNA (P3F.s2, Fig. 4D). Thus, PAX3-FKHR is responsible for the elevated FGFR4 mRNA levels in a fusion-positive rhabdomyosarcoma cell line.

It is worth noting that our study might provide more accurate data on PAX3-FKHR–dependent regulation for *MET*. In contrast with a previous report showing that the *MET* promoter was regulated by PAX3 (32), our data identified two PAX3-FKHR binding sites in the introns of *MET*. Enhancer reporter assays showed that both were activated by PAX3 and PAX3-FKHR (Table 1; Supplementary Fig. S4). As these sequences are selectively bound by PAX3-FKHR in rhabdomyosarcoma cells, the results indicate that *MET* is regulated by PAX3 or PAX3-FKHR via intronic enhancers.

Identification of PAX3-FKHR–dependent enhancers at *ALK* and *MYCN*

Of the genes containing proximal or internal binding sites for PAX3-FKHR, Ingenuity Pathway analysis (Ingenuity Systems) revealed the strongest disease association with developmental, skeletal, and muscular disorders, among which MYCN was implicated (Supplementary Fig. S5). In addition to amplification in neuroblastomas, *MYCN* was shown to be amplified in ~20% of both rhabdomyosarcomas associated with adverse outcome (33). However, its expression was significantly higher in ARMS than in embryonal rhabdomyosarcoma. Moreover, an increase in MYCN expression was associated with worse prognosis only in ARMS, not embryonal rhabdomyosarcoma. Our ChIP-seq data showed a binding site located at 96.8 kb downstream of the transcription start site that could serve as a PAX3-FKHR–dependent enhancer (Table 1). Furthermore, protein analysis in a panel of rhabdomyosarcoma cell lines showed the highest levels of MYCN only in PAX3-FKHR fusion–positive rhabdomyosarcoma cells (Fig. 5C), and it is downregulated with a shRNA targeting PAX3-FKHR (Fig. 5D), suggesting a role of PAX3-FKHR in the expression of MYCN in rhabdomyosarcoma.

Although originally identified in translocations in lymphoma, activating mutations in *ALK* were also recently associated with neuroblastoma (34–37). Our ChIP-seq data again showed a strong PAX3-FKHR site in the third intron of *ALK*, which was a very potent PAX3-FKHR–dependent enhancer (Table 1). Infecting Rh4 cells with a lentivirus containing a shRNA (s2) for PAX3-FKHR led to a significant reduction of ALK mRNA (Fig. 4D). Thus, *ALK* expression could also be regulated by PAX3-FKHR.

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PAX3-FKHR directly upregulates IGF1R, which might serve as a biomarker for anti-IGF1R therapy

PAX3 is known to be important for the proliferation and survival of muscle progenitor cells (38, 39). Although it is not known how PAX3 accomplishes these functions, it is possible that PAX3-FKHR targets genes important for growth and survival, and for the development of rhabdomyosarcoma. Ingenuity Pathway analysis of PAX3-FKHR targets also revealed IGF1R in the developmental disorder network (Supplementary Fig. S5). The *IGF1R* has a PAX3-FKHR binding site in the second intron (Fig. 5A). When cloned into the enhancer reporter, it exhibited specific enhancer activity that was dependent on either PAX3 or PAX3-FKHR (Fig. 5B). Analysis of IGF1R protein levels in rhabdomyosarcoma cell lines showed an apparent correlation with PAX3-FKHR fusion proteins, in which all four cell

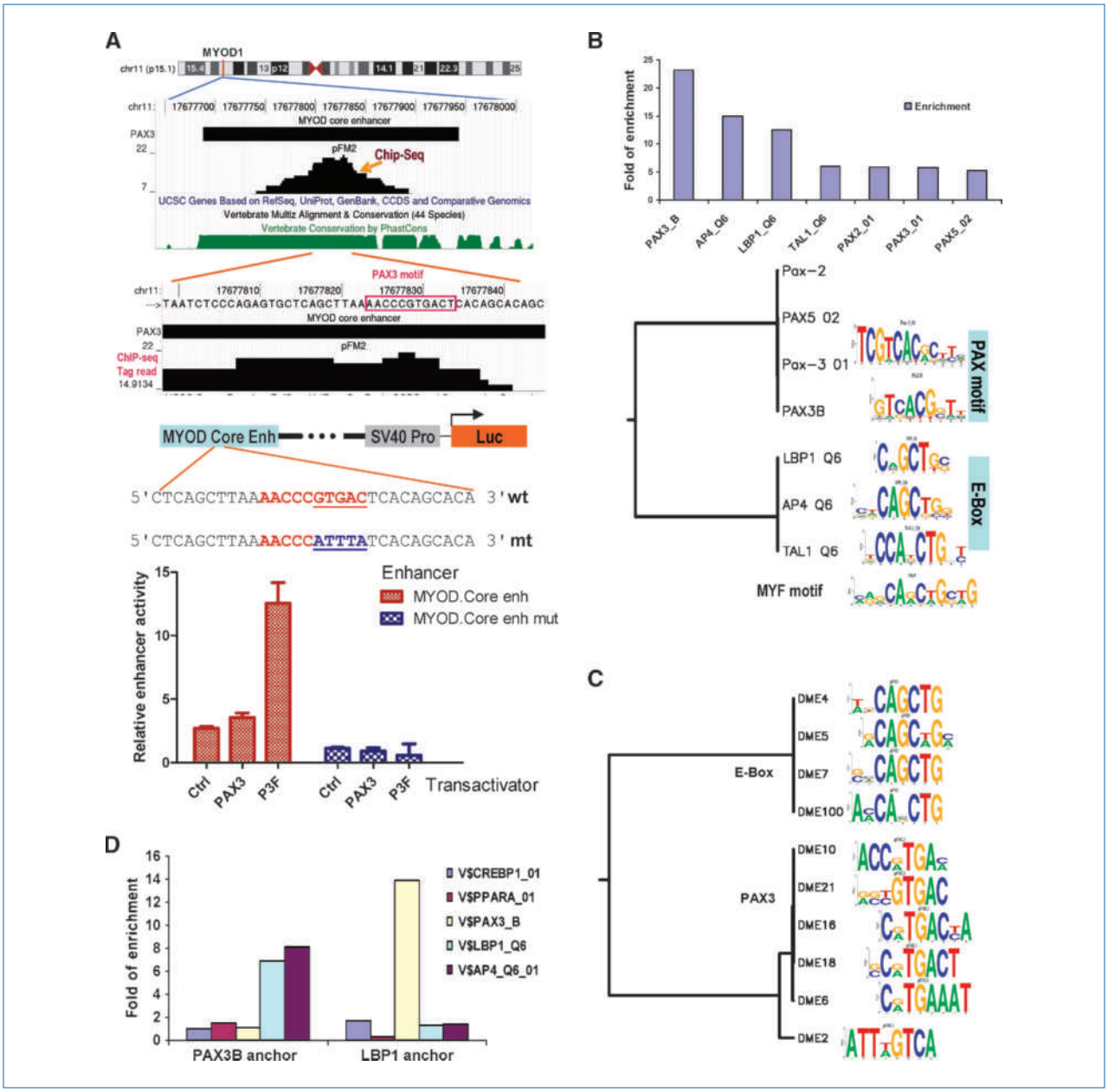

Figure 3. PAX3 recognition sequence in the *MYOD* core enhancer and the coenrichment of PAX3 and E-box motifs in the binding sites. A, the identification of a novel PAX3 recognition sequence at the *MYOD* core enhancer and site-directed mutagenesis study of the site in transfected A204 rhabdomyosarcoma cells. B, enrichment of transcription factor motifs in PAX3-FKHR sites and clustering of the top enriched motifs. C, results of a *de novo* motif search in which 9 of the top 10 motifs were shown to be related to either E-box or PAX3 motifs (see Supplementary Table S6 for details). D, coenrichment of PAX3 and E-box motifs among PAX3-FKHR sites. Either PAX3B or LBP1 was anchored, and the relative enrichment of other sites within 100 bp of the anchor.

Published OnlineFirst July 27, 2010; DOI: 10.1158/0008-5472.CAN-10-0582

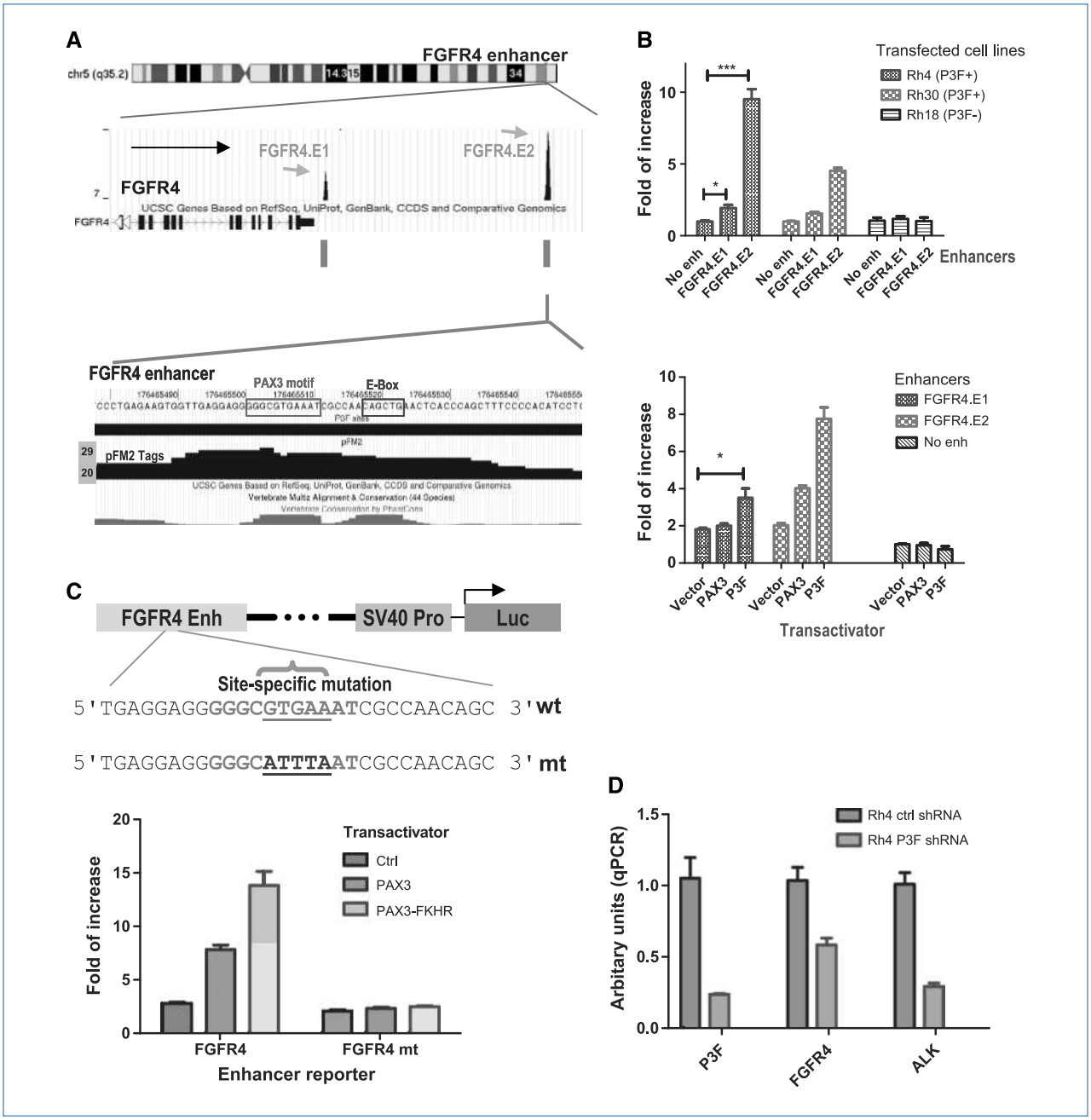

Figure 4. Fine-mapping of a PAX3-FKHR–dependent enhancer for *FGFR4* gene. A, ChIP-seq results identified two PAX3-FKHR binding sites downstream of *FGFR4*. The peak of the FGFR4.E2 binding signal is associated with a conserved PAX3 sequence. B, enhancer activity for both binding sites in PAX3-FKHR (P3F)–positive and –negative cells, and with cotransfection *PAX3* or *PAX3-FKHR* expression plasmid in RD cells. C, site-directed mutagenesis of PAX3 recognition sequence FGFR4.E2 enhancer resulted in a loss of transactivation by PAX3 or PAX3-FKHR in RD cells. D, RT-qPCR data showing downregulation of PAX3-FKHR with a lentiviral shRNA (s2) vector led to reduced ALK and FGFR4 mRNA in Rh4.

lines with the fusion protein had high levels of IGF1R (Fig. 5C). To show the requirement of PAX3-FKHR for elevated IGF1R expression, Rh4 cells were infected with two shRNA lentiviral vectors targeting PAX3-FKHR. The results showed that in addition to downregulating PAX3-FKHR, these RNAi vectors also reduced IGF1R protein levels, similar to that of MET (Fig. 5D), whereas the levels of ERK and AKT were constant. The results indicate that PAX3-FKHR could directly upregulate IGF1R.

Discussion

In this study, we present a high-resolution whole genome map of PAX3-FKHR binding sites in ARMS. To our knowledge,

Published OnlineFirst July 27, 2010; DOI: 10.1158/0008-5472.CAN-10-0582

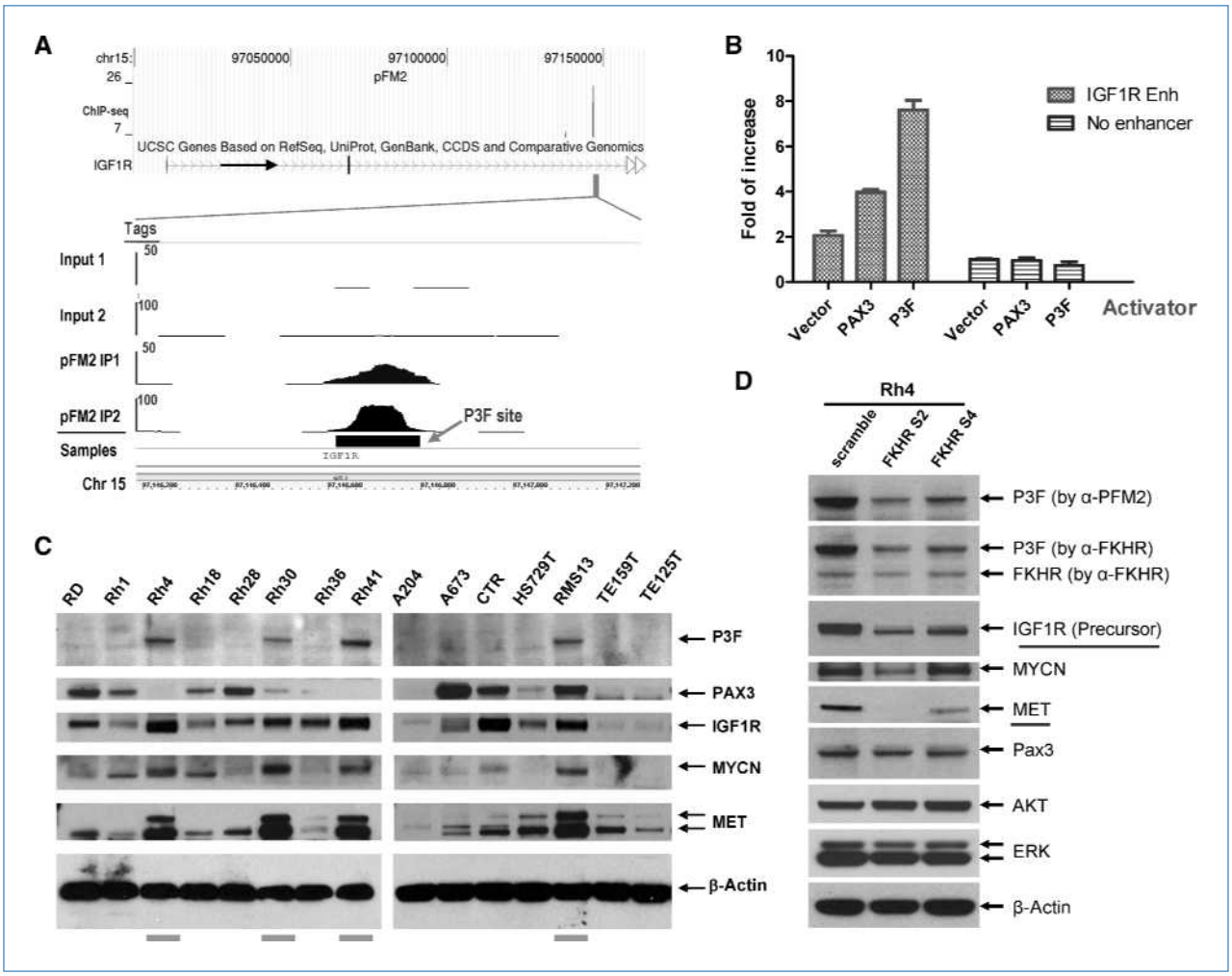

Figure 5. *IGF1R* as a target for PAX3-FKHR transactivation. A, identification of a PAX3-FKHR binding site in the 2nd intron of *IGF1R*. ChIP-seq results of input and PFM2 immunoprecipitated DNA from two experiments. B, PAX3 and PAX3-FKHR–dependent activation of the *IGF1R* enhancer in cotransfected RD cells. C, immunoblots of IGF1R, MYCN, MET, and PAX3-FKHR in a panel of rhabdomyosarcoma cell lines. D, shRNA lentiviral vectors against PAX3-FKHR results in the downregulation of IGF1R, MYCN, and MET in Rh4.

this is the first such map for a PAX protein. Understanding how PAX3-FKHR acts to drive ARMS tumorigenesis depends on developing a comprehensive catalog of the genes which are directly regulated by this aberrant oncogenic transcription factor. Previous expression array analysis of PAX3-FKHR–regulated genes provided information on the genes that were differentially expressed (40–42). However, that approach has significant limitations due to its inability to identify direct targets and to link the genes with specific cis-acting sequences for follow-up investigations. Promoter analysis, although used frequently for the study of putative PAX3-FKHR–regulated genes, is entirely constrained to sequences immediately adjacent to the transcription start sites. Development studies of PAX3 targets reveal that PAX3 regulates myogenic initiating genes via distal enhancers (15–18, 30). Although our PAX3-FKHR binding site results show concise binding on the PAX3-dependent enhancers of *MYF5* and *MYOD*, which were identified with transgenic animal models, our data failed to provide support for nearly all previous reports on the characterization of PAX3-FKHR–mediated gene regulation as they mostly focused on limited promoter sequences (43–45). In one instance, we showed two intronic enhancers for *MET* which were not at the previously described *MET* promoter. PAX3 motifs are rather degenerate and our data shows that only ~1 in 1,000 of the predicted PAX3 motifs in the genome were actually bound by PAX3-FKHR (Supplementary Table S5). We believe that the traditional plasmid-based report assay is necessary, but not sufficient, to establish the roles of the regulatory sequences, whereas the binding of PAX3-FKHR to these sequences *in vivo* is essential. Overall, in our genome-wide analysis, only 0.4% of the binding sites are within 1 kb upstream of the transcription start site. The binding sites are conserved distal elements, enriched for PAX3 motifs, present in genes implicated in the developmental processes, and associated with genes induced by PAX3-FKHR in rhabdomyosarcoma cells and tumors. Our whole genome map of

Published OnlineFirst July 27, 2010; DOI: 10.1158/0008-5472.CAN-10-0582

PAX3-FKHR binding sites nominates numerous candidates for a role in the transforming activity of ARMS, and strongly suggests that the action of this fusion oncogene is achieved through a network of pathways affecting myoblast differentiation, migration, proliferation, and survival (Table 1). Thus, a map of PAX3-FKHR binding sites should provide the framework for future studies of its downstream targets and pathogenesis mechanisms.

Our study also provides a genomic overview in understanding PAX3 or PAX3-FKHR–mediated gene regulation. It was previously established that *MYF5* (E-box protein) and *PAX3* were genetically required for proper *MYOD* expression (27). Our genome-wide analysis of PAX3-FKHR sites shows the colocalization of PAX3 and E-box motifs at these sites, suggesting the potential coregulation of many target genes by PAX3 and MYF-like E-box proteins. The roles of such coregulation in development and cancer are questions for future investigations.

The mechanism of PAX3-FKHR action in ARMS could also be viewed as a form of aberrant muscle development. Sustained expression of other developmental transcription factors as a result of PAX3-FKHR is likely to be an important aspect of ARMS pathogenesis. In addition to the myogenic E-box factors, we also identified a number of homeobox genes as PAX3-FKHR targets. Our data showed that PAX3-FKHR binds to the enhancers of *MEOX1, MEOX2,* and *PRRX1*, and is responsible for their expression. Mesenchyme homeobox genes 1 and 2 (*MEOX1* and *MEOX2*) are expressed in somites that give rise to connective tissue, cartilage, muscle, and the axial skeleton. Both genes have been implicated in axial skeleton and limb muscle development (46). PAX3 and MEOX2 were coexpressed in most migrating myoblasts and MEOX2 was absent from limbs in homozygous $Pax3^{Sp}$ mice (47). Similarly, *PRRX1* is implicated in limb development. Mice homozygous for mutant *Prrx1* died soon after birth and exhibited defects in skeletogenesis (48). PRRX1 was expressed at a significantly reduced level in homozygous $Pax3^{Sp}$ mice (49). Thus, our work identified new developmental transcription factors driven by PAX3-FKHR in ARMS (and likely by PAX3 in normal development). Determining which of these transcriptional regulators are important in establishing the ARMS phenotype and how they act are important questions for future research.

Identification of the genes and pathways directly regulated by PAX3-FKHR provides an opportunity to better understand the molecular pathogenesis of ARMS, to mine new therapeutic targets, and to provide biomarkers for the development of novel agents. In this connection, our study identifies *ALK, FGFR4, IGF1R*, and *MYCN* as direct targets for PAX3-FKHR, which may have important roles in the pathogenesis of rhabdomyosarcomas. Previous studies showed that MYCN amplification and overexpression were associated with adverse prognosis in ARMS (33) and was downstream of PAX3-FKHR (50). Our data identifies a PAX3-FKHR–dependent regulatory sequence in *MYCN*. In addition, our data indicates *ALK* as a target of PAX3-FKHR in rhabdomyosarcoma, opening the possibility of follow-up investigations on the roles of *ALK* in rhabdomyosarcoma. Finally, the comprehensive map of PAX3-FKHR targets provided by this study will facilitate the search for therapeutic approaches for rhabdomyosarcoma. Although it is currently not possible to target PAX3-FKHR itself, selectively targeting one or more critical proteins regulated by PAX3-FKHR such as IGF1R or FGFR4 might improve the outcome of patients with this aggressive cancer. Our results provide the first evidence of the regulation of *FGFR4* by PAX3-FKHR in ARMS, which was recently suggested to contribute to *in vivo* tumor growth and lung metastasis (31). Our data further shows that PAX3-FKHR regulates *IGF1R* expression in rhabdomyosarcoma. We previously showed that its elevated expression was directly correlated with rhabdomyosarcoma sensitivities to a therapeutic antibody against IGF1R, and inhibiting IGF1R resulted in a profound reduction of AKT signaling both *in vitro* and *in vivo* (19). Thus, the systematic identification of PAX3-FKHR targets provides a blueprint for the evaluation of targeted therapeutics against signaling pathways driven by the essential causative genetic aberration in this tumor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Fred Barr and Michael Anderson for *PAX3* and *PAX3-FKHR* plasmids, Andrew Smith for assistance with DME analysis, and Zhigang Kang and Sean Davis for technical assistance.

Grant Support

The Intramural Research Program of the NIH, National Cancer Institute, and in part with federal funds from the NIH, under contract N01-CO-12400. The contents of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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Received 02/16/2010; revised 05/03/2010; accepted 06/12/2010; published OnlineFirst 07/27/2010.

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Published OnlineFirst July 27, 2010; DOI: 10.1158/0008-5472.CAN-10-0582

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Genome-Wide Identification of PAX3-FKHR Binding Sites in Rhabdomyosarcoma Reveals Candidate Target Genes Important for Development and Cancer

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Cancer Res 2010;70:6497-6508. Published OnlineFirst July 27, 2010.