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ZBED6, a Novel Transcription Factor Derived from a Domesticated DNA Transposon Regulates IGF2 Expression and Muscle Growth

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Abstract

A single nucleotide substitution in intron 3 of IGF2 in pigs abrogates a binding site for a repressor and leads to a 3-fold up-regulation of IGF2 in skeletal muscle. The mutation has major effects on muscle growth, size of the heart, and fat deposition. Here, we have identified the repressor and find that the protein, named ZBED6, is previously unknown, specific for placental mammals, and derived from an exapted DNA transposon. Silencing of Zbed6 in mouse C2C12 myoblasts affected Igf2 expression, cell proliferation, wound healing, and myotube formation. Chromatin immunoprecipitation (ChIP) sequencing using C2C12 cells identified about 2,500 ZBED6 binding sites in the genome, and the deduced consensus motif gave a perfect match with the established binding site in Igf2. Genes associated with ZBED6 binding sites showed a highly significant enrichment for certain Gene Ontology classifications, including development and transcriptional regulation. The phenotypic effects in mutant pigs and ZBED6-silenced C2C12 myoblasts, the extreme sequence conservation, its nucleolar localization, the broad tissue distribution, and the many target genes with essential biological functions suggest that ZBED6 is an important transcription factor in placental mammals, affecting development, cell proliferation, and growth.

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Competing Interests: A patent has been filed concerning the possible commercial use of the ZBED6 gene and protein.

Abbreviations: ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; QTL, quantitative trait locus; QTN, quantitative trait nucleotide; RT-PCR, real-time PCR; SILAC, stable isotope labeling of amino acids in culture; siRNA, small interfering RNA; TSS, transcription start site

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Introduction

Strong selection for lean growth in the domestic pigs during the last 60 years has resulted in increased muscle growth and reduced fat deposition. Quantitative trait locus (QTL) mapping using an intercross between the European Wild Boar and Large White domestic pigs identified the most important locus that has responded to this selection pressure as a paternally expressed QTL colocalized with the gene for insulin-like growth factor 2 (IGF2) [1]. The allele present in the domestic pig increases muscle growth and heart size, and reduces subcutaneous fat deposition. The causative mutation for this QTL is a single nucleotide substitution in intron 3 of IGF2 [2]. The mutation is located in a CpG island that is well-conserved among mammals, and 16 bp including the mutated site showed 100% sequence identity among eight placental mammals. This quantitative trait nucleotide (QTN) is one of the rare examples in which a single base substitution underlying a complex trait has been identified and the mechanism of action is partially understood [2].

The IGF2 mutation, a G to A transition, disrupts the interaction with an unknown nuclear factor, a repressor, and leads to a 3-fold up-regulation of IGF2 expression in skeletal muscle. Elevated
Author Summary

The molecular identification of genes and mutations affecting complex traits and disorders has proven to be very challenging in humans as well as in model organisms. These so-called quantitative traits arise from interactions between two or more genes and their environment, and can be mapped to their underlying genes via closely linked stretches of DNA called quantitative trait loci (QTL). Previously, we identified a single nucleotide substitution in a noncoding region of the insulin-like growth factor 2 gene (IGF2) in pigs that is underlying a major QTL affecting muscle growth, heart size, and fat deposition. The mutation disrupts interaction with an unknown nuclear protein acting as a repressor of IGF2 transcription. In the present study, we have isolated a zinc finger protein of unknown function and show that it regulates the expression of IGF2. The protein, which we named ZBED6, is encoded by a domesticated DNA transposon that was inserted into the genome prior to the radiation of placental mammals. ZBED6 is exclusive to placental mammals and highly conserved among species. Our functional characterization of ZBED6 shows that it has a broad tissue distribution and may affect the expression of thousands of other genes, besides IGF2, that control fundamental biological processes. We postulate that ZBED6 is an important transcription factor affecting development, cell proliferation, and growth in placental mammals.

Results

Identification of the IGF2 Repressor Using Oligonucleotide Capture and Mass Spectrometry

Our previous electrophoretic mobility shift assay (EMSA), as well as transient transfection experiments with luciferase reporters, demonstrated that the unknown repressor is expressed in mouse C2C12 myoblasts [2]. To isolate the IGF2 repressor, we employed affinity capture using nuclear extracts from C2C12 cells and biotinylated oligonucleotides corresponding to the wild-type (q) and mutant (Q) sequence where only the former binds the repressor. Two different nuclear extracts were prepared using stable isotope labeling of amino acids in culture (SILAC) technique [4], in which the “heavy” extract proteins contained the stable-isotope-labeled amino acids lysine and arginine, whereas the “light” extract proteins contained the natural versions of these amino acids. The wild-type and mutant oligonucleotides were incubated with heavy and light extracts, respectively. Captured heavy and light proteins were mixed and separated with SDS-PAGE. Gel slices were digested with trypsin, and the resulting peptides were analyzed by liquid chromatography mass spectrometry (LCMS). The acquired spectra were searched against the RefSeq database containing mouse protein sequences to identify proteins present in the sample. Ratios of the amount of each protein enriched by the q and Q constructs were computed by comparing the mass spectral signals from the heavy and light versions of each identified peptide composing the protein.

The protein demonstrating the highest fold enrichment by q (9.0±1.2-fold; Figure 1A) corresponded to a transcript annotated as an alternative splice form of the poorly characterized \(\zeta^{3}\)h11a gene. ZC3H11A belongs to a large family of zinc finger proteins with 58 known members in mouse [5]. However, a closer examination revealed that the captured peptide is encoded by an intronless gene located in intron 1 of \(\zeta^{3}\)h11a (Figure 1B). The gene contains an open reading frame of more than 900 codons and encodes a protein with no sequence similarity to ZC3H11A. The encoded protein contains two BED domains and an hATC dimerization domain (Figure 1C). The BED domain was originally identified by a bioinformatic analysis using two chromatin-boundary-element-binding proteins from Drosophila, BEAF and DREF, as seeds for homology search [6]. We named our protein ZBED6 because it is the sixth mammalian protein with one or more BED domains (Figure 1C and 1D). ZBED6 is related to the hAT superfamily of DNA transposons, named after hobo from Drosophila, Activator from maize, and Tam3 from snapdragon [7]. For instance, the active Hermes transposase from the houyhnesian BED domain and a carboxyterminal hATC domain (Figure 1C).

The mammalian ZBED proteins showed high sequence divergence outside the BED and hATC domains and represent divergent members of the hAT superfamily (unpublished data). The two BED domains of ZBED6 are more closely related to each other than to any other mammalian BED sequence, implying an internal duplication after the integration in the genome (Figure S1). The ZBED6 protein is highly conserved among placental mammals, and in particular, the DNA-binding BED domains show 100\% or close to 100\%, sequence identity among 26 different species, including the pig (Figure S2); the only sequence differences compared with the consensus sequence for placental mammals occurred in species with low-coverage genome sequences and may therefore represent sequence errors.

To address whether ZBED6 is the bona fide repressor binding the QTN region in IGF2 intron 3, we produced a truncated mouse Zbed6 protein including the two DNA-binding BED domains and used this in EMSA with the q and Q oligonucleotides differing only at the QTN position. EMSA revealed a highly specific interaction with the wild-type q oligonucleotide, and a 100-fold excess of mutant Q oligonucleotide could not outcompete the interaction (Figure 1E). A polyclonal anti-ZBED6 antibody was developed by immunizing rabbits with this recombinant protein containing the two BED domains. A supershift was obtained when nuclear extracts from C2C12 cells were incubated with this anti-ZBED6 antibody, providing further biochemical evidence for ZBED6 as the elusive IGF2 repressor (Figure 1E).

Zbed6 Has a Broad Tissue Distribution and Is Coexpressed with Zc3h11a

Northern blot analysis (Figure 2A) and real-time PCR (RT-PCR) analysis (Figure 2B) showed that Zbed6, like IGF2, has a broad...
tissue distribution in mouse and is expressed in skeletal muscle, consistent with it being the \textit{Igf2} repressor. Northern blot analysis (Figure 2A) and RT-PCR amplification and sequencing (unpublished data) revealed that \textit{Zbed6} is coexpressed with \textit{Zc3h11a} as an 13-kb splice variant of \textit{Zc3h11a}, retaining the genomic region from exon 1 to exon 4 (including introns 1, 2, and 3 with \textit{Zbed6} located in intron 1) spliced to the remaining \textit{Zc3h11a} exons (exons 5–18).
An examination of chromatin immunoprecipitation (ChIP) sequencing data for the human genome provided by the ENCODE consortium showed that there is one major RNA polymerase II binding site located just upstream of exon 1 of ZC3H11A, which apparently constitutes the common promoter for ZC3H11A and ZBED6 (Figure 2C). This is further supported by the perfect colocalization with a 5' cap analysis gene expression (CAGE) tag. This promoter region contains binding sites for the Max, Myc, Fos, Jun, and NF-E2 transcription factors, all with a central role in regulating cell proliferation and associated with cancer development.

ZBED6 and ZC3H11A proteins were detected in the nuclei of postnatal muscle and brain tissue from mouse by using immunohistochemistry (Figure 3). ZBED6 expression was also confirmed in skeletal muscle (longissimus dorsi) cell nuclei from a 6-mo-old pig (Figure 3K–3N), i.e., at the age at which a highly significant effect of the pig IGF2 mutation is documented [1,2].

The ZBED6 Protein Shows a Nucleolar Localization in Mouse C2C12 Cells

Western blot analysis of proteins from mouse C2C12 cells revealed two different isoforms, denoted ZBED6a and ZBED6b, with apparent molecular weights of 122 and 116 kDa, respectively (Figure 4A). These isoforms most likely correspond to the use of two alternative start codons in the open reading frame of ZBED6, as demonstrated by comigration with recombinant proteins representing the two isoforms (Figure 4A).

Bioinformatic analysis revealed bipartite nuclear localization signals in two regions of ZBED6, amino acid residues 61–80 and 231–248. We used two different GFP-fusion constructs to confirm a nuclear localization in C2C12 cells (Figure 4B). The GFP-BED1/2x protein (residues 47–384) containing both regions with nuclear localization signals was primarily associated with granular structures in the nucleus, suggesting a nucleolar localization. This was confirmed by double immunofluorescence staining of C2C12 cells (Figure 4C) showing colocalization of endogenous ZBED6 and nucleophosmin 1, a well-known marker for the nucleolus [8]. However, ZBED6 did not show a complete localization to the nucleolus since some dispersed staining throughout the nucleus was evident (Figure 4C). Interestingly, the GFP-BED1/2y fusion protein (residues 90–384) also showed nuclear localization, but as an exclusion from the nucleolus (Figure 4B). This implies that the nuclear localization signal comprising residues 61–80 constitutes or contains a nucleolar localization signal. This lysine- and arginine-rich sequence (KKKRKKGLRIKGKRRRKKLI) is highly positively charged and resembles a nucleolar localization signal previously identified in the myogenic regulatory factor Myf5.
Figure 3. Immunohistochemistry of ZBED6 and ZC3H11A in brain and muscle tissue from mouse and in pig muscle. (A–J) Immunofluorescence analysis of mouse tissues stained with anti-ZBED6 or anti-ZC3H11A antibodies. (A and D) Coronal brain overview images assembled from pictures taken at 4 x magnification. (B, C, E, and F) Higher magnification images of boxed areas indicated in (A and D) with anti-ZBED6 (B and C) or anti-ZC3H11A (E, F) immunopositive signals in red. (G–J) Images of anti-ZBED6 or anti-ZC3H11A antibody stainings of section from mouse thigh muscle. Nuclear localization of ZBED6 and ZC3H11A is confirmed by colabeling with DAPI (blue) in both brain and muscle as indicated by arrowheads, whereas nuclei lacking ZBED6- or ZC3H11A-positive signals are indicated by arrows. (K–N) Immunofluorescence analysis of pig muscle tissue (longissimus dorsi) stained with anti-ZBED6 (K and L) or anti-ZC3H11A (M and N). α-Bungarotoxin fused to a fluorescent dye (αBTX, green) was used to confirm identification of skeletal muscle tissue since this toxin binds cholinergic receptors in neuromuscular junctions. Scale bars indicate 480 μm in (A and D), 22 μm in (B, C, E, and F), and 14 μm in (G–N).

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The nucleolar localization of ZBED6 is of considerable interest in relation to the phenotype of IGF2-mutant pigs because the function of the nucleolus is associated with regulation of cell growth and proliferation [10].

Silencing of Zbed6 Confirms Specific Interaction with the IGF2 QTN Site

Zbed6 was silenced in C2C12 cells by using siRNA to obtain further insight into its functional significance. Quantitative PCR revealed a >75% decrease in detected Zbed6 mRNA, and immunocytochemistry further confirmed a highly efficient silencing at the protein level (Figure 5A). Zbed6-silenced and control C2C12 cells were used to repeat our previously described luciferase assay including a reporter construct containing either the wild-type or mutant sequence of the QTN region fused with the IGF2 P3 promoter (Figure 5B). An assay based on C2C12 control cells transfected with scrambled oligonucleotide replicated our previous results, since a construct containing the wild-type QTN region repressed luciferase expression in comparison with a construct containing P3 alone, whereas a construct including the mutant QTN region was associated with no or only minor repression (Figure 5B). In contrast, transfection experiments using three different silencing oligonucleotides directed against Zbed6 mRNA completely abolished repression with the wild-type q construct (Figure 5B).

Silencing of Zbed6 Is Associated with Elevated IGF2 Expression, Increased Cell Proliferation, and a Faster Wound Healing Process

ZBED6 function was further investigated by specific gene silencing in C2C12 cells that were induced to differentiate by changing from growth to differentiation medium. IGF2 mRNA expression was low in both control and Zbed6-silenced cells the first days after differentiation was induced (Figure 5D). However, at day...
factors and certain families of transcription factors were particu-
larly abundant among the putative ZBED6 targets (Figure 6F).

Human Orthologs of Mouse ZBED6 Targets Are
Significantly Overrepresented among Disease-Causing
Genes

The ChiP-seq data indicated that ZBED6 takes part in the
regulation of genes associated with basal functions in placental
mammals. Therefore, we decided to perform an

Ingenuity Pathways Analysis (Ingenuity Systems, http://www.
ingenuity.com) to test whether putative ZBED6 targets are
overrepresented among disease-associated genes in humans.
This was accomplished by first downloading a table with all established
mouse–human orthologs (Mouse Genome Informatics, http://
www.informatics.jax.org/orthology.shtml) and then this list of
human orthologs was used for the analysis. The results showed a
highly significant association between our putative ZBED6 target
genomes and a number of diseases (Figure 6G). The most significant
association was observed for developmental disorders, consistent
with the Gene Ontology analysis, followed by cancer, cardiovas-
cular disease, and neurological disease.

Discussion

Our quest for the nuclear factor binding the QTN site in the
porcine Igf2 gene has been driven by the vision that this factor
must be important, since disruption of the interaction with one of its
target sites alters body composition and promotes cardiac growth in
pigs. Our previous experiments revealed a highly specific interaction
between the factor and its target site in Igf2 [2], but it was not until
we used the ultrasensitive SILAC technology that we could take
advantage of this specificity and isolate ZBED6. The results
presented in this study have conclusively demonstrated that ZBED6 is the bona fide repressor binding the QTN site in pig Igf2. This
conclusion is based on (i) EMSA with recombinant ZBED6 protein,
(ii) supershift of EMSA complex using an anti-ZBED6 antibody, (iii)
abolishment of the repressor function in a luciferase assay after
siRNA silencing, and (iv) ChiP data. The biological significance of
ZBED6 was underscored in this study as siRNA silencing in C2C12
cells led to faster myotube formation and wound healing, and
increased cell proliferation.

The difference between less complex eukaryotes like Caenohab-
ditis elegans and more complex eukaryotes, such as human, is
related not to the number of protein-coding genes, but rather to
the complexity of the gene regulatory networks. A large proportion
of vertebrate genomes is composed of transposable elements,
and their integration in the genome has contributed to the evolution of
regulatory networks [11]. The majority of these transposable
elements are retrotransposons, but 5%–10% are derived from
DNA transposons. In the initial analysis of the first human genome
assembly, Lander et al. [12] identified 47 human genes derived
from transposable elements, as many as 43 of these are derived
from DNA transposons, and in fact, one of the genes listed in
Table 13 of the human genome paper corresponds to ZBED6.
However, ZBED6 has never been appropriately annotated in any
mammalian genome, despite the fact that it constitutes an
approximately 1,200 bp open reading frame and that part of the
ZBED6 protein is extremely well conserved among placental mammals. A bioinformatic
analysis of other vertebrate genomes did not reveal the
presence of a functional ZBED6 gene outside the placental
mammals. We found evidence for a nonfunctional ZBED6
sequence at the orthologous positions in the Platypus and opossum
genomes, but these genomes did not contain an extended open
reading frame for ZBED6 (unpublished data). This implies that the
integration of ZBED6 happened before the divergence of the
monotremes from the other mammals, but that the gene has been
inactivated or lost in monotremes and marsupials. Thus, ZBED6
must have evolved its essential function in the time span after the
split between marsupial and placental mammals, but before the
radiation of different orders of placental mammals. An interesting
topic for future research will be to reveal what advantage the
development of ZBED6 as a new regulatory protein has provided
to the placental mammals.
Figure 6. ChIP sequencing using mouse C2C12 cells. (A) ChIP-sequencing peak around the quantitative trait nucleotide (QTN) position in an intron of Igf2. (B) ZBED6 binding motif in Igf2 and consensus ZBED6 motif based on peaks within 5 kb of transcription start sites (TSS) and with at least 25 overlapping fragments but excluding the binding site at Igf2. (C and D) ChIP-sequencing peaks are preferentially located in the near vicinity of (C) TSS and (D) CpG islands. (E) Gene Ontology categories highly enriched among the genes bound by ZBED6. Ontology terms are shown on the y axis; p-values for the significance of enrichment are on the x axis. (F) Overrepresented transcription factor families bound by ZBED6, including homeobox proteins (HOX), paired-like (PAX), HOX factors (MEIS), hairy and enhancer of split protein (HES), krueppel C2H2 zinc finger (KLF), Forkhead box (FOX), cbp/p300-interacting transactivator (CITED), SNO and SKI oncogenes (SNO/SKI), atonal homolog (ATOH), basic helix-loop-helix (BHLH), LIM homeobox (LHX), POU domain containing classes 3 and 4 (POU), NK transcription factor related (NKX), early B-cell factor (EBF), nuclear receptor (NR), SRY box (SOX), T-box (TBX), and Iroquois homeobox protein (IRX). The number of transcription factor genes associated with ZBED6 binding sites is
**ZBED6** is an apparent example of a domesticated transposon that has lost its ability to transpose, because it occurs as a single copy gene at the same location in intron 1 of ZC3H11A in all placental mammals for which at least a partial genome sequence is available. ZBED6 has evolved an essential function in this group as implicated by the observation that the two DNA-binding BED domains (about 100 amino acids together) show near 100% amino acid identity across 26 placental mammals (Figure S2). The two BED domains in ZBED6 have apparently evolved by internal duplication because the two copies are more similar to each other than to any other mammalian BED sequence. The mechanism by which ZBED6 acts as a repressor remains to be determined.

Chromatin remodeling is an obvious possibility since other members of the ZBED family have this function. For instance, the *Drosophila* Dref protein, a BED domain protein, is found in complex with the NURF chromatin remodeling complex and its human ortholog ZBED1 interacts with MI2, a chromatin remodeling factor, and PC2, a Polyclomb group protein involved in heterochromatin formation [13]. The ability of ZBED6 to interact with chromatin and affect transcriptional regulation is most likely a function derived from the ancestral transposase. The nucleolar localization of ZBED6 (Figure 4C) suggests that it may mediate transcriptional silencing by moving the IGF2 locus and other targets to the nucleolus.

Our ChIP-sequencing experiment using mouse C2C12 myoblasts revealed more than 1,000 genes putatively regulated by ZBED6 in the mouse. We assume that a majority of these binding sites are true positives, because (i) we were able to generate a consensus binding motif (Figure 6B) with a perfect match with the established IGF2 binding site using both peaks with high and low enrichment levels, (ii) the majority of the binding sites occurred in the vicinity of TSS (Figure 6C), (iii) most of the binding sites occurred within or near CpG islands (Figure 6D), in line with the established binding site in IGF2, and (iv) the highly significant enrichment of certain Gene Ontology terms (Figure 6E). Thus, although we are certain that ZBED6 interacts with a majority of the genes listed in Table S1, transcriptome analysis will be required to assess the importance of ZBED6 for transcriptional regulation of these putative targets. In this context, it is worth emphasizing that disruption of the interaction between ZBED6 and the IGF2 QTN in pigs leads to a 3-fold up-regulation of IGF2 mRNA in skeletal muscle and altered body composition. Interestingly, our data indicated that the Zbed6 gene itself was bound by ZBED6 (Table S1), implying autoregulation of its expression.

About 1,200 of the ZBED6 binding sites in C2C12 cells occurred within 5 kb of the TSS of an annotated gene. The analysis of Gene Ontology terms associated with these genes revealed a highly significant enrichment for a number of important biological processes such as development, transcriptional regulation, and cell differentiation (Figure 6E). As many as 262 of the putative target genes encode transcription factors, 36 containing the homeobox domain, 26 members of the basic helix-loop-helix (bHLH) family, ten belonging to the FOX family, eight nuclear receptors, and seven members of the SOX family (Figure 6F). Many of these putative ZBED6 targets have a crucial role during development, and the results suggest that ZBED6 is an important regulator of development, cell proliferation, and growth. The binding of ZBED6 to its target sites in IGF2 leads to repression of IGF2 expression both in pig skeletal muscle [2] and in mouse C2C12 cells (this study). It may appear surprising that genes associated with neurogenesis were much more overrepresented in our peak list than genes associated with muscle development (Figure 6E), given the fact that we used mouse C2C12 myoblasts in this experiment. However, this pattern is expected if ZBED6 is primarily a repressor that silence genes not being part of the developmental program of a certain cell type. Another intriguing observation was the clear trend that ZBED6 preferentially binds downstream of the transcription start site which appears logical for a repressor (Figure 6C).

IGF2 is an imprinted gene, but our list of top hits did not indicate any overrepresentation of imprinted genes. In this respect, it is noteworthy that the QTN mutation in pigs does not result in loss of imprinting, but rather exclusively increases the transcription from the paternal IGF2 allele [2]. Thus, ZBED6 is unlikely to be a regulator of imprinting. However, one of the identified ZBED6 targets is the gene for growth factor receptor-bound protein 10 (Ghr10), also denoted Meg1 (maternally expressed gene 1), that is maternally expressed and a potent growth inhibitor [14]. GRB10 binds to the insulin receptor (INSR) and the IGF1 receptor (IGF1R), and inhibits the growth-promoting activities of insulin (INS), IGF1, and IGF2.

The list of genes associated with ZBED6 binding sites (Table S1) includes additional members, besides IGF2, of the IGF-signaling pathway, namely the genes for the IGF1 receptor (Igf1r), IGF2 binding protein 2 (Igf2bp2), IGF binding protein 3 (Igfbp3), and IGFBP-like protein 1 (Igfpl1), suggesting that ZBED6 is an important regulator of IGF signaling. Furthermore, Ghr10, as mentioned above, also takes part in the regulation of IGF signaling [14].

**Genome Wide Association (GWA) studies** have revealed a number of loci in the human genome associated with multifactorial disorders [Office of Population Genomics; http://www.genome.gov/26525384]. An examination of this database showed that the region harboring ZBED6 is not one of the associated regions in any of the studies published so far. This means that the current GWA screens for different multifactorial disorders have not revealed any common ZBED6 variants associated with disease. This does not exclude the possibility of rare sequence polymorphism in ZBED6 affecting disease susceptibility in certain families. However, the ChIP-sequencing data indicated that ZBED6 has a fundamental role in regulating several biological processes. Mutations altering ZBED6 function or expression may therefore have severe pleiotropic effects through the many downstream targets. This notion is consistent with the near 100% conservation of the BED domains among placental mammals.

Our current model for ZBED6 function is summarized in Figure 7. Our data on the IGF2 locus indicate that ZBED6 acts primarily as a repressor, likely with a modulating effect, although it is fully possible that it acts as a transcriptional activator under some circumstances.

First, germline or somatic mutations at target sites may lead to transcriptional up-regulation as demonstrated for the IGF2 locus in pigs [2]. Our findings that the mammalian genome contains thousands of putative ZBED6 targets and that these are enriched among genes associated with disease suggest that sequence polymorphism at ZBED6 target sites may contribute significantly to variation in disease susceptibility in humans. Furthermore, the ZBED6 binding motif contains a CpG dinucleotide so we expect...
to find genetic polymorphisms as CpG sites are associated with a high rate of C→T and G→A transitions [15], as exemplified by the pig IGF2 QTN. Gain or loss of ZBED6 binding sites may also have contributed to phenotypic evolution in placental mammals.

Second, our data suggest that ZBED6 targets can be released from repression by epigenetic activation. This is implied by the finding that EMSA using an autosomal CpG site with a methylated CpG site was not bound by ZBED6 [2]. Interestingly, the pig QTN had no effect on IGF2 transcription in liver, and the QTN region was shown to be methylated in this tissue, whereas it was undermethylated in skeletal muscle where the QTN had a drastic effect on IGF2 expression [2]. Thus, epigenetic regulation of the access of ZBED6 to its target sites may play an important role during development and cell differentiation.

Third, ZBED6 targets can be released from repression by down-regulation of ZBED6 expression, as demonstrated by siRNA experiments in the present study. Finally, loss-of-function mutations in ZBED6 are expected to up-regulate many target genes. Our finding that Zbed6 silencing in C2C12 cells leads to faster cell proliferation and wound healing combined with the identification of a large number of cancer-associated downstream targets by ChIP sequencing implies that further studies of ZBED6 function is of considerable interest for tumor biology.

Data reported here suggest that ZBED6 has an essential role in a number of crucial gene regulatory networks. Thus, the discovery of ZBED6 opens up many avenues for research that may have profound implications for human medicine.

Materials and Methods

Proteomics

Two sets of murine C2C12 myoblast cells (ATCC CRL-1772) were used. Cultures were grown according to standard cell-culture procedures in SILAC-light and SILAC-heavy labeled Dulbecco’s Modified Eagle Medium (DMEM, ThermoFisher) containing 10% dialyzed fetal bovine serum (FBS) and 150 mM NaCl, 0.25 mM EDTA, 0.5 mM DTT; 0.1% Tween-20, 0.5 mg/ml BSA, 200 μg/ml poly-dl-dC) in a total volume of 700 μl. Mixtures were incubated for 45 min at room temperature (RT) on a rotator. Ten microliters of streptavidin-coated magnetic beads (Dynal) was added to each tube, and the mixture was further incubated for 30 min at RT on a rotator. Beads were spun for 5 s at 1,000g and then captured using a magnetic pull-down system. Beads were washed 3×700 μl in binding buffer without poly-dl-dC and then 4×700 μl in binding buffer without poly-dl-dC or BSA. The supernatant was discarded, and proteins were eluted by boiling in Laemmli buffer (+10 mM DTT). Protein eluates were mixed 1:1 by volume.

Proteins were separated on an SDS-PAGE gel (4%-12% NuPage), stained with Coomassie Blue, and the entire lane was cut into ~20 bands. Each band was reduced, alkylated, digested with trypsin according to standard proteomics practices [17], and the resulting peptides were analyzed by LCMS on an Orbitrap (ThermoFisher) mass spectrometer as described [18]. Database searching was performed by Mascot against the REFSEQ database of mouse proteins as of June 2006. SILAC quantification was performed using msQuant [19].

Expression of Recombinant Protein and Antibody Production

RT-PCR, using poly-A–enriched RNA extracted from C2C12 cells, was used to obtain nucleotides 1–2943 and 139–2943 of mouse Zbed6 transcripts (named Zbed6a and Zbed6b, respectively; EMBL Bank accession number FM882123); the two constructs begin at the two alternative start codons. These constructs, containing a Kozak sequence for efficient initiation of translation, were cloned into pcDNA3 (Invitrogen) and verified by DNA sequencing.

PCR was used to subclone a fragment encoding the two BED domains, amino acid residues 90–384 of Zbed6a, into pGEX-5X-3 (GE Healthcare). GST and GST-BED1/2 fusion protein was purified from BL21 (DE3)pLysS bacteria using glutathione Sepharose 4B or GSTrap FF columns (GE Healthcare), according to the manufacturer’s instructions.

Polyclonal antibody production was performed by Agrisera AB. Shortly, GST-BED1/2 was used to immunize one rabbit. Polyclonal anti-ZBED6 antibodies were affinity purified by first passing serum over a HiTrap NHS-activated HP column (GE Healthcare) coupled with GST, whereafter the flow-through was applied to a column coupled with GST-BED1/2. Anti-ZBED6 antibodies were eluted with 0.2 M glycine (pH 2.5) and dialyzed against 20 mM HEPES (pH 7.4) and 150 mM NaCl.

EMSA

Nuclear extracts from C2C12 cells were prepared using the NucBuster Protein Extraction kit (Novagen). EMSAs were performed as previously described with minor modifications [2]. The following oligonucleotides were annealed in 1× NEB2 buffer (NEB) q/Q-fwd AGATCCCTTCGGCCTAGGCGTAACACGGCGGAGGCGA-3’

IGF2-Q: 5’-Biotin-GATCCTTCGGCCTAGGCGTAACACGGCGGAGGCGA-3’

The complementary (+)-strands were also synthesized, and the pairs were annealed prior to use. One picomole of ds-q oligonucleotide was mixed with 1.4 mg of heavy nuclear extract and 1 pmol of ds-Q oligonucleotide was mixed with 1.4 mg of light nuclear extract in binding buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.25 mM EDTA, 0.5 mM DTT; 0.1% Tween-20, 0.5 mg/ml BSA, 200 μg/ml poly-dl-dC) in a total volume of 700 μl. Mixtures were incubated for 45 min at room temperature (RT) on a rotator. Ten microliters of streptavidin-coated magnetic beads (Dynal) was added to each tube, and the mixture was further incubated for 30 min at RT on a rotator. Beads were spun for 5 s at 1,000g and then captured using a magnetic pull-down system. Beads were washed 3×700 μl in binding buffer without poly-dl-dC and then 4×700 μl in binding buffer without poly-dl-dC or BSA. The supernatant was discarded, and proteins were eluted by boiling in Laemmli buffer (+10 mM DTT). Protein eluates were mixed 1:1 by volume.

Proteins were separated on an SDS-PAGE gel (4%-12% NuPage), stained with Coomassie Blue, and the entire lane was cut into ~20 bands. Each band was reduced, alkylated, digested with trypsin according to standard proteomics practices [17], and the resulting peptides were analyzed by LCMS on an Orbitrap (ThermoFisher) mass spectrometer as described [18]. Database searching was performed by Mascot against the REFSEQ database of mouse proteins as of June 2006. SILAC quantification was performed using msQuant [19].

Figure 7. Proposed model for ZBED6 action. ZBED6 repression may be released by (i) germline or somatic mutations at target sites, (ii) methylation (Me) of the CpG site in the binding motif, (iii) down-regulation of ZBED6 expression, or (iv) loss-of-function mutations in ZBED6.

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The C2C12 mouse myoblast cell line (ATCC-CLR-1772) was cultured at 37°C in a humidified atmosphere of 5% CO₂ using DMEM (ATCC-30-2002) supplemented with 10% FBS (Invitrogen) and 1× Antibiotic-Antimycotic solution (Invitrogen). The cultures were split every 2 to 3 d. C2C12 cells were differentiated by growing the cells in differentiation media, DMEM with 2% horse serum.

Cell Culture
The C2C12 mouse myoblast cell line (ATCC-CLR-1772) was cultured at 37°C in a humidified atmosphere of 5% CO₂ using DMEM (ATCC-30-2002) supplemented with 10% FBS (Invitrogen) and 1× Antibiotic-Antimycotic solution (Invitrogen). The cultures were split every 2 to 3 d. C2C12 cells were differentiated by growing the cells in differentiation media, DMEM with 2% horse serum.

Reactions were supplemented with 4 pmol (100-fold molar excess) unlabelled ds-oligonucleotide. After the addition of 40 fmol end-labeled 3²P-dCTP ds-oligonucleotide, reactions were incubated at RT for 30 min. In EMSAs including supershift reaction, incubation for 20 min at RT preceded the addition of 2 μl of purified polyclonal anti-ZBED6 antibody (0.13 μg/μl) and incubation then continued for an additional 20 min at RT. Complexes were separated on a 1.5-mm 5% native 29:1 polyacrylamide gel in 0.5× TBE at 70V for 3–4 h.

Northern Blot
A mouse multiple-tissue Northern blot panel 4 (MN-MT-1) and a mouse developmental tissue skeletal muscle panel (MN-102-D from Zyagen) was used. Partial Zbed6 and Zc3h11a coding sequences were cloned into a vector and probe template amplified by PCR including either SP6 or T7 sequence from the vector. A purified probe template (200 ng) was used for 3²P-labeled RNA probe synthesis using the MaxiScript Kit (Ambion). Mouse β-actin (Ambion) was amplified by PCR from C2C12 CDNA, sequenced, and used as template for 3²P-labeled DNA probe synthesis using the Amersham Megaprime DNA labeling system (GE Healthcare). Hybridizations were done at 68°C (RNA probes) or 42°C (DNA probe) using the ULTRAhyb buffer (Ambion) followed by washes in 2× SSC+0.1% SDS and 0.1× SSC+0.1% SDS at 60°C (RNA probes) or 60°C (DNA probe). Autoradiographs were exposed for a few hours to several days.

Immunohistochemistry
Mouse and pig tissue were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 2–4 h on ice, cryoprotected in 30% sucrose, followed by embedding in Tissue-Tek O.C.T. compound (A/S Chemi-Teknik) and cryostat sectioning. Fluorescent immunohistochemistry was performed on cryosections from mouse hind limb muscle postnatal day 3 (p3), mouse brain (p22), and pig muscle (longissimus dorsi) (6 mo). Rabbit antibodies against ZC3H11A (A301-525A, Bethyl Laboratories) and ZBED6 were diluted to 0.2 μg/ml in PBS containing 1× blocking reagent (Roche), 0.3% Triton X-100 (Sigma), and incubated at 4°C overnight. In the case of the anti-ZC3H11A antibody, a commercially available blocking peptide (Bethyl Laboratories) was used at 20 μg/ml and incubated with the anti-ZC3H11A antibody for 2 h at RT prior to application on tissue. The following day, slides were washed in PBS and incubated with a secondary anti-rabbit IgG antibody conjugated to Alexa 594 (Invitrogen) and DAPI (Sigma) in PBS for 1 h at RT. Slides were washed in PBS and mounted with 2.5% DABCO (Sigma) in glycerol containing 0.1M Tris (pH 8.6). For pig muscle tissue, sections were also incubated with Alexa 488-conjugated α-bungarotoxin (1:1,000, Invitrogen). Staining was analyzed with a confocal microscope (Olympus BX61W1) or a confocal microscope (Zeiss LSM 510 META). Images were adjusted for contrast and brightness in Adobe Photoshop (Adobe).

Gene Silencing Using siRNA
A total of 5×10⁴ C2C12 cells or 30%–50% confluent cells were transfected with 50 pmol of negative control siRNAs (Ambion) or ZBED6 siRNAs (Ambion) with 6 μl of Lipofectamine 2000 Reagent (Invitrogen) in 1.5 ml of Opti-MEM I (Invitrogen) per well in 6-well plates. The following pooled Silencer Select siRNA sequences (Ambion) were used to silence Zbed6 expression in C2C12 cells: duplex 1 sense, 5’-CUUCACUCUCAAGCCGA-CAT-3’; duplex 2 sense, 5’-UGUUGGAUCAUGCAUCAAAT-3’; duplex 3 sense, 5’-GGGCGUGGUGGCAACAAAGA-3’. The dincleotide tt (indicated in lower case letters) was added to all oligonucleotides to improve the stability of siRNA after transfection. After 24 h, medium was changed to fresh DMEM with 10% FBS. Biological triplicates were used for each siRNA treatment.

Luciferase Assay
Silencing was performed 2 d prior to transient transfection with luciferase reporters. Previously described [2] constructs containing the porcine IGF2 QTN region, the porcine IGF2 P3 promoter and the firefly luciferase reporter gene (P3, qP3 and P3+qP3) were used. Zbed6 silenced C2C12 cells or negative control siRNA treated cells grown in 12-well plates were transfected with a total of 2 μg of DNA and 6 μl of Lipofectamine 2000 Reagent (Invitrogen). One microgram of firefly luciferase construct and 20 ng of Renilla luciferase vector as control (ph-RG, Promega) and empty pcDNA3 vector (Invitrogen) up to 2 μg of DNA were used. Transfections were performed in opti-MEM (Gibco), and medium changed to growth medium (DMEM supplemented with 10% FBS) after 4 h. Cells were harvested 24 h posttransfection, and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and an Infinite M200 luminometer (Tecan).

Cell Proliferation Assay
At 48 h post-siRNA transfection, the cells were incubated 3–4 h with medium containing 10% Alamar blue (Invitrogen). The
Scratch Wound Assay

At 72 h post-siRNA transfection, cells reached almost confluence, and medium was replaced with fresh DMEM supplemented with 0.1% FBS. A surface wound was created by scraping a pipette tip across the confluent cell monolayer. Twenty-four hours after scraping, the number of cells in the scratch was counted and cells treated with negative control siRNAs and Zbed6 siRNAs were compared. Statistical analysis was performed using a Student t-test.

RNA Isolation and Real-Time (RT) PCR Analysis

RNA was isolated from tissue samples from six C57BL/6 mice (three males and three females) either by RNaseasy mini kit (QIAGEN) or acidic phenol extraction as described [20]. RNA from C2C12 cell samples was isolated using the RNaseasy Mini Kit (QIAGEN), then all samples were subjected to reverse transcription using cDNA high capacity kit (Applied Biosystems). mRNA transcripts were measured by quantitative PCR analysis using TaqMan Gene Expression master mix (Applied Biosystems) on a 7900HT Fast RT-PCR System (Applied Biosystems); probes and primers used are given in Table S2. Data were analyzed with a threshold set in an individual protein are indicated by a hyphen followed by a +/. Statistical analysis was performed using a Student t-test.

Immunocytochemical Staining of Cytospins of C2C12 Cells

Cells were trypsinized by 0.25% trypsin EDTA (Invitrogen). After blocking with 10% FBS and washing twice in PBS (Invitrogen), 2 to 3 million cells were resuspended in 1 ml of PBS with 20% FBS and centrifuged as cytospins for 3 min at 800 rpm. The cytospins were dried at RT overnight and then fixed with acetone for 10 min and hybridized with 250 μl of anti-ZBED6 antibody (0.2 μg/ml) for 20 min, followed by staining protocol described by Human Protein Atlas (http://www.proteinatlas.org).

Chromatin Immunoprecipitation (ChiP) Analysis and Sequencing

Normal ChiP was performed as previously described [21], whereas ChiP sequencing was performed as follows. Chromatin was immunoprecipitated from approximately 10^7 subconfluent C2C12 cells. Protein-DNA cross-links were made in RT for 10 min with 0.37% formaldehyde, and cells were lysed in RIPA buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Sonication was done to generate DNA fragments mainly in the 100–300-bp range using a Covaris instrument to get optimal fragment sizes. Library construction was done according to the manufacturer’s protocol (AB SOLiD v3.0 fragment library) with eight rounds of amplification. Sequencing was done on a quarter of a slide and gave 50 million 50-bp reads. Alignment was done in two steps, first the AB pipeline (mapreads) was used to align full-length reads with up to four mismatches, and subsequently, the remaining reads was truncated to 35 bases and realigned with four mismatches using ZOOMI [22]. This gave 18±6.5 million uniquely placed alignments at 23 million unique positions. Each read was extended to 200 bases and overlap profiles were used to identify regions of enrichment. FindPeaks 3.1.92 [23] was used to estimate the false discovery rate (FDR), giving a probability of <0.001 at 15 overlapping fragments. Peaks falling within 100 kb of centromeric gaps or overlapping with Satellite and rRNA repeats were removed to reduce nonrandom false-positive peak calls. This gave 2,499 peaks, which were then associated with the nearest TSS and CpG island (Table S1). We noted that the additional sonication done before sequencing gave larger regions with enrichment compared to what is normally seen in size-selected ChiP-seq dataset, therefore, we additionally filtered the peaks to contain only the highest scoring peak within 5 kb before performing de novo motif finding using BioProspector [24]. We searched for motifs of length 8 bp both in the full list of peaks and in subsets of peaks divided by enrichment and location, and used mouse sequences 1 kb upstream of transcription start sites (TSS) as background. We used the DAVID software for Gene Ontology analysis [25,26].

Supporting Information

Figure S1 Alignment of BED domains from the human ZBED family of proteins. Multiple copies of BED domains in an individual protein are indicated by a hyphen followed by a numerical. Identical amino acid residues (>50%) in a specific position are boxed. Amino acid residues belonging to the same group in a specific position (>50%; hydrophobic, hydrophilic, acid, or basic) are shaded in gray. The four zinc-chelating residues are shown in red. Accessions numbers are NM_001726819 (ZBED1), NM_024508 (ZBED2), NM_032367 (ZBED3), NM_014338 (ZBED4), BC047754 (ZBED5), and XM_001726819 (ZBED6).

Found at: doi:10.1371/journal.pbio.1000256.s001 (0.27 MB TIF)

Supporting Information

Figure S2 Alignment of ZBED6 zinc finger domains from 26 mammalian species. (A) BED domain 1 and (B) BED domain 2 (amino acid residue numbering as in mouse ZBED6 (FM821213). Accession numbers: pig (Sus scrofa; CU655999), human (Homo sapiens; XM_001726819), chimpanzee (Pan troglodytes; AACZ02012362), orangutan (Pongo abelii; ABGA01056271), rhesus monkey (Macaca mulatta; AANU0124087), dog (Canis familiaris; AAEX02008140), cat (Felis catus; AANG01029813), rat (Rattus norvegicus; AAHX01075149), cattle (Bos taurus; AAFC03020996), northern tree shrew (Tupaia belangeri; AAPY01699446), thirteen-lined ground squirrel (Spermophilus tridecemlineatus; ABAQ01297623), common shrew (Sorex araneus; AALT01236730), philippine tarsier (Tarsius syrichta; ABRT010393906), Orkd’s kangaroo rat (Dipodomys ordii; ABRQ01075989), gray mouse lemur (Microcebus murinus; ABDC01073199), large flying fox (Pteropus vampyrus; ABRP01175854), alpaca (Lama pacos; ABRRO1306061), horse (Equus caballus; AAWR02036736), nine-banded armadillo (Dasypus novemcinctus; AAGV02036736), little brown bat (Myotis lucifugus; AAPE01620944), bottle-nosed dolphin (Tursiops truncatus; ABRN01226209), rock hyrax (Procavia capensis; ABQQ01152452),

URL

Information on genomes is available at http://www.genome.ucsc.edu.
guinea pig (Cavia porcellus; AAKN02017923), European hedgehog (Erinaceus europaeus; AANN01531372), and American pika (Ochotona princeps; AAYZ01471625). Initial alignment of the corresponding DNA sequences revealed single nucleotide gaps/insertions in some sequences originating from the Whole-Genome-Shotgun Sequence database at http://www.ncbi.nlm.nih.gov/ (posted date: October 24, 2008, 2:23 AM). These positions have been treated as sequence artifacts and have therefore been corrected to give a continuous reading frame before translating to protein sequence. A single nucleotide insertion has been removed from American pika and thirteen-lined ground squirrel. A missing base (N) has been introduced at codon 173 in alpaca and at codon 306 in northern tree shrew. The Gln-residue (Q) at position 131 in rock hyrax is possibly caused by two sequence errors (an insertion of a C followed by a deletion of an A), but remains unedited. Dashes indicate sequence identity to the master sequence [pig]. Blanks indicate missing data.

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Figure S3 Differentiation of scrambled and Zbed6-knockdown myoblasts into myotubes on day 6. C2C12 myoblasts treated with scrambled and Zbed6 siRNA were submitted to a differentiation medium with 0.1% horse serum in myoblasts treated with scrambled and Zbed6 siRNA were submitted to a differentiation medium with 0.1% horse serum in

All peaks with at least 15 overlapping reads are listed and sorted according to the number of reads. Dist_CpG, distance to closest CpG island in base pairs; Dist_TSS, distance to transcription start site in base pairs; GeneID, gene name; overlaps, number of overlapping extended reads.

Found at: doi:10.1371/journal.pbio.1000256.s004 (0.17 MB PDF)

Table S2 Primers and probes for real-time PCR analysis of Zbed6, Igf2, and Zc3h11a.

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: JDJ JRZ KK CW KLT GA GH LA. Performed the experiments: EM LJ JDJ JRZ KK CW KLT GA GH LA. Analyzed the data: EM LJ JDJ JRZ KK CW KLT GA GH LA. Contributed reagents/materials/analysis tools: SS FP ESL SC. Wrote the paper: All authors contributed to the writing of this paper.