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Relationships between p63 Binding, DNA Sequence, Transcription Activity, and Biological Function in Human Cells

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Summary

Using tiled microarrays covering the entire human genome, we identify ~5800 target sites for p63, a p53 homolog essential for stratified epithelial development. p63 targets are enriched for genes involved in cell adhesion, proliferation, death, and signaling pathways. The quality of the derived DNA sequence motif for p63 targets correlates with binding strength binding in vivo, but only a small minority of motifs in the genome is bound by p63. Conversely, many p63 targets have motif scores expected for random genomic regions. Thus, p63 binding in vivo is highly selective and often requires additional factors beyond the simple protein-DNA interaction. There is a significant, but complex, relationship between p63 target sites and p63-responsive genes, with ΔNp63 isoforms being linked to transcriptional activation. Many p63 binding regions are evolutionarily conserved and/or associated with sequence motifs for other transcription factors, suggesting that a substantial portion of p63 sites is biologically relevant.

Introduction

In the classical view of gene regulation and functional genomics, DNA sequence motifs dictate the specific binding of transcriptional regulatory proteins, either activators or repressors, and these bound regulators activate or repress the expression of the corresponding structural gene. This paradigm has been the basis for interpreting numerous experiments over the past three decades, but the relationships between DNA sequence motif, protein binding in vivo, and transcriptional activity have rarely been examined in an unbiased manner. The combination of chromatin immunoprecipitation (ChIP) and high-density, tiled microarrays covering entire genomes (or mechanistically unbiased portions such as whole chromosomes) makes it possible to map transcription-factor binding sites in an unbiased fashion.

Genome-wide identification of in vivo targets of transcription factors in mammalian cells has been technically difficult due to the very large size of the genome. To circumvent this issue, several studies have employed microarrays that contain selected genomic regions such as CpG islands (Weinmann et al., 2002), promoter regions of annotated genes (Odom et al., 2004; Zhang et al., 2005), or 10 kb regions surrounding the transcription start site of annotated genes (Boyer et al., 2005). Although such studies have been extremely valuable, they do not represent an unbiased approach. Alternatively, binding sites for several transcription factors (Myc, Sp1, p53, NF-κB, CREB, and estrogen receptor) have been identified in a relatively unbiased manner by using tiled arrays representing all nonrepetitive sequences on human chromosomes 21 and/or 22 (Martone et al., 2003; Cawley et al., 2004; Euskirchen et al., 2004; Carroll et al., 2005). Finally, by combining ChIP with SAGE-based approaches, CREB and p53 target sites were identified on a genome-wide basis (Impey et al., 2004; Wei et al., 2006). However, due to the limitations of sequencing enough tags, these SAGE-based analyses were not comprehensive and favored the identification of high-affinity sites.

Most unexpectedly, all studies using unbiased genomic approaches have shown that transcription factors bind specifically to a surprisingly large number of genomic regions (extrapolated to 2,000–25,000 depending on the protein). The majority of these in vivo targets do not map near the 5′ ends of protein-coding genes and, hence, would not be identified by more biased approaches. The human genome expresses a remarkably large number of noncoding RNAs (Kapranov et al., 2002; Bertone et al., 2004; Carninci et al., 2005; Cheng et al., 2005), and many in vivo targets of these transcription factors are associated with such noncoding RNAs. These observations suggest that many in vivo targets of transcription factors are involved in the expression of noncoding RNAs and conversely that expression of noncoding RNAs is regulated by similar factors and mechanisms as utilized for classical protein-coding genes (Cawley et al., 2004).

Previous studies on the relationship between DNA sequence motif, protein binding in vivo, and transcriptional activity in human cells have some significant limitations. First, with the exception of experiments on the general transcription factor TAF1, which were used to identify Pol II promoters (Kim et al., 2005), comprehensive identification of in vivo target sites has never been described at the level of the entire human genome. Second, few studies have attempted to define DNA sequence motifs ab initio from in vivo binding sites, and the relationship between sequence motif and in vivo binding has not been investigated. Third, and most important, the finding of numerous protein binding sites in unexpected places has prompted the question of whether such sites are biologically functional. In this regard, in vivo targets have rarely been examined for their evolutionary conservation or for their effects on gene expression in cells depleted for the relevant factor.

Here, we address these relationships with genome-wide, unbiased identification and analysis of target sites for p63 (also TP73L/p51/KET), a homolog of the p53

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tumor suppressor. p63 shares strong structural and functional similarities to p53 and a third homolog, p73 (Yang et al., 2002; Harms et al., 2004), but all three members of the p53 family possess distinct physiological roles. In particular, p63 has been linked to the maintenance of epithelial stem cells and morphogenesis of skin, breast, prostate, bladder, and related tissues (Mills et al., 1999; Yang et al., 1999). Additional studies implicate p63 in canonical p53 pathways governing growth arrest and apoptosis (Yang et al., 2002; Harris and Levine, 2005), but the mechanism and molecular details of these interactions remain unclear.

Efforts to understand p63 biology are complicated by the existence of multiple, naturally occurring isoforms encoded by the p63 gene. *trans*-activating (TA) isoforms possess an acidic, N-terminal transcriptional activation domain similar to that of p53. ΔNp63 isoforms lack this activation domain and are presumed to be transcriptional repressors with possible dominant-negative effects on transactivation by the p53 family (Yang et al., 1998). ΔNp63 variants are predominant in stratified epithelial tissues and cell lines (Yang et al., 1998; Nylander et al., 2002; Westfall et al., 2003), suggesting that these isoforms are responsible for p63's role in epithelial morphogenesis and related disease syndromes (van Bokhoven and McKeon, 2002).

In the present work, we combine a global interrogation of p63 binding sites with de novo motif identification and analysis of site behavior, sequence conservation, association with other transcription factor motifs, and transcriptional profiling of p63-depleted cells. Together, these strategies render a comprehensive, functional genomics view of p63 activity and its physiological targets, thus providing a platform for understanding its role in cancer and developmental processes.

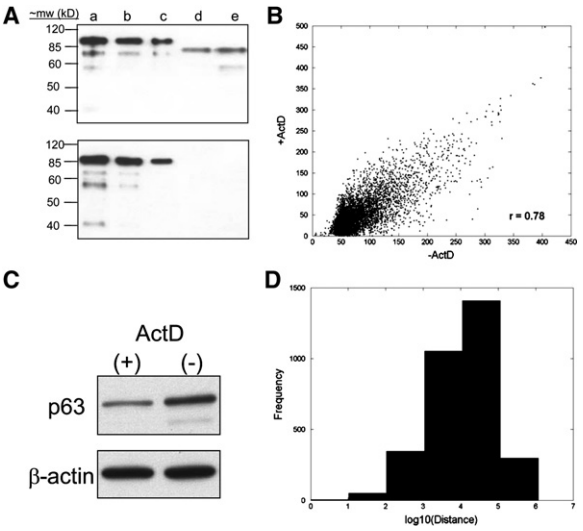

Figure 1. Genome-Wide Identification of p63 Binding Sites in ME180 Cells

(A) Immunoblotting with 4A4 anti-p63 (top) and 6E6 anti-TAp63 (bottom) antibodies shows that ΔNp63 isoforms are predominant in epithelial cell lines. Lane a, recombinant TAp63α 2 ng; lane b, recombinant TAp63α 1ng; lane c, recombinant TAp63α 0.5 ng; lane d, human foreskin keratinocyte whole-cell lysate; and lane e, ME180 whole-cell lysate. Abbreviations: mw, molecular weight; kD, kilodaltons.

(B) Binding enrichment scores of (−) ActD defined sites in (−) Act D and (+) Act D samples along with the Pearson correlation coefficient (r = 0.78).

(C) p63 protein expression in the presence or absence of ActD.

(D) Distance from the midpoint of p63 binding regions located in the vicinity of genes to the closest transcription start site (TSS).

Results

Genome-Wide Identification of p63 Binding Sites

p63 expression is predominant in stratified epithelial cells, with significantly lower levels detected in other cell types (Yang et al., 1998). To create a high-resolution, global map of in vivo interactions between p63 and DNA, we analyzed the ME180 cervical carcinoma cell line that expresses abundant p63. ME180 cells were grown in the absence (−) or presence (+) of the DNA-intercalating agent actinomycin D (Act D), based on reports that genotoxic damage can influence p63 expression and activity (Liefer et al., 2000). Crosslinked chromatin from ME180 cells was immunoprecipitated with the 4A4 anti-p63 antibody that recognizes all p63 isoforms (Yang et al., 1998, 1999). ME180 cells do not contain detectable levels of the TA isoform (Figure 1A), consistent with ΔNp63 being the primary isoform expressed in epithelial cells (Yang et al., 1998; Nylander et al., 2002; Westfall et al., 2003); hence, the immunoprecipitated protein-DNA complexes are almost exclusively those involving ΔNp63 isoforms. DNA from the resulting samples was amplified and hybridized to a set of 14 high-density oligonucleotide arrays interrogating the nonrepetitive sequences of the entire human genome at 35 bp resolution.

Using genomic positions with a significance threshold of $p \leq 10^{-5}$, 5807 and 3688 binding sites were identified for (−) and (+) Act D samples, respectively (Table S1 in the Supplemental Data available with this article online). The Pearson correlation between binding enrichment scores of the two samples is 0.78 (Figure 1B and Figure S1), indicating a very high degree of similarity. We tested a few "best" candidates for potential differential binding (i.e., sites identified in one sample that have very low scores in the other sample) and found that all of them are due to false positives in one sample. In general, p63 binding for the targets tested is slightly lower in the (+) Act D samples (Figure 1B and Figure S1), and Act D treatment causes an ~2-fold decrease in p63 protein levels (Figure 1C). Thus, Act D does not affect the specificity of p63 binding but rather causes a slight decrease in p63 levels and genome occupancy that results in an apparent decrease in the number of sites that pass a defined cutoff.

Thirty-seven out of forty-one randomly selected targets in the (−) ActD sample, representing the range of p values, were validated by quantitative PCR analysis (Table S2), resulting in a false discovery rate (FDR) for the 5807 targets of ~9% (Table S3). At more stringent cutoffs, we identified 4730 sites at an FDR of 4% and 3397 sites at an FDR of 1%. Although these more stringent cutoffs improve the accuracy of defining p63 targets, they significantly increase the number of false negatives (i.e., true targets that miss the cutoff); hence, choosing a cutoff for further analysis is largely arbitrary and involves a trade off between false positives and

false negatives. In fact, we estimate that there are ~500–1000 additional p63 sites that did not pass our original cutoff. For most of the analyses, we used the list of 5807 targets, although more stringent lists were employed in certain cases.

3159 of the 5807 sites can be mapped between the region encompassing 5 kb upstream to 1 kb downstream of well-characterized genes, a significant enrichment beyond random expectation (Table S4). Furthermore, p63 preferentially associates with promoter regions, first introns, and CpG islands. However, 56% of the gene-associated p63 binding sites are located more than 10 kb from mRNA start sites (Figure 1D). This observation is consistent with previous analyses of Myc, Sp1, p53, estrogen receptor, CREB, and NF-κB (Martone et al., 2003; Cawley et al., 2004; Euskirchen et al., 2004; Carroll et al., 2005). Approximately 80% of the remaining p63 sites are located within 5 kb upstream to 1 kb downstream of the various transcript annotations compiled from the UCSC Genome Browser database. We suspect that p63 sites will increasingly be linked to mRNA transcripts as the mammalian transcriptome becomes better characterized.

The Quality of the p63 Motif Correlates with the Strength of p63 Binding, but the Vast Majority of Motifs Are Unbound by p63 In Vivo

Applying de novo motif discovery algorithms MEME (Bailey and Elkan, 1994) and AlignACE (Hughes et al., 2000), we identified a dyad-symmetric motif composed of two direct repeats (Figure 2A and data not shown) that is highly specific to the p63-bound sequences and shows a 4.1-fold enrichment of occurrences over expected frequency from genomic background. As expected, each half-site of the motif bears a resemblance to the p53 consensus sequence.

We directly investigated the relationship between the quality of the p63 DNA sequence motif and the extent of p63 binding in vivo. A motif score was assigned to each binding region according to the degree of similarity to the position weight matrix, and a clear relationship between motif and binding enrichment scores is observed (Figure 2B). However, only 8% of the very best p63 motifs and only 1%–3% of typical p63 motifs (i.e., those present in the majority of p63-bound regions) are bound in vivo (Figure 2C). Conversely, roughly 22% of the actual p63 target regions have low motif scores (<10) that are comparable to those of randomly selected genomic regions (Figure 2D), and eight out of ten such low motif targets were confirmed by quantitative PCR analysis (Table S2). Thus, p63 can selectively bind to ~1000 low motif targets, even though there are millions of such sequences in the human genome. Taken together, these results indicate that the quality of the p63 motif contributes to, but is a poor predictor of, p63 binding in vivo. Nevertheless, p63 binding in vivo is highly selective in that the 5807 target sites constitute a very small proportion of the human genome.

p63 Binds All Members of the p53 Gene Family

p63 associates with the promoter of *p53*, introns 3 and 4 of *p73*, and introns 1, 3, and 4 of the *p63* gene itself (Figure S2). As both *p63* and *p73* contain an alternative transcription start site in intron 3, giving rise to the ΔN

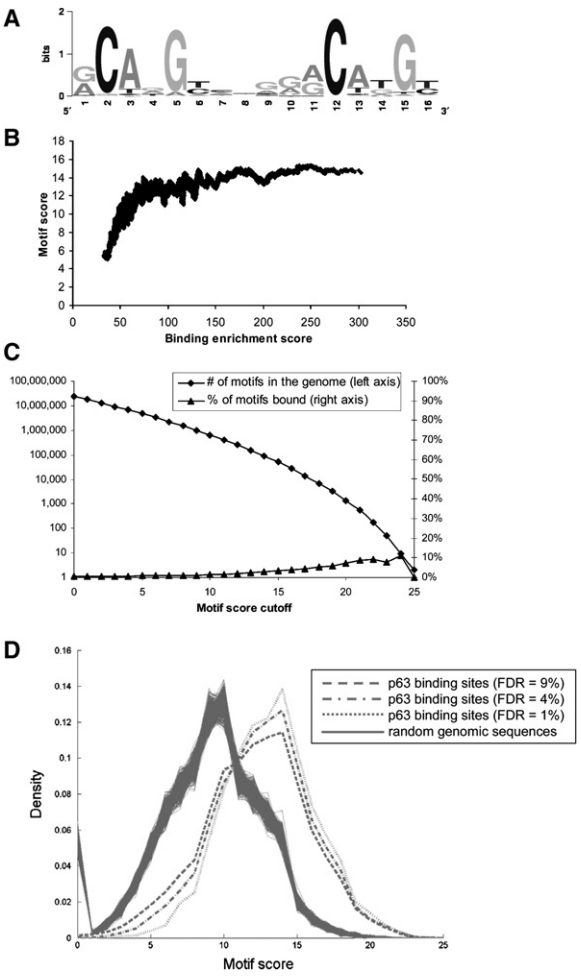

Figure 2. p63 Binding and Sequence Determinants

(A) Motif identified de novo from the p63-bound sequences.

(B) Relationship between motif and binding enrichment scores (moving average, window size = 50).

(C) The number of motif instances in the genome (primary y axis) at various score cutoffs and proportions bound in ME180 cells (secondary y axis).

(D) Motif score distribution for p63-bound sequences at various FDR cutoffs and randomly selected comparable genomic sequences (1000 groups).

isoforms (Yang et al., 1998, 2000), the positions of p63 binding sites at genes of *p53* family are located in the promoter and/or first intron of transcripts encoding the full-length or ΔN isoforms. Thus, p63 may regulate its own expression as well as crossregulate expression of both *p53* and *p73*.

Relationship between p63 and p53 Target Sites

p63 and p53 have similar DNA binding domains and can regulate common genes (Harms et al., 2004). Based on a genomic analysis of p53 binding in 5-fluorouracil-stimulated HCT116 cells using ChIP-PET technology, 327 high-confidence p53 binding sites were identified (Wei et al., 2006). Sixty-two of these overlap with the p63-bound sequences described here, far exceeding random expectation ($p \approx 2.4 \times 10^{-70}$; Figure S3A). Importantly, the motif scores of the common p53/p63 sites are significantly better than those of typical p63

sites ($p \approx 4.7 \times 10^{-13}$), suggesting that such common sites are due to direct interaction with these sequence elements by the homologous p53 and p63 DNA binding domains. However, 61% of the p53 sites score poorly in terms of p63 binding enrichment (<20; Figure S3B). These observed differences in p53 and p63 targets either reflect true differences between p53 and p63 and/or differences in cell-type specificity.

p63 Binding Sites Are Evolutionarily Conserved

The observation that p63 interacts with a broad array of genomic loci, including many in noncanonical locations, raises the question of whether they are all biologically relevant. Evolutionary conservation is a well-recognized property for functional elements. To determine if p63-bound sequences are evolutionarily conserved, we examined the eight-way alignments of human, chimpanzee, mouse, rat, dog, chicken, zebrafish, and fugu (Blanchette et al., 2004). Based on the percentage of nucleotide identity, we found a significantly ($p < 0.001$) stronger conservation for p63-bound sequences in mouse, rat, dog, and (to a lesser extent) chicken as compared with randomly selected genomic sequences (Figure 3A).

With respect to mouse, 65.6% of the sites show higher conservation than random sequences, indicating that 31.2% are conserved beyond expectation and thus may be functional. On average, a p63 binding site shows 38.6% nucleotide identity, far exceeding that from comparable random sets (25.6%, $p < 1.1 \times 10^{-16}$; Figure 3B). Those located near well-characterized genes (40.7% identity), and especially the ones within 1 kb of the initiation site (49.4% identity), are even more conserved. Importantly, p63 sites distant from current gene annotations are still significantly more conserved, although to a slightly lesser extent (36.1% identity). These observations not only suggest a functional role for many of the identified p63 binding sites but also raise the possibility of p63 regulation at these loci across a wide range of species.

p63-Bound Regions Are Enriched with DNA Sequence Motifs for Other Transcriptional Regulatory Proteins

As transcriptional regulatory regions often contain multiple transcription-factor binding sites in locally dense clusters, we asked whether the p63-bound sequences are preferentially associated with human DNA sequence motifs in the TRANSFAC database (Wingender, 2004) (release 6.1). Although TRANSFAC motifs are not generated in an unbiased manner and hence are unlikely to be completely accurate, they nevertheless represent a good description of DNA sequences recognized by transcription factors. In fact, inaccuracies in the TRANSFAC motifs should introduce randomness into the analysis and hence underrepresent the potential association of other transcription factors with the p63-bound regions.

Many of the TRANSFAC position weight matrices appear significantly overrepresented in p63 binding sites relative to genomic background. We also permutated these matrices and found seven distinct motifs that occur at a significantly higher frequency in p63-bound sequences over their shuffled "counterfeits" (Figure 4), suggesting they are the most likely candidates for functional partners of p63. Depending on the motif, about 5%–25% of the p63 sites contain additional copies of a given motif, when compared to randomly sampled genomic sequences. Although the proportion of p63 binding regions containing additional TRANSFAC motifs cannot be measured accurately, many, and probably most, p63 binding regions contain additional TRANSFAC motifs, and hence putative transcription factor binding sites, than expected by chance.

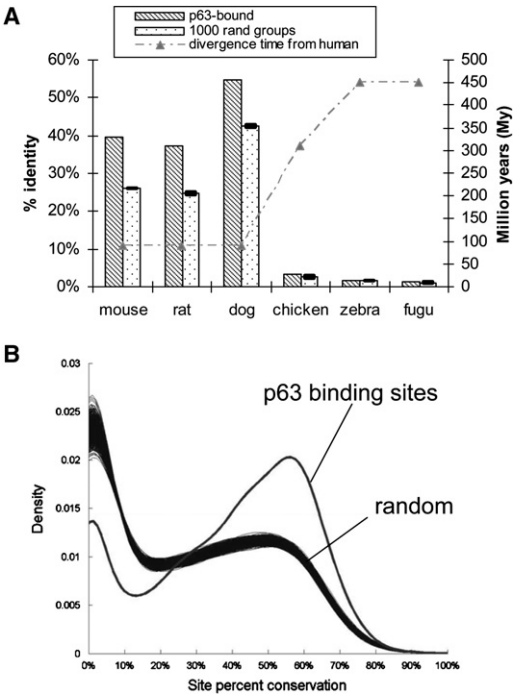

Figure 3. Sequence Conservation of p63 Binding Sites

(A) Total percent identities of p63-bound sequences and 1000 groups of randomly selected comparable genomic sequences across multiple species. Error bars correspond to standard deviations from 1000 randomly sampled groups.

(B) The distribution of percent identity with mouse per site. The dashed line depicts p63 binding sites, whereas the thick black line represents random genomic sequences (1000 groups).

p63 Binding Is Correlated with p63-Dependent Transcriptional Activity, but the Relationship between Binding and Gene Expression Is Complex

Using arrays covering ~20,000 human genes, we investigated the transcriptional effects of p63 binding by analyzing ME 180 cells depleted ~10-fold for all p63 isoforms via expression of a small hairpin RNA (shRNA) targeting the p63 oligomerization domain (Figure 5A). The average differential expression rank for p63-bound genes was better than any of 10,000 random groups, indicating a significant correlation between p63 binding and p63-dependent changes in mRNA expression (Figure 5B). Similar results were observed with a stepwise approach (Figure 5C).

Although there is a significant correlation between p63 binding and p63-dependent transcriptional activity, this relationship is complex. First, depending on the FDR cutoff, only about 14%–27% of the downregulated and 12%–15% of the upregulated genes show p63 binding

Motif Logo	Binding Factor (Accession Number)	P Value Relative to Permutated Motifs	Percentage of Sites with More Motif beyond Expectation
	NF-AT (M00302)	<0.001	6.3
	c-Ets-1 (M00339)	<0.001	25.5
	STAT5 (M00457)	<0.001	5.4
	Bach1 (M00495)	<0.001	5.5
	Lmo2 complex (M00277)	0.001	20.2
	YY1 (M00059)	0.004	18.3
	NF-1 (M00193)	0.008	25.5

Figure 4. Overrepresented TRANSFAC Motifs in p63-Bound Sequences

The sequence logos of the TRANSFAC motif were produced by the World Wide Web Service at http://www.bio.cam.ac.uk/cgi-bin/seqlogo/logo.cgi. The height of each letter is proportional to its frequency of occurrence in the binding-site matrix, times the information content at each position. The percentage of sites with more motif beyond expectation was relative to random genomic sequences. See the Experimental Procedures for details.

in the vicinity (Table 1 and Table S5). Although additional p63-responsive genes may be directly regulated by p63 bound at large distances from the coding region, this observation likely reflects indirect effects of p63 depletion. As a key regulator of epithelial cells, p63 deficiency yields dramatic phenotypic effects (Yang et al., 1999; Mills et al., 1999), and p63-depleted ME180 cells undergo detachment and apoptosis shortly after the time when RNA was isolated (Figure S4).

Conversely, only 15%–20% of p63-bound genes exhibit concomitant changes in mRNA expression in $p63^{shRNA}$ versus control cells. It is likely that many more p63 target sites are transcriptionally competent, but not detected due to various technical and biological reasons (see Discussion). In this regard, 37% (46 out of 124) of p63-responsive genes identified by differential expression in p63-transfected cells (Osada et al., 2005) contain p63 sites defined here ($p = 2.8 \times 10^{-10}$; Table S6). Interestingly, binding sites for the subset of p63 targets that are associated with p63-dependent transcriptional effects are significantly more conserved through evolution than typical p63 sites.

Unexpectedly, p63 binding is significantly correlated with p63-dependent activation of gene expression, indicating that p63 behaves as a direct transcriptional activator for many targets in vivo (Table 1; $p < 1.1 \times 10^{-6}$). In contrast, we only observe a marginal relationship between p63 binding and p63-dependent repression of gene expression. Similar trends were seen for p63-bound genes upon keratinocyte differentiation, during which p63 levels decrease dramatically (F. Pinto, Z.Z., and F.M., unpublished data, and data not shown). As ΔNp63 isoforms are predominant in epithelial cells and the only isoforms detected in ME180 cells, these results strongly suggest that, despite lacking a canonical activation domain, ΔNp63 proteins directly activate many genes in vivo.

p63 Functions in Adhesion, Cell Proliferation, Death, and Signaling Pathways

The p63 targets uncovered in our study include genes previously linked to p63, such as *cdkn1a/p21*, *bbc3/puma* (Harms et al., 2004), and *dst/bpag* (Osada et al., 2005). The subset of p63 targets showing p63-dependent expression is enriched with genes involved in cell cycle, cell death, and cell proliferation (Table S7), and many p63-bound genes have protein kinase activity ($p = 2.1 \times 10^{-8}$). At least 24 p63 targets are associated with Notch signaling, and p63 also binds multiple components of the Wnt and TGFβ signaling cascades (Table S8). The Notch, Wnt, and TGFβ pathways are implicated in epithelial morphogenesis and stem-cell biology (Lefort and Dotto, 2004; Molofsky et al., 2004), and effects on these pathways may contribute to the molecular basis underlying the phenotypes of p63 deficiency. *rac1* is an intriguing p63 target, because inactivation of *rac1* causes a loss of epithelial stem cells analogous to that seen with p63 deficiency (Yang et al., 1999; Benitah et al., 2005). Additional components of Rac1 signal transduction, such as *pak1*, are also bound by p63.

Finally, an unbiased pathway mapping of p63-bound targets using the Kyoto Encyclopedia of Genes and Genomes (KEGG) further revealed their overrepresentation in tight junction ($p = 2.5 \times 10^{-6}$), adherens junction ($p = 4.7 \times 10^{-6}$), and focal adhesion ($p = 1.6 \times 10^{-5}$) genes (Table S9). These findings point to an important biological function for p63 consistent with previous links between *perp*, a p63 target gene, and cell-adhesion complexes (Ihrie et al., 2005). Our results significantly expand this notion by uncovering a set of targets that likely mediate p63's effect on cellular-adhesion and communication pathways.

Discussion

Complex Relationships between DNA Sequence Motifs, DNA Binding In Vivo, and Transcriptional Regulation: Implications for Functional Genomic Analysis

Functional genomic analyses typically assume that DNA sequence motifs dictate binding of transcription factors, and bound transcription factors regulate gene expression. However, the relationships between DNA sequence motifs, in vivo binding, and transcriptional

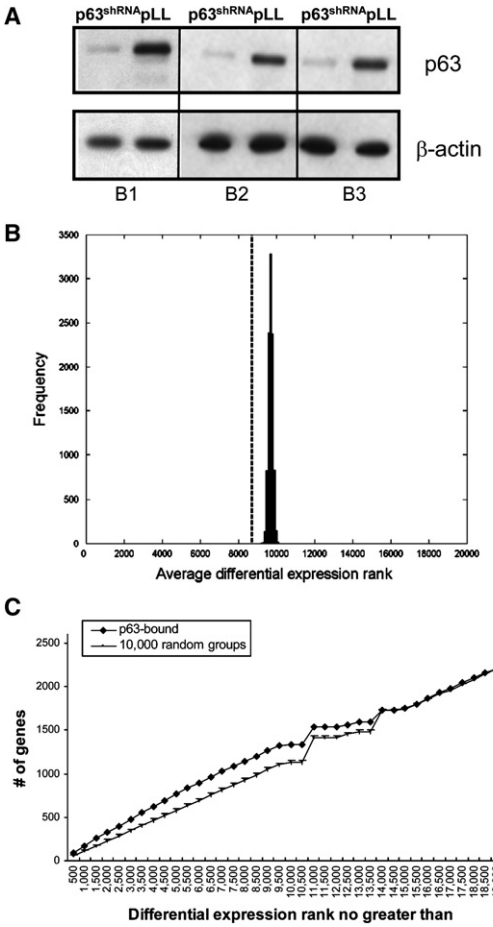

Figure 5. Correlation between Binding and p63-Dependent Expression Change

(A) Depletion of p63 protein levels in cells expressing $p63^{shRNA}$ or containing the pLL vector (B1, B2, and B3, biological replicates).
(B) Average differential expression rank for p63-bound genes (dash) and 10,000 randomly selected groups (histogram).
(C) Differential expression rank for p63-bound genes (black) and 10,000 randomly selected groups (gray) falling into top of the list using a step size of 500.

Table 1. Identification of Direct Targets for p63

FDR Cutoff		Differentially Expressed Genes[a]	p63 Bound[b]	Both	P Value[c]
0.05	Down[d]	175		47	2.1×10^{-8}
	Up[d]	395		49	3.3×10^{-1}
0.10	Down[d]	458		88	1.1×10^{-6}
	Up[d]	577	2247	75	1.6×10^{-1}
0.15	Down[d]	1230		226	1.7×10^{-13}
	Up[d]	760		109	1.0×10^{-2}
0.20	Down[d]	2454		372	6.1×10^{-9}
	Up[d]	1002		144	3.4×10^{-3}

[a] Genes identified as differentially expressed by either RP (Breitling et al., 2004) or SAM (Tusher et al., 2001) method at the specified FDR cutoff.
[b] Genes bound by p63 anywhere from 5 kb upstream to 1 kb downstream and with RNAi expression data available.
[c] The probability of obtaining at least the observed overlap by chance, given the number of p63-bound, differentially expressed, and total genes in the genome. It was calculated by using hypergeometric distribution.
[d] Wild-type versus shRNA.

activity are poorly understood and have not been systematically investigated. In accord with the classical view, the level of in vivo binding is significantly correlated with the similarity to the p63 motif.

However, in striking contrast to this view and to the situation in *E. coli* where the presence of a LexA motif virtually guarantees DNA binding in vivo (Wade et al., 2005), the vast majority of p63 motifs are not bound in vivo. Only 8% of the very best p63 motifs and only 1%–3% of typical p63 motifs are actually bound in vivo. Conversely, ~15%–20% of the actual p63 targets have motif scores that are typical of random genomic regions. Taken together, these observations demonstrate the dual notions that p63 binding in vivo requires considerable information beyond direct binding to the DNA sequence motif and that DNA binding in vivo is highly selective. Although 5800 p63 target sites appear to be a large number, this actually reflects a great deal of selectivity given that there are greater than 10^9 potential binding locations in the human genome. In fact, this level of selectivity is comparable to, and in fact somewhat greater than, the 50 targets of LexA repressor in *E. coli* cells (Wade et al., 2005).

Our results strongly suggest that p63 binding in vivo requires accessible chromatin and/or additional transcriptional regulatory proteins that cooperatively associate with DNA. In yeast cells, promoter regions are preferentially accessible to coding regions, and in many cases, this is due to intrinsically poor interactions between histones and promoter DNA (Sekinger et al., 2005). In mammalian cells, it is unknown whether accessible chromatin is due primarily to differences in intrinsic histone-DNA interactions or cooperative recruitment of chromatin-modifying activities by multiple DNA binding proteins. As p63 target regions are significantly enriched in TRANSFAC motifs, cooperative interactions with other transcription factors likely help p63 select a limited number of genomic targets out of the vast number of potential sites.

The relationship between in vivo binding and transcriptional activity is also complex. In accord with conventional views, there is a significant relationship between p63 binding in vivo and p63-dependent transcriptional activity. However, about 75% of the p63-responsive genes do not show p63 binding in the vicinity of the genes, suggesting that p63-dependent regulation in many of these cases does not reflect a direct function of p63. Conversely, only 10%–20% of the p63-bound sites show p63-dependent regulation in ME180 cells depleted for p63.

Although only a minority of p63 targets appear to show p63-dependent effects on transcription, this is likely to be a considerable underestimate for technical and biological reasons. From a technical perspective, some p63 targets might regulate transcription at a long distance from the binding site and, hence, would be mischaracterized with respect to transcriptional function. In addition, some p63 targets might regulate noncoding or other RNAs that are not assayed on the transcriptional profiling arrays. Lastly, subtle p63-dependent transcriptional effects are difficult to measure.

There are at least three biological reasons for the apparent absence of p63-dependent transcriptional

effects at many p63 targets. First, functional redundancy is a property of many eukaryotic enhancers, such that a p63 binding site might contribute to transcriptional activity, yet removal of this binding site or p63 itself might not produce a significant transcriptional effect. Functional redundancy may also arise from related proteins (e.g., p53 and p73) that recognize common target sites. Second, enhancers typically require the combined action of multiple factors, such that a protein(s) may bind to an enhancer under a given condition, whereas transcription only occurs when the remaining factors bind. Third, DNA binding and transcriptional activation are separable properties of activator proteins, and proteins bound to promoter regions in vivo are often transcriptionally active only under certain environmental or developmental conditions. In yeast cells, most transcription factors bind under "nonactivating" conditions, and a great deal of transcriptional regulation is not mediated at the level of DNA binding (Harbison et al., 2004); classifying such nonactivating target sites as nonfunctional is profoundly misleading. For all of these reasons, the inability to detect a transcriptional effect under a single experimental condition does not indicate that the p63 binding site is nonfunctional.

Taken together, these observations have significant implications for interpreting the results of large-scale functional genomic experiments. Although it is likely that the conventional relationships between DNA sequence motif, protein binding in vivo, and transcriptional function will occur with statistical significance, these conventional relationships actually represent the minority of individual cases. Given the overall validity of the data, the apparent discordance from conventional relationships reflects physiological reality, and not experimental error, in the vast majority of cases. As such, these results indicate that it is inappropriate to use the presence of DNA sequence motifs or transcriptional function to validate in vivo binding.

A Substantial Number of p63 Binding Sites Are Biologically Relevant

The unexpected finding that mammalian transcription factors bind specifically to a surprisingly large number of genomic regions has prompted the question of whether many (or most) of these target sites are biologically functional. This is a difficult issue, because biological function can be defined in various ways, and the concept of a biologically irrelevant target that is bound with high selectivity in living cells under physiological conditions is rather nebulous. A very conservative view is that in vivo binding sites are presumed to be nonfunctional unless one can demonstrate a transcriptional effect or mutant phenotype. By this criterion, ~15% of the p63 binding sites are associated with genes whose level of transcription is affected by p63. However, this view not only ignores many well-known complexities of eukaryotic gene regulation described above and imposes a highly restricted definition of biological function, but it also reverses a centuries-old and continuously justified view that discrete and highly specific phenomena that occur in living cells are likely to be biologically relevant.

For reasons discussed above, it is highly likely that many of the p63 target sites that do not show transcriptional effects under the single condition tested will mediate transcriptional effects under other conditions. Nevertheless, in the absence of transcriptional analyses under other conditions, we employed two other methods to assess biological function. First, we show that about 30% of p63 target regions are evolutionarily conserved beyond chance expectation. Evolutionary conservation is a well-established approach for assessing biological function. Interestingly, the subset of p63 target regions associated with p63-dependent transcriptional effects is more conserved than typical p63 sites, indicating that conservation and transcriptional effects are related. Second, functional enhancers and silencers typically involve multiple proteins and target sites, and many p63 target regions contain significantly more DNA sequence motifs associated with transcription factors (TRANSFAC motifs) than expected. Although the TRANSFAC motifs were not generated in an unbiased manner and have limitations and inaccuracies, these deficiencies should actually introduce significant randomness into the analysis. Thus, the combined transcriptional, evolutionary, and motif-clustering analyses suggest that many, and probably the significant majority of, p63 binding sites are biologically functional. Although our conclusions are strictly limited to p63 and comparable analyses have yet to be performed on other proteins, it seems likely that many, and perhaps most, in vivo targets of transcriptional regulatory proteins will have biological significance in mammalian cells.

Implications for p63 Activity and Biological Function

Our comprehensive and unbiased identification of in vivo targets for p63 across the entire human genome confirms and extends previous notions that p63 exhibits strong similarities to p53 with regard to DNA motif and binding. Indeed, p63 targets were enriched for cell cycle and apoptosis, sharing many of the canonical p53 effectors in these pathways. Furthermore, p63 binds to all three genes of the p53 family, suggesting additional functional interactions and potential crossregulatory mechanisms.

Unexpectedly, our identification of numerous direct transcriptional targets of p63 reveals that transcriptional activation is a physiological and common mechanism of action for ΔNp63 isoforms. These ΔN isoforms have been traditionally viewed as repressors or dominant-negative regulators (Yang et al., 1998; Westfall et al., 2003), and the few reports hinting at the potential for transactivation have been limited to single gene or reporter studies in transfected cells. In the absence of an obvious transactivation domain, it is likely that ΔNp63 proteins exert these effects in conjunction with other factors, possibly linked to the additional DNA motifs found in many p63 binding sites.

Lastly, it has been suggested that adhesion complexes involving Perp, a p63 target, may be linked to the role of p63 in epithelial homeostasis (Ihrie et al., 2005). Our results indicate that p63's function in cell adhesion extends far beyond that mediated by PERP, and they suggest that cell-adhesion defects may underlie the epithelial phenotypes caused by p63 deficiency. Finally, cell adhesion has also been linked to cell motility and tumor metastasis (Flores et al., 2005), suggesting that p63's cell-adhesion targets are involved in this

process. Thus, together with p63 targets in other essential biological pathways, our work provides a valuable platform for elucidating p63's function in cancer and development.

Experimental Procedures

Unbiased, Genome-Wide Identification of p63 Binding Sites

ME180 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in the presence of absence of 5 nM actinomycin D (+Act D). Immunoblotting and ChIP were performed by standard procedures using the 4A4 anti-p63 monoclonal antibody, which recognizes all p63 isoforms (Yang et al., 1998). Input and ChIP DNA was amplified by two rounds of primer extension followed by PCR. The resulting samples were hybridized to a 14 array set of high-density, tiled whole-genome arrays (Affymetrix) covering essentially all of the nonrepetitive DNA sequences of the human genome with one oligonucleotide pair (PM and MM probes) every 35 bp.

A binding p value for each genomic position was determined by the Wilcoxon rank sum test (Cawley et al., 2004), and p values from three biological replicates were combined by using Stouffer's sum-of-z's method (Whitlock, 2005). Binding sites were identified by imposing a composite p value cutoff of 10^{-5} and merging genomic positions separated by less than 500 bp. We excluded positions where the composite value was dominated by a single replicate or that had an unusually high density of probes. Finally, each binding site was extended to 500 bp on each side to account for the smoothing effect of using a window-based approach.

p63 targets were validated by real-time quantitative PCR. Fold enrichment for a genomic region was determined relative to a nonenriched region (exon 3 of the histone H3 gene), and occupancy units were defined as the fold-enrichment value minus background (H3 reference value set to 1). For each biological replicate, we normalized occupancy values based on a positive control region (p21 promoter). Based on p63 occupancy for various control negative regions, we defined validated targets as those regions showing greater than 2.5 occupancy units. To determine an overall false discovery rate (FDR), we adopted a weighted approach in which FDRs were determined for sites sorted into "bins" of binding enrichment scores. We determined the FDR for each bin: $FDR_{bin} = 1 - [\text{number of sites validated in bin / total number of sites assayed in bin}]$. By calculating the percentage of total sites for each bin, we then determined the $FDR_{total} = \sum_{bin}$ (percentage of sites in bin × FDR_{bin}), where percentage of sites = number of sites in bin/total number of sites (Table S3). Details for these procedures are described in the Supplemental Data.

Discovery of a p63 Sequence Motif and Analysis of the Relationship between Motif Score and the Level of p63 Binding

MEME and AlignACE were used to search for enriched sequence motifs among repeat-masked sequences within the 500 bp region centered at the genomic position(s) with the highest binding p value for p63 binding. In consideration of computational time, we performed the search with 500 top sequences. The specificity of the motifs and binding-enrichment scores for each target region was assessed by ScanACE (Hughes et al., 2000). Motif presence/absence call was determined by a cutoff of one standard deviation below the mean of the scores for each of the aligned sites used to define the motif. The probability of finding at least the observed number of motif occurrences was calculated with a one-tailed binomial test. We generated 1000 randomized motifs by shuffling the columns (i.e., positions) of its weight matrix and counted their instances in the p63-bound sequences. Both methods independently identify a dyad-symmetric motif that is highly specific to the p63-bound sequences ($p < 2.2 \times 10^{-16}$) and is significantly overrepresented when compared to randomized motif matrices ($p < 0.001$; z score = 13.5). Using a variety of motif binding algorithms, we were unable to identify a common DNA sequence motif shared by p63 targets lacking a noncanonical motif. For each binding site, a binding enrichment score was generated from a smoothed "peak" estimator using the five genomic positions with the highest binding p values in the region and one-step Tukey's biweight algorithm. A motif score was assigned based on the motif occurrence with the highest ScanACE score (Hughes et al., 2000) (no thresholding) in the sequence, or 0 if the score was negative. Details of the statistical analysis are provided in the Supplemental Data.

Sequence Conservation Analysis

Based on the eight-way alignments (Blanchette et al., 2004), we generated overlaid versions of the human genome with corresponding sequences from the other seven species. In cases of more than one multiple alignment for a given human region (e.g., with different indels), we selected the one with the best alignment score. Percentage of sequence identity was calculated by counting the proportion of nucleotides in the p63-bound sequences with exact matches in the overlaid genome. Statistical significance was assessed with 1000 randomly sampled groups of the same number of sequences of the same length from the same chromosomes as p63 binding sites (Supplemental Data). Assuming all "functional" sites and (by definition) half of the nonfunctional sites are more highly conserved than random genomic sequences, we derived the percent of functional sites (i.e., those conserved beyond expectation) is $2(Z - 50\%)$, where Z is the proportion of binding sites that have a higher level of conservation than random sequences.

Identifying Overrepresented TRANSFAC Motifs in the p63 Target Regions

As many TRANSFAC matrices are similar to each other, we clustered them using the Tree program at a cutoff of 0.70 (Hughes et al., 2000). For each "distinct" motif, PATSER (Hertz and Stormo, 1999) (v. 3e) was used to search p63-bound and 1000 groups of randomly selected genomic sequences (same lengths and same chromosomes as p63 sites) for matches to TRANSFAC position weight matrices corresponding to human factors (Zhu et al., 2005). We also generated 1000 randomized versions of each motif by shuffling the columns (i.e., positions) of its weight matrix and compared their occurrences in p63-bound sequences with that of the "true" motif. The proportion of p63 binding sites containing more copies of "partner" motifs than random sequences was determined as described in the Supplemental Data, and the percent of sites with more partner motif beyond expectation was determined as described for the conservation analysis.

Identification of p63-Responsive Genes

An shRNA for p63 was cloned into the pLL3 lentiviral expression vector (F. Pinto), and viral production and transduction were performed, as previously described (Rubinson et al., 2003). ME180 cells were transduced with viral supernatant containing pLL $p63^{shRNA}$ or empty vector (control) and harvested ~65 hr later. Total RNA was purified with Trizol (Invitrogen), converted to cDNA, transcribed in vitro to generate biotinylated cRNA, and hybridized to the Affymetrix HG-U133 plus 2.0 GeneChip, according to manufacturer's instructions. Data from three biological replicates were normalized, and genes showing differential expression in p63-depleted and control cells were determined by a rank product method (Breitling et al., 2004). A permutation-based estimation procedure with 100 random "experiments" was then used to estimate FDR. See the Supplemental Data for additional details.

Gene Ontology and KEGG Assignments and Statistics of Enrichment

Standard gene ontology vocabulary for description of biological processes at the fourth level was retrieved by using the webtool FatiGO (Al-Shahrour et al., 2004). KEGG pathway IDs and associated gene lists were downloaded from ftp://ftp.genome.jp/pub/kegg/pathways/hsa. Statistical significance for functional category enrichment was determined by hypergeometric distribution (Tavazoie et al., 1999), and correction for multiple hypothesis testing was conducted by using the Q value package, which employs an FDR method and has increased power over Bonferroni-type approach (Storey and Tibshirani, 2003). We only reported the enriched categories with corresponding FDR < 0.05.

Supplemental Data

Supplemental Data include four figures and nine tables and can be found with this article online at http://www.molecule.org/cgi/content/full/24/4/593/DC1/.

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Accession Numbers

Raw (cel files) and processed (p value files) data files can be found at http://genetics.med.harvard.edu/~zzhu/p63/p63.html. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible under GEO Series accession numbers GSE5993 and GSE6132 for the RNAi and ChIP-Chip data, respectively.