A Functional Map of Genomic HIF1 α -DNA Complexes in The Eye Lens Revealed Through Multiomics Analysis

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Abstract

Background: During eye lens development the fetal vasculature regresses leaving the lens without a direct oxygen source. Both embryonically and throughout adult life, the lens contains a decreasing oxygen gradient from the surface to the core that parallels the natural differentiation of immature surface epithelial cells into mature core transparent fiber cells. These properties of the lens suggest a potential role for hypoxia in the regulation of genes required for mature lens structure and function. Since HIF1 α is a master regulator of the hypoxic response, these lens properties also implicate HIF1 α as a potential requirement for lens formation and homeostasis. Here, we employed a multiomics approach combining CUT&RUN, RNAseq and ATACseq analysis to establish the genomic complement of lens HIF1 α binding sites, genes activated or repressed by HIF1 α and the chromatin states of HIF1 α -regulated genes.

Results: CUT&RUN analysis revealed 8,375 HIF1 α -DNA binding complexes in the chick lens genome. 1,190 HIF1 α -DNA binding complexes were significantly clustered within chromatin accessible regions (χ^2 test $p < 1 \times 10^{-55}$) identified by ATACseq. Formation of the identified HIF1 α -DNA complexes paralleled the activation or repression of 526 genes, 116 of which contained HIF1 α binding sites within 10kB of the transcription start sites. Some of the identified HIF1 α genes have previously established lens functions while others have novel functions never before examined in the lens. GO and pathway analysis of these genes implicate HIF1 α in the control of a wide-variety of cellular pathways potentially critical for lens formation, structure and function including glycolysis, cell cycle regulation, chromatin remodeling, Notch and Wnt signaling, differentiation, development, and transparency.

Conclusions: These data establish the first functional map of genomic HIF1 α -DNA complexes in the eye lens. They identify HIF1 α as an important regulator of a wide-variety of genes previously shown to be critical for lens formation and function and they reveal a requirement for HIF1 α in the regulation of a wide-variety of genes not yet examined for lens function. They support a requirement for HIF1 α in lens development, structure and function and they provide a basis for understanding the potential roles and requirements for HIF1 α in the development, structure and function of more complex tissues.

Background

During eye lens development the fetal vascular vasculature regresses leaving the lens without a direct oxygen source [1–4]. Both embryonically and throughout adult life, the lens contains a decreasing oxygen gradient from the surface to the core [2, 4] paralleling the natural differentiation of immature surface epithelial cells into mature core transparent fiber cells [5]. These properties of the lens suggest a potential role for hypoxia in the regulation of genes required for mature lens structure and function.

Hypoxia has profound effects on cellular physiology and function through regulation of the hypoxia inducible factor (HIF) family of transcription factors (HIFs) [6, 7]. Three HIFs have been identified, including HIF1, HIF2 and HIF3 [8]. In most cells, the master regulator of the hypoxic response is HIF1 [6–10]. Although primarily investigated as an essential regulator of vascularization [11], emerging evidence

suggests that HIF1 plays key roles in the regulation of a wide variety of cellular processes required for the development, homeostasis and/or disease states of a wide variety of tissues [12–19].

HIF1 is a heterodimer made up of two subunits, HIF1 β /ARNT and HIF1 α . The HIF1 β /ARNT subunit is expressed constitutively but the protein level of HIF1 α is tightly regulated in response to environmental oxygen levels. Under normoxic (21% oxygen) conditions, HIF1 α is regulated through post-translational hydroxylation of prolines in its oxygen dependent domain (ODD) by members of the 2-oxoglutarate-dependent dioxygenase superfamily of prolyl hydroxylases (PHD1, PHD2 and PHD3) [6, 7]. Proline hydroxylation promotes interaction with the von Hippel-Lindau tumour suppressor protein, targeting HIF1 α for proteasomal degradation [6, 7]. Additional hydroxylation of an asparaginyl moiety in the C-terminus inhibits binding of HIF1 α to its co-factors, p300 and CREB binding protein (CBP), directly inhibiting its transcriptional activity [6, 7, 9]. Under hypoxic conditions, oxygen-dependent PHDs are inactivated, allowing HIF1 α to bind to nuclear pores and translocate to the nucleus where it dimerizes with HIF1 β /ARNT and recruits CBP and p300 to regulate transcription [6, 7, 9, 20].

To date, the range and spectrum of genes controlled by HIF1 α , particularly in primary cells, has not been fully elucidated. A major limitation has been the availability of easily obtainable cells from actual tissues and organs. One ideal system is the ocular lens, which consists of a surface layer of cuboid epithelial cells that embryologically and throughout adult life differentiate to form a core of highly elongated organelle-free fiber cells [5]. This process parallels a naturally occurring lens oxygen gradient from the surface to the core [1–4]. During early lens formation, the lens receives oxygen from a network of capillaries but as the lens develops this capillary network is eliminated, leaving the lens without a direct oxygen source [1–3]. The results in 2% oxygen levels at the lens surface [2, 4] decreasing to 0.1% oxygen levels in the lens core [4] and resulting in a hypoxic microenvironment where lens fiber cell differentiation takes place. We propose that hypoxia is required for key features of lens fiber cell differentiation and that this process depends on transcriptional regulation by HIF1 α .

Since HIF1 is expressed in the lens at 120-fold higher levels than HIF2, and because HIF3 was not detected in the lens [21], we propose that HIF1 α is the major regulator of the lens hypoxic response. Consistently, lenses lacking HIF1 α degenerate shortly after birth and their analysis reveals extensive vacuolization and fiber cell dysmorphology consistent with aberrant lens fiber cell differentiation and structure [22]. In addition, we recently discovered that elimination of non-nuclear organelles, a key step in lens fiber cell differentiation program, is dependent on hypoxia and HIF1 α -dependent activation of the mitophagy gene BNIP3L [23].

Collectively, studies suggest an important role for HIF1 α in the regulation of a wide-variety of lens gene expression events. To identify the range and spectrum of lens genetic events regulated by HIF1 α , we employed Cleavage Under Targets and Release Under Nuclease (CUT&RUN) [24, 25] to map genome-wide HIF1 α -DNA binding complexes in a well-characterized primary embryonic chick lens cell model system. This model system has been used to successfully investigate a multiple lens properties and functions including lens cell differentiation, gene expression, autophagy/mitophagy [26, 27], PI3K signaling [28],

TGFB signalling [29, 30] and gap junction expression and function [31]. We then mapped the positions of the identified HIF1 α -DNA binding complexes relative to transcription start sites of nearest neighbor genes and used RNAseq to examine the HIF1 α -regulated expression profiles of these genes. We also examined the relationship between HIF1 α -DNA binding, gene activation or repression and chromatin configuration through analysis of Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq) data [32, 33]. We chose CUT&RUN over ChIP-seq analysis since only a few thousand cells are required for technical success, whereas ChIP-seq requires millions of cells for the same output level [24]. Moreover, CUT&RUN assesses DNA-binding in the native state and does not require DNA-protein cross-linking, which can introduce artefacts [24].

CUT&RUN analysis using a HIF1 α -specific antibody identified over 8000 HIF1 α -DNA specific complexes in primary lens cells following HIF1 α activation. As validation, motif analysis of the identified binding sites confirmed enrichment of the HIF1 α consensus sequence. 1190 of the identified binding complexes were contained in chromatin accessible regions identified by ATAC-seq [33]. Activation of HIF1 α was required for activation or repression of 526 genes identified by RNA sequencing. 116 of these genes contained HIF1 α -DNA binding complexes within 10kB of the transcription start sites (TSS's) of coding genes. Some of the identified HIF1 α genes have previously established lens functions while others have novel functions not before examined in the lens. GO and pathway analysis of these genes implicate HIF1 α in the control of a wide-variety of cellular pathways potentially critical for lens formation, structure and function including glycolysis, cell cycle regulation, chromatin remodeling, Notch and Wnt signaling, differentiation, development, and transparency.

These data establish the first functional map of genomic HIF1 α -DNA complexes in the eye lens. They identify HIF1 α as an important regulator of a wide-variety of genes previously shown to be critical for lens formation and function and they reveal a requirement for HIF1 α in the regulation of a wide-variety of genes not yet examined for lens function. They support a requirement for HIF1 α in lens development, structure and function and they provide a basis for understanding the potential roles and requirements for HIF1 α in the development, structure and function of more complex tissues.

Methods

Primary lens cell culture

Primary chick lens epithelial cell cultures were prepared from the lenses of Embryonic day 10 (E10) White Leghorn embryonated chicken eggs (Charles River Laboratories, Storrs, CT) as previously described [26]. Briefly, primary lens cells were isolated from chicken lenses by trypsinization and agitation. Cells were plated onto dishes coated with mouse laminin (Invitrogen, prod no: 23017-015) and cultured in Medium 199 (Invitrogen, prod no: 11150) supplemented with 10% FBS (Invitrogen, prod no: 16140-071) and penicillin-streptomycin antibiotic mix (50 units/ml; Invitrogen, prod no: 15140-122). The resulting primary lens cells were incubated for 2 days at 37°C in the presence of 5% CO₂ before treatment with DMOG as described below.

CUT&RUN on DMOG-treated lens cells and Bioinformatics analysis

Primary lens cells were treated with the HIF1 α activator dimethyloxallyl glycine (DMOG, Santa Cruz Biotechnology, prod no: sc200755) (1 mM) for 4 hours at 37°C. DMOG is a cell permeable competitive inhibitor of prolyl hydroxylase domain-containing proteins such as the PHDs. DMOG has been shown to activate HIF1 α , under normoxic (21% O₂) conditions in a wide variety of cell types [93, 94] including lens cells [23]. Cleavage Under Targets & Release Using Nuclease (CUT&RUN) [24, 25] was performed on unfixed permeabilized primary lens cells incubated with a HIF1 α -specific ChIP grade antibody (Abcam, ab2185) and an IgG negative control antibody (Invitrogen, prod no: 10500c) to elucidate genome-wide HIF1 α -DNA binding complexes. The protocol was adapted from Meers et al., 2019 [95]. Briefly, primary lens cells were harvested, counted, and pooled into biological duplicates of 500,000 cells each and resuspended in 1.5 ml Wash Buffer without Digitonin (20mM HEPES pH 7.5, 150mM NaCl, 0.5mM Spermidine, Protease Inhibitor). After three rounds of washing and centrifuging for 3 minutes at 600 g, the resuspended cells were washed in 1 ml wash buffer and ConA-coated magnetic beads (Bangs Laboratories, prod no: BP531, 10 μ l per sample) were added followed by gentle rotation at room temperature for 10 minutes. Each sample was divided into two equal volumes and the supernatants removed using a magnet stand. 150 μ l of antibody buffer with 0.1% final concentration digitonin was added to each aliquot with gentle agitation to permeabilize the cells. For each biological sample, one aliquot of ConA-bound cells was incubated with ChIP-grade HIF1 α antibody and the other aliquot was incubated with IgG antibody at a 1:100 dilution and rotated at 4°C overnight. HIF1 α antibody specificity was confirmed by western analysis of primary lens cell nuclear extract and by ChIP-qPCR published in a previous study [23]. Antibody-bound ConA-bound cells were washed twice with 1 ml wash buffer with 0.1% digitonin. The supernatant was removed and 150 μ l of Protein A-MNase (700ng/mL final concentration, Addgene #123461)[95] in wash buffer with digitonin was added to the pellet with gentle agitation and the mix rotated at 4°C for 1 hour. Cells were washed twice with 1 ml of digitonin wash buffer and once with 1ml low salt rinse buffer (20mM HEPES pH 7.5, 0.5mM Spermidine, 0.05% Digitonin). The supernatant was removed and 200 μ l of ice-cold incubation buffer (3.5mM HEPES pH 7.5, 10mM CaCl₂, 0.05% Digitonin) added with gentle agitation. The resulting samples were incubated on ice for 5 minutes before removing the supernatant using the magnet stand followed by the addition of 200 μ l STOP buffer (170mM NaCl, 20mM EGTA, 0.05% Digitonin, 50ug/mL RNase A, 25 μ g/mL Glycogen) with gentle agitation. The samples were incubated for 30 minutes at 37°C to release CUT&RUN fragments. Following incubation, the supernatant containing CUT&RUN fragments was transferred to a fresh microcentrifuge tube. CUT&RUN fragments were treated with 2 μ l 10% SDS and 2.5 μ l Proteinase K (20 mg/ml) for 1 hour at 50°C. DNA was isolated by standard Phenol-Chloroform extraction using Qiagen MaXtract tubes, and the DNA pellet resuspended in 30 μ l 1 mM Tris-HCl pH 8 0.1 mM EDTA. Library prep was carried out using the NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB E7645) following the manufacturer's instructions [96]. Samples were resuspended in 15 μ l of 0.1x TE Buffer. Libraries were pooled and PE50 sequencing on an Illumina Hiseq platform was performed.

Reads were trimmed with Trimmomatic [97] with the following settings (ILLUMINACLIP: 2:15:4:4:true SLIDINGWINDOW:4:15 MINLEN:25) and aligned to the galgal6 genome (ensemble version 96) using Bowtie2 [98] with the following settings: (–end-to-end,–dove-tail,–phred33). Samtools [99] was used to retain fragments < 120bp, and to remove reads in mitochondrial DNA and remove reads overlapping low-complexity regions in the Galgal6 genome as defined by the RepeatMasker [100] track in the UCSC genome browser. HIF1 α -DNA binding complexes were identified using MACS2 [101, 102] by pooling the HIF1 α BAM files versus the pooled IgG BAM files using the following settings: (narrowPeak setting, q value cutoff 0.05, –SPMR, –keep-dup auto). MACS2 on CUT&RUN samples identified 8,375 HIF1 α -DNA binding complexes in the primary chick lens cells (**Table S1**).

RNAseq on DMOG-treated lens cells and Bioinformatics analysis

Biological triplicates of primary chick lens cells were incubated in the presence or absence of 1 mM DMOG for 4 hours at 37°C. Total RNA was isolated from primary lens cells using TRIZOL® reagent (Invitrogen, prod no: 15593018) according to the manufacturer's instructions. Libraries were prepared following Illumina's TruSeq-stranded-total-RNA-sample preparation protocol. Paired-end sequencing was performed on Illumina's NovaSeq 6000 platform. Cutadapt [103] was used to remove adaptor-contaminated reads, low quality bases and undetermined bases. Reads were mapped to galgal6 genome (ensemble version 96) using Bowtie2 [98] and HISAT2 [104]. The mapped reads were assembled using StringTie [105]. Comprehensive transcriptomes were generated using gffcompare and StringTie [105]. The R package edgeR [106] was used to estimate the expression levels of all transcripts and to identify differentially expressed genes (log2 fold change FPKM > 0.6 or <-0.6) with parametric F-test comparing nested linear models (p < 0.05). 526 genes were found to be differentially expressed in DMOG-treated primary lens cells relative to untreated primary lens cell samples (**Table S2**).

Additional Bioinformatics methods

MACS2 generated bedgraph files for the HIF1 α and IgG CUT&RUN samples were converted to BIGWIG format and visualized in UCSC Genome Browser (https://genome.ucsc.edu/s/jdisatha/CUTRUN_Lens_HIF1A) [107]. Genomic coordinates of chick lens open chromatin regions mapped by ATAC sequencing [33] were lifted to galgal6 using UCSC Genome Browser liftover tool. HIF1 α -DNA binding complexes identified by CUT&RUN that are also contained within previously identified open chromatin regions [33] are noted in **Table S1**.

The Bedtools [108] Shuffled function was used to generate a negative control background of HIF1 α -DNA binding complexes randomly distributed across the galgal6 genome using default settings.

The ChIPseeker [109], bedtools [108] and Closestbed tools were used to identify relative distances of HIF1 α -DNA binding complexes to the nearest gene transcription start sites. (**Table S1**).

Deeptools [110], plotProfile and plotHeatmap functions default settings were used to generate the profile and heatmap in **Fig. 2A**.

MEME-suite [111] DREME tool [112] default settings were used to identify short-ungapped overrepresented sequence patterns in the HIF1 α -DNA binding complexes at genomic regions identified by CUT&RUN.

HIF1 α -dependent genes identified by our multiomic analysis were cross-referenced with hypoxiadb [43] to elucidate previously known hypoxia-dependent genes compared to novel HIF1 α -dependent genes.

Enrichr [113] and Msigdb [114] tools were used to identify significantly enriched processes and pathways associated with the identified HIF1 α -dependent genes.

All heatmaps shown were generated with the Morpheus Bioinformatics tool (<https://software.broadinstitute.org/morpheus/>).

Statistical analysis

Chi-squared goodness-of-fit test was performed in R against the null hypothesis of no significant difference between the genomic distribution pattern of HIF1 α -DNA binding complexes identified by CUT&RUN compared to the distribution pattern of randomly distributed HIF1 α -DNA binding complexes generated by bedtools ShuffleBed (Fig. 2D and 2F).

Chi-squared test was performed in R for all other statistical comparisons. Bonferri corrected p-values are reported.

Results

CUT&RUN identifies 8,375 HIF1 α -DNA binding complexes in the lens genome

Multiple lines of evidence suggest an essential role for HIF1 α in regulating gene expression pathways leading to mature lens cell structure and function. The lens resides in a hypoxic environment [1–4], lens deletion of HIF1 α disrupts lens cell structure and development [22] and hypoxia and HIF1 α are required for the elimination of non-nuclear organelles during mature lens cell formation through activation of the *BNIP3L* gene [23]. Based on these data, we hypothesized that HIF1 α could regulate a wide-variety of genes involved in multiple genetic pathways important for mature lens cell structure and function. To establish the range and spectrum of lens genes regulated by HIF1 α , we first sought to establish the genomic-level occupancy of HIF1 α -DNA binding complexes in primary lens cells upon HIF1 α activation using Cleavage Under Targets & Release Using Nuclease (CUT&RUN) (Figs. 1A-B). HIF1 α was activated in primary embryonic chick lens cells by exposure to 1mM DMOG for 4 hours. This 4-hour DMOG treatment was selected based on previous whole lens studies [23] showing this time of exposure resulted in robust binding of HIF1 α to its cognate site of the BNIP3L gene promoter detected by ChIP-qPCR and robustly activated BNIP3L transcription detected by RT-PCR [23]. BNIP3L is an established HIF1 α target in multiple

cell-types [23, 34, 35]. In this study, exposure of primary lens cells to 1mM DMOG for four hours also resulted in a greater than 2-fold increase in *BNIP3L* mRNA (Figs. 1C-D).

Biological duplicates of primary chicken lens cell preparations were treated with 1mM DMOG for 4 hours to activate HIF1 α followed by *in situ* HIF1 α CUT&RUN [24, 25]. Following Illumina sequencing, small chromatin fragments were mapped across the Galgal6 genome and interrogated for enrichment relative to an IgG isotype control CUT&RUN. There were 8,375 HIF1 α -DNA binding complexes across the Galgal6 genome with high resolution and specificity (**Table S1**). These exhibited specific binding within narrow regions of chromatin. A heatmap of all identified HIF1 α -DNA binding complexes is shown in **Fig. 2A**. The data reveal the presence of a highly enriched HIF1 α signal relative to the control IgG signal at genomic regions containing identified HIF1 α -DNA binding complexes. To validate the binding identified by CUT&RUN, we analyzed nucleotide patterns in DNA sequences underlying genomic regions bound by HIF1 α using *de novo* motif discovery to determine enrichment of transcription factor footprints at these loci. The most significant motif identified was the canonical HIF1 α motif (5'-ACGTG-3'; $E < 3.6 \times 10^{-111}$, **Fig. 2B**), confirming the presence of the HIF1 α binding sequence in DNA regions identified to be bound to HIF1 α by CUT&RUN.

The majority of identified HIF1 α -DNA binding complexes were located within 100kbp of the nearest gene transcription start site (Fig. 2C), indicating a potential role in transcriptional regulation. To determine whether the observed distribution could have occurred by chance, we compared binding patterns of HIF1 α -DNA complexes identified by CUT&RUN on DMOG-treated primary lens cells to a computer-generated random distribution of HIF1 α -DNA binding complexes across the genome. We found that the 8,375 HIF1 α DNA binding sites clustered closer to transcription start sites of genes compared to the computer-generated random distribution model of HIF1 α DNA binding (χ^2 test $p < 2. \times 10^{-16}$, **Fig. 2D**). Specifically, HIF1 α -DNA binding complexes were clustered more within 10kb of transcription start sites and clustered less at ranges greater than 10kb compared to the computer-generated model of randomly distributed HIF1 α -DNA binding complexes. 1,359 HIF1 α -DNA binding complexes (16.2%) were mapped within 3kb of transcription start sites, 3,708 HIF1 α -DNA binding complexes (44.3%) were found to be within gene bodies, and 3,308 HIF1 α -DNA binding complexes (39.5%) were mapped to intergenic regions greater than 3kb away from transcription start sites of genes (**Fig. 2E**). HIF1 α -DNA binding complexes were also found to be positively associated with proximal promoter regions (+/-3kbp region around transcription start sites) and negatively associated with gene body regions relative to the computer-generated random distribution of HIF1 α -DNA binding complexes ($p < 1 \times 10^{-11}$, **Fig. 2F**)).

Active gene regulatory regions often cluster in regions of accessible chromatin comprised of low nucleosome density [36–38]. Notably, our previous study identified the canonical HIF1 α motif as one of the top enriched motifs contained within open chromatin regions identified in the embryonic chick lens genome by ATAC-seq [33]. We therefore compared our HIF1 α DNA binding complexes identified by CUT&RUN to these previously identified open chromatin regions. The analysis revealed 1,190 HIF1 α -DNA binding complexes (14.2%) identified by CUT&RUN that were also contained within genomic regions that exist as open chromatin regions in embryonic chick lens genome (**Table S1**). HIF1 α -DNA binding

complexes were also found to be positively associated with open chromatin regions and negatively associated with closed chromatin regions compared to the computer-generated random distribution of HIF1 α -DNA binding complexes (χ^2 test $p < 1 \times 10^{-55}$). We also sought to determine if the distance between the HIF1 α -DNA binding complex and the transcription start sites of genes was significantly associated with binding to open chromatin regions. This analysis found that HIF1 α -DNA binding complexes were positively associated with open chromatin regions and negatively associated with closed chromatin regions, irrespective of distance from the transcription start site compared to the computer-generated random distribution of HIF1 α -DNA binding complexes (**Fig. 3A**). These data suggest that HIF1 α binding to regions near transcription start sites in the genome is biologically specific.

Such proximity to promoters and enrichment of binding within gene bodies and open chromatin regions, indicated with high probability that HIF1 α is likely to be a direct transcriptional regulator of a large proportion of bound genes. Consistently, CUT&RUN identified the HIF1 α -DNA binding complex previously discovered by ChIP-qPCR in the 5'-proximal upstream region of *BNIP3L* (**Fig. 3B**) [23] that also overlaps with open chromatin regions previously identified by ATACseq of embryonic chick lenses [33]. Additional visualizations of HIF1 α CUT&RUN signal tracks and open chromatin regions in the Galgal6 genome can be found at the UCSC genome browser link (https://genome.ucsc.edu/s/jdisatha/CUTRUN_Lens_HIF1A).

HIF1 α binding induces expression of numerous genes in lens cells

To determine the range and spectrum of gene transcriptional events corresponding with HIF1 α DNA binding occupancy in lens cells, we performed RNAseq on primary lens cells under the same conditions performed for the CUT&RUN analysis. This activation of HIF1 α resulted in upregulation of 219 genes and downregulation of 309 genes (log2FC at least 0.60 in either direction at $p < 0.05$) (**Fig. 4A, 4D, Table S2**). To determine if there was a direct correlation between HIF1 α binding and changes in gene expression, each HIF1 α DNA-binding loci was mapped to the transcription start site (TSS) of nearest neighbor genes. This analysis revealed that upon HIF1 α activation, 40% (212 of 528) of differentially expressed genes were bound by HIF1 α ($p < 0.001$ χ^2 test compared to genes not regulated by DMOG) (**Fig. 4B**). We also sought to determine whether this significant association was specifically dependent on gene induction or repression. This analysis found that upon HIF1 α activation, both 46.1% (101 of 219) of upregulated genes and 36.2% (111 of 309) of downregulated genes were bound by HIF1 α ($p < 0.001$ and < 0.01 , respectively by χ^2 test relative to unresponsive genes) (**Fig. 4E, 4G**). Additionally, genes exhibiting HIF1 α -DNA binding had greater magnitude fold changes (abs(log2FC), $p < 1 \times 10^{-7}$ Mann-U Whitney test) compared to genes not bound by HIF1 α . Taken together, these data indicated a significant role for HIF1 α binding in lens cell gene regulation.

We next sought to determine whether the distance between the HIF1 α -DNA binding complex and the TSS of genes was correlated with gene induction or repression in HIF1 α -activated primary lens cells. A significantly higher percentage of differentially expressed genes were bound by HIF1 α within 3kb (61 of 526 (11.6%), χ^2 test $p < 0.001$), 10kb (55 of 526 (10.5%), χ^2 test $p < 0.001$), and 100kbp (86 of 526 (16.3%),

χ^2 test $p < 0.01$) from the transcription start site compared to non-differentially expressed genes (940 of 16777 (5.6%), 1191 of 16777 (7.1%), 2366 of 16777 (14.1%) respectively) (**Fig. 4C**). This indicated that proximity of HIF1 α binding to the TSS correlates with activation or repression of gene transcription. Consistently, upregulated genes were significantly enriched in HIF1 α -binding within 3kb of the TSS compared to both downregulated and non-responsive genes (Fig. 4F, 4H). Conversely, down-regulated genes were significantly enriched in HIF1 α binding between 3kbp-10kbp away from the TSS relative to non-responsive genes (Fig. 4F, 4H). Thus, genes induced by HIF1 α were more likely to exhibit proximal HIF1 α binding near the TSS and genes repressed by HIF1 α were more likely to be bound by HIF1 α at a greater distance from the TSS (**Fig. 4I**).

Since there was a statistically significant association between altered expression of genes upon HIF1 α activation and HIF1 α binding to proximal regions within 100kbp of the transcription start site (**Fig. 4C**) but not at distances greater than 100kbp of the transcription start site, we defined a cutoff distance of 100kbp to mark genes whose expression levels are at least in part regulated by the activation and binding of HIF1 α to proximal regulatory sites. Thus, 96 of the 219 upregulated genes and 106 of the 307 downregulated genes contain HIF1 α -DNA binding complexes within 100kbp of the transcription start site (**Fig. 4J**).

Collectively, these data establish a significant relationship between HIF1 α -DNA binding complexes to proximal gene regulatory regions and control of specific gene expression by HIF1 α in lens cells. Interestingly, HIF1 α acts as both an activator and suppressor of gene expression in lens cells consistent with the features of lens cell differentiation that include both activation and repression of specific genes [21, 33, 39].

Integrated CUT&RUN and RNAseq analysis reveals a requirement for HIF1 α in the regulation of previously established and novel genetic pathways implicated in lens differentiation, homeostasis, structure and function.

HIF1 α and hypoxia are known to control numerous cellular processes including glycolysis, cell cycle and apoptosis [40–42]. Based on the integrated analysis of HIF1 α -DNA binding complexes identified by CUT&RUN and transcriptional regulation by RNAseq, we defined genes as HIF1 α -dependent targets if they were significantly induced or repressed upon HIF1 α activation and contained at least one HIF1 α -DNA binding complex within 100kbp of the TSS. Employing these criteria, 202 genes were HIF1 α -dependent. Of these genes, 62 were previously established as regulated by hypoxia and/or HIF1 α (**Fig. 5A, Table S3**) in non-lens tissues [43] while 140 genes were novel (**Fig. 5B, Table S3**).

To elucidate the cellular processes and genetic pathways specific for HIF1 α activation in the lens, we used enrichR and MSigDB to identify significantly enriched processes and pathways associated with HIF1 α -activated and repressed genes. Analysis of HIF1 α -dependent activation pathways revealed a wide variety of previously established key lens pathways associated with lens homeostasis and maturation including activation of glycolysis [44], TNF-alpha signaling via NF κ B [45, 46], reactive oxygen species

pathway [26], mTORC1 signaling [27], epithelial-mesenchymal transition [47–50], heme metabolism [51, 52], UV response [44], and apoptotic pathways [53–55] (**Fig. 6A and B, Table S4**). By contrast, analysis of HIF1 α -dependent repressed pathways revealed a wide variety of previously established key lens pathways associated with Notch signaling [56–58], Wnt signaling [59–61], and maintenance of epithelial phenotype [39, 62] (**Fig. 6A and B, Table S4**). The identification of these pathways suggests a novel HIF1 α -dependent regulation of critical lens pathways governing lens homeostasis and maturation.

In addition to these previously established lens pathways a large number of novel pathways without previously established lens functions were also revealed. Of the upregulated genes, the novel enriched pathways included but are not limited to the IL-2/STAT5 signaling pathway while the novel repressed pathways included those involved in adipogenesis, telomerase, KRAS, and FoxO family signaling (**Fig. 6A and B, Table S4**).

In addition to novel pathways a wide-variety of specific HIF1 α -induced genes were also identified including ENO1, PANK4, and BHLHE40. ENO1 is a multifunctional protein that is both a lens structural crystallin and is involved in glucoses metabolism [63, 64] (**Fig. 6C**). PANK4 has been linked to autosomal dominant congenital cataracts [65] (**Fig. 6D**) and BHLHE40 is a transcriptional repressor also involved in NOTCH signaling (**Fig. 6E**). Other HIF1 α -induced genes include ANAPC16 that coordinates lens differentiation through formation of the anaphase-promoting complex [66] and BMP4 that plays a well-established role in lens development [60, 67]. Finally, the chromatin remodeling enzymes HDAC1, KDM3A KDM5A were activated by HIF1 α and their induction could be important for the lens differentiation program (**Table S3**). Future functional analysis of these genes and pathways will likely reveal novel requirements in essential HIF1 α -regulated lens processes and their identification here provides insight into their potential roles in other more complex tissues.

Discussion

A major feature of the developing and adult eye lens is the presence of an oxygen gradient that parallels the naturally occurring surface to core differentiation of immature lens epithelial cells into mature transparent fiber cells [4]. We therefore hypothesized that hypoxia-activation of HIF1 α could regulate lens gene expression levels required to achieve the mature form and function of lens cells. Consistently, a previous study showed disrupted lens structure upon lens-specific deletion of HIF1 α that included dissociation of lens fiber cells, extensive lens vacuolization and disintegration of the entire lens shortly after birth [68]. Consistent with an essential role for HIF1 in lens structure and function, we have established that both hypoxia and activation of HIF1 α are required for the elimination of non-nuclear organelles during lens fiber cell formation through HIF1 α -dependent activation of the mitophagy protein BNIP3L [23].

Although the HIF1 α -dependent lens phenotype is well established, the range and spectrum of lens genetic pathways governed by HIF1 α have not been identified. To identify these pathways and their sub-components, we employed a multiomics approach using CUT&RUN [24, 25] to identify and map the

genomic complement of HIF1 α -DNA binding complexes in the lens, RNA seq to identify the range and spectrum of gene expression changes directly corresponding with specific HIF1 α -DNA binding complexes and ATAC sequencing data to examine the chromatin accessibility of the identified genes [33].

Our analysis identified 8,375 HIF1 α -DNA binding complexes in the lens genome. HIF1 α -DNA binding complexes were more likely to cluster near TSS compared to a computer-generated random distribution of HIF1 α -DNA binding complexes ($p < 2.2 \times 10^{-16}$). 2,576 (30.8%). HIF1 α -DNA binding complexes were mapped to regions within 10kbp of the nearest TSS. Consistent with the specificity of these HIF1 α -DNA binding complexes, the HIF1 α consensus sequence motif (5'-RCGTG-3') was highly enriched in the identified HIF1 α -bound genomic regions ($E < 3.6 \times 10^{-111}$). Collectively, these results suggest an important role for HIF1 α in the regulation of multiple lens pathways and genes. Consistent with HIF1 α playing an important role in lens gene regulation, 1,186 (14.2%) HIF1 α -DNA binding complexes were localized to regions of open chromatin and the HIF1 α consensus sequence motif was enriched in the identified open chromatin regions [33].

CUT&RUN has been shown to outperform traditional ChIP-seq methods in resolution, efficiency, and data quality [24]. Therefore, we chose CUT&RUN as a robust method of detecting and mapping HIF1 α -DNA binding complexes across the chick lens genome. We are confident that our CUT&RUN mapping is accurate since it replicated our previous finding using ChIP-qPCR that HIF1 α binds to a 5' proximal regulatory region of the *Gallus gallus BNIP3L* gene upon HIF1 α activation [23]. Analysis of all HIF1 α -DNA binding complexes indicated an enriched HIF1 α signal and motif analysis of the identified HIF1 α -DNA binding complexes confirmed the HIF1 α binding consensus sequence as the most significantly enriched motif. To our knowledge, this study is the first CUT&RUN analysis of HIF1 α .

Of the genes containing HIF1 α -DNA binding complexes within 100kbp from the TSS 96 genes were transcriptionally activated in association with HIF1 α binding ($\log_2FC > 0.6$, $p < 0.05$) while 106 genes were transcriptionally repressed in association with HIF1 α binding ($\log_2FC < -0.6$, $p < 0.05$). This relationship was statistically significant for both upregulated genes (χ^2 test $p < 0.001$) and downregulated genes (χ^2 test $p < 0.01$). Many of the genes identified to be activated by HIF1 α in lens cells are associated with pathways critical for lens homeostasis, structure and transparency including glycolysis [44], TNF-alpha signaling via NFkB [45, 46], reactive oxygen species pathway [26], mTORC1 signaling [27], epithelial-mesenchymal transition [47–50], heme metabolism [51, 52], UV response [44], and apoptotic pathways [53–55].

A striking feature of the data is that an almost equal number of lens genes are repressed upon HIF1 α binding suggesting that HIF1 α acts as both an activator and a repressor of lens gene expression. Although many previous studies have identified HIF1 α as a transcriptional repressor of individual genes [69–72], to our knowledge this is the first study to identify HIF1 α as a genome-wide repressor [73].

Many of the HIF1 α -repressed genes identified in this study are associated with Wnt signaling, lipid metabolism, and cell adhesion (**Table S3**). These pathways have previously been implicated in lens cell

differentiation and homeostasis [48, 60, 61, 74–78]. This novel finding suggests a role for HIF1 α -dependent repression of these pathways could regulate lens differentiation. Consistently, previous studies have shown that HIF1 α represses specific genes associated with Wnt signaling in skeletal muscle and osteoblasts [79, 80] and other studies have shown that both HIF1 and HIF2 reduce expression of enzymes needed for fatty acid breakdown in lipid metabolism [81].

In addition to establishing HIF1 α as both an activator and a repressor in lens cells, our analysis also identified a novel association between gene regulation and the distance of HIF1 α -DNA binding complexes relative to TSS's of activated or repressed genes. Specifically, HIF1 α binding to regions within 3kbp of gene TSS's is significantly associated with gene activation. Conversely, HIF1 α binding to more distal regions between 3kbp-10kbp of gene TSS's is significantly associated with gene repression. This difference has implications for the mechanisms underlying HIF1 α control of gene expression in the lens and likely a wide-variety of other tissues. It is possible that HIF1 α -DNA binding complexes within 3kbp of TSS's promote open complex formation through interactions with other transcription factors and transcriptional co-activators that might be required for HIF1 α activation including EP300, CBP, and HIF1 β [6, 7, 9]. Conversely, distant HIF1 α -DNA binding complexes more than 3kbp from TSS's may repress open complex formation of gene expression by recruiting these transcriptional regulators away from TSS's. These possibilities are consistent with previous studies linking the action of transcription factors with proximal or distal binding [82, 83]. To our knowledge, the present study is the first to-date to find a statistically significant association between genome wide HIF1 α binding site distances and activation or repression of multiple genes.

Many of the identified HIF1 α -dependent genes also have well established functions critical for lens cell metabolism, structure, transparency, differentiation, and development. An autosomal dominant mutation in the identified HIF1 α -activated PANK4 gene is associated with congenital posterior cataract [65] and is therefore critical for lens transparency. The HIF1 α -activated ENO1 gene (tau crystallin) is both a lens structural protein and a glycolytic enzyme [63]. BNIP3L, BMP4, GATA3, CDKN1B, HES5, JAG1, and VEGFA all play important roles in lens cell development and differentiation (**Table S3**) [23, 56, 60, 67, 84–91]. Therefore, this study suggests that activation of HIF1 α in the lens plays a crucial role in maintaining these important lens-specific functions.

In addition to identifying known HIF1 α -dependent genes, the present analysis also identified 151 novel HIF1 α -dependent genes. These include but are not limited to *JARID2*, *BMP4*, *SIX4*, *GATA3*, *ARID5A*. These genes are involved in metabolism, chromatin remodeling, development and differentiation of a variety of tissues and cell types (**Table S3**). Their identification suggests novel HIF1 α -dependent roles of the genes in lens and other tissues and suggest novel functions for HIF1 α .

Although 202 genes showed a direct relationship between binding of HIF1 α and gene activation or repression, an additional 324 genes exhibited activated or repressed gene expression upon HIF1 α activation in the absence HIF1 α binding. These results suggest that HIF1 α acts to regulate downstream events that control expression of these genes including downstream transcription factors or chromatin

remodeling proteins encoded by *BHLHE40*, *HDAC1*, *JARID2*, *KDM3A*, and *KDM5B* (**Table S3**) detected in the present report and previously established to be regulated by HIF1 α [43]. These downstream factors could activate or repress expression of these genes by altering chromatin accessibility.

4,509 genes that exhibited HIF1 α binding did not exhibit altered expression levels even though they were the nearest neighbor to HIF1 α binding sites extending out to 100kbp away from cognate TSS's. This is not uncommon, as many previous studies have found that not all transcription factor binding sites are associated with active changes in gene expression [83, 92] and it is possible that activation or repression of these genes by HIF1 α is dependent on the presence of additional transcription factors and/or co-activators/co-repressors not present or active under the conditions of the present study. Alternatively, the data could indicate a role for HIF1 α in chromatin structure and/or maintenance and future studies will be needed to address these possibilities.

Conclusions

Collectively, the present report establishes a functional genomic map of novel HIF1 α -regulated genes of the eye lens and supports the hypothesis that HIF1 α activation is critical for achieving mature lens structure and transparent function. They also establish HIF1 α as both an activator and repressor of lens gene expression. The data also reveal novel HIF1 α -DNA complex features, confirm previously established HIF1 α targets and identify novel HIF1 α targets that function in the lens and likely other tissues.

Abbreviations

CUT&RUN: Cleavage Under Targets and Release Under Nuclease

ATACseq: Assay for Transposase-Accessible Chromatin sequencing

HIF1 α : Hypoxia inducible factor 1 alpha

TSS: Transcription start site

ChIP-qPCR: Chromatin immunoprecipitation coupled with quantitative polymerase chain reaction

FPKM: Fragments per kilobase of exon per million fragments mapped

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Raw sequencing reads for the CUT&RUN experiment have been deposited at NCBI-GEO series accession number GSE166626. Raw sequencing reads for the RNAseq experiment have been deposited at NCBI-GEO series accession number GSE166632.

ATACseq data were obtained from supplemental files from [32].

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

JD Conceived of the studies, performed experiments, analyzed data and participated in writing the manuscript as part of his requirements for graduating from the Florida Atlantic University Integrative Biology-Biomedical Science PhD program; LB Conceived of the studies, performed experiments, analyzed data, participated in writing the manuscript and funding acquisition; DC analyzed data and participated in writing the manuscript; JK analyzed data and participated in writing the manuscript; BA analyzed data and participated in writing the manuscript; MK Conceived of the studies, analyzed data, participated in writing the manuscript, funding acquisition and directed the work.

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Figures

Figure 1

Multiomic identification of functional HIF1α DNA binding targets in the lens genome (A) Duplicate pools of 500,000 primary chick lens cells were cultured with or without the HIF1α activator DMOG (1mM) for 4 hours. Samples were analyzed by CUT&RUN and RNAseq analysis to identify genome-wide HIF1α-DNA binding complexes and corresponding HIF1α-dependent gene expression changes. Results were compared with ATACseq data [33] to identify the chromatin accessibility of identified HIF1α-DNA binding complexes. Integrated bioinformatics analysis was used to identify the pathways and functions specific for identified HIF1α-targets. (B) HIF1α is rapidly degraded under normoxic conditions but the presence of DMOG, inhibits prolyl hydroxylase resulting in HIF1α stabilization, DNA binding and transcriptional modulation in lens cells. (C-D) RT-PCR showing increased levels of the BNIP3L control transcript relative to β-actin in lens cells incubated in the presence of 1mM DMOG for 4 hours. *p < 0.05 t-test n = 3.

Figure 2

The spectrum and specificity of HIF1α-DNA binding complexes of the lens genome. (A) Heatmap of HIF1α binding signal compared to IgG signal at all genomic regions containing HIF1α-DNA binding complexes identified by CUT&RUN. (B) De novo motif analysis of HIF1α-DNA binding complexes identified by CUT&RUN. The canonical HIF1α consensus sequence was the most significantly enriched motif (E-value 3.6×10^{-111}). (C) Percent of the 8,375 HIF1α-DNA binding complexes identified by CUT&RUN that were found within the indicated distances from the nearest gene transcription start site. (D) Relative percentage difference between the distribution of HIF1α-DNA binding complexes identified by CUT&RUN compared to computer generated HIF1α-DNA binding complexes randomly distributed across the genome and sorted by distance to the nearest gene transcription start site. (X2-test, $p < 2.2 \times 10^{-16}$). (E) Percent of the 8,375 HIF1α-DNA binding complexes identified by CUT&RUN that were found within proximal promoter regions (+/- 3kbp of gene transcription start sites), genebodies, or intergenic regions. (F) Same as (D) but HIF1α-DNA binding complexes are categorized by genomic loci within proximal promoter regions (+/- 3kbp of gene transcription start sites), genebodies, or intergenic regions. (X2-test, $p < 1 \times 10^{-11}$)

Figure 3

HIF1α-DNA binding complexes identified by CUT&RUN cluster in open chromatin regions (A) HIF1α-DNA binding complexes were separated into those bound in open chromatin regions and those bound in closed chromatin regions and then sorted into distances away from the transcription start site (0-3kbp, 3-10kbp, 10-100kbp, over 100kbp). The results were compared to what is expected from the computer generated randomly distributed HIF1α-DNA binding complexes. HIF1α-DNA binding complexes were positively associated with open chromatin regions and negatively associated with closed chromatin regions at 0-3kbp, 3-10kbp, 10-100kbp, and over 100kbp distances away from the transcription start site ($***p < 1 \times 10^{-6}$) compared to the computer-generated random distribution of HIF1α-DNA binding complexes. (B) CUT&RUN tracks of HIF1α (red) and IgG negative control (black) within the genomic region of Chr22 encompassing the *Gallus gallus* gene *BNIP3L*. The vertical light blue highlight and light blue box indicates a HIF1α-DNA binding complex in the proximal promoter of the gene. The orange track shows the combined chromatin accessibility map representing a combination of the differentiation stage-specific chromatin accessibility tracks obtained from [33] indicating regions of open chromatin in whole chick lens tissue.

Figure 4

HIF1 α binds to and regulates the expression of over 200 lens genes: (A) Differential gene expression analysis by RNAseq reveals 219 upregulated genes (log₂ fold change FPKM > 0.6, p < 0.05) and 307 downregulated genes (log₂ fold change FPKM < -0.6, p < 0.05) in primary lens cells treated with the HIF1 α activator DMOG (1mM) relative to control untreated cells. (B) Percent of differentially expressed genes (DEG) (|log₂ fold change FPKM| > 0.6, p < 0.05) and non-differentially expressed genes (NON-DEG) (p >

0.05) that have a HIF1 α -DNA binding complex. Differentially expressed genes are more significantly associated with HIF1 α binding compared to non-differentially expressed genes (X2-test, ***p < 0.001). (C) Same analysis as (B) but separated by distance between the transcription start site (TSS) and the HIF1 α -DNA binding complex. (X2-tests, ***p < 0.001, **p < 0.01, ns p > 0.05). (D) Heatmap of the top 100 differentially expressed genes sorted by hierarchical clustering. (E) Percent of upregulated genes, downregulated genes and NON-DEG that have a HIF1 α -DNA binding complex. Upregulated and downregulated genes are significantly associated with HIF1 α -DNA binding complexes. (X2-test, ***p < 0.001, **p < 0.01). (F) Same analysis as (E) but separated by distance between the transcription start site (TSS) and the HIF1 α -DNA binding complex. (X2-test, ***p < 0.001). (G) Heatmap of differentially expressed (DEGs) and non-differentially expressed (Non-DEGs) genes with a HIF1 α -DNA binding complex (DEGs). (H) Same as (G) but separated by distance from the transcription start site (0-3kbp, 3-10kbp, 10-100kbp, and over 100kbp). (I) HIF1 α CUT&RUN coupled to RNAseq analysis elucidates a significant relationship between HIF1 α binding within 3kbp of the TSS and gene upregulation. In contrast, HIF1 α binding between 3-10kbp of the TSS is significantly associated with gene downregulation. (J) Overlap of number of genes that are significantly upregulated or significantly downregulated and genes with HIF1 α -DNA binding complexes within 100kbp of the transcription start site.

Figure 5

Integrated CUT&RUN and RNAseq analysis identifies previously established and novel HIF1 α -dependent lens targets (A) Heatmap of significantly upregulated ($\log_2FC > 0.7$, $p < 0.05$) and downregulated ($\log_2FC < -0.7$, $p < 0.05$) genes with a HIF1 α -DNA binding complex that are known to be regulated by hypoxia from [43] (B) Heatmap of significantly upregulated ($\log_2FC > 0.7$, $p < 0.05$) and downregulated ($\log_2FC < -0.7$, p

< 0.05) genes with a HIF1α-DNA binding complex that are not previously known to be regulated by hypoxia from [43]

Figure 6

CUT&RUN and RNAseq analysis identifies HIF1α-dependent pathways critical for lens structure, homeostasis, metabolism, and differentiation: (A) Genes with a HIF1α-DNA binding complex that are significantly downregulated ($\log_2FC < -0.7$, $p < 0.05$) were input into enrichR to elucidate significantly

associated biological pathways and processes. The top 50 most statistically significant non-redundant pathways are shown. (B) Genes with a HIF1 α -DNA binding complex that are significantly upregulated ($\log_2FC > 0.7$, $p < 0.05$) were input into enrichR to elucidate significantly associated biological pathways and processes. The top 50 most statistically significant non-redundant pathways are shown. (C-E) CUT&RUN tracks of HIF1 α (red) and IgG negative control (black) within the genomic region encompassing the *Gallus gallus* genes ENO1 (C), PANK4 (D), and BHLHE40 (E). The vertical light blue highlight and light blue box indicates a HIF1 α -DNA binding complex in the proximal promoter of the gene. The orange track shows the combined chromatin accessibility map representing a combination of the differentiation stage-specific chromatin accessibility tracks obtained from [33] indicating regions of open chromatin in whole chick lens tissue.

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- [TableS3HIF1aDependentGenesSummary.xlsx](#)
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