Acute Targeting of General Transcription Factor IIB Restricts Cardiac Hypertrophy via Selective Inhibition of Gene Transcription

Danish Sayed, MD, PhD; Zhi Yang, PhD; Minzhen He, MD; Jessica M. Pfleger; Maha Abdellatif, MBChB, PhD

Background—We previously reported that specialized and housekeeping genes are differentially regulated via de novo recruitment and pause-release of RNA polymerase II, respectively, during cardiac hypertrophy. However, the significance of this finding remains to be examined. Therefore, the purpose of this study was to determine the mechanisms that differentially regulate these gene groups and exploit them for therapeutic targeting.

Methods and Results—Here, we show that general transcription factor IIB (TFIIB) and cyclin-dependent kinase 9 are upregulated during hypertrophy, both targeted by microRNA-1, and play preferential roles in regulating those 2 groups of genes. Chromatin immunoprecipitation-sequencing reveals that TFIIB is constitutively bound to all paused, housekeeping, promoters, whereas de novo recruitment of TFIIB and polymerase II is required for specialized genes that are induced during hypertrophy. We exploited this dichotomy to acutely inhibit induction of the latter set, which encompasses cardiomyopathy, immune reaction, and extracellular matrix genes, using locked nucleic acid–modified antisense TFIIB oligonucleotide treatment. This resulted in suppression of all specialized genes, while sparing the housekeeping ones, and, thus, attenuated pathological hypertrophy.

Conclusions—The data for the first time reveal distinct general TFIIB dynamics that regulate specialized versus housekeeping genes during cardiac hypertrophy. Thus, by acutely targeting TFIIB, we were able to inhibit selectively the former set of genes and ameliorate pressure overload hypertrophy. We also demonstrate the feasibility of acutely and reversibly targeting cardiac mRNA for therapeutic purposes using locked nucleic acid–modified antisense oligonucleotides. (Circ Heart Fail. 2015;8:138-148. DOI: 10.1161/CIRCHEARTFAILURE.114.001660.)

Key Words: general transcription factors ■ microRNA ■ posttranscriptional gene silencing ■ transcription factor IIB

Regulation of gene expression is fundamental to organogenesis and pathogenesis, including cardiac hypertrophy. During hypertrophy, there are 2 distinct transcriptional events that occur. One, there is a generalized increase in total RNA synthesis that underlies the 30% to 50% increase in cell volume.1–3 Two, superimposed on this, there is a more substantial increase in the expression of specific genes, such as atrial natriuretic factor (Nppa) and α-skeletal actin (Acta1).4,5 The latter mode of transcription has been studied in more detail as reports show that it involves regulation by specific transcription factors and enhancers.6,7 This ultimately requires transcription factor IIB (TFIIB) for the recruitment of polymerase II (pol II), promoter assembly of the preinitiation complex, and transcriptional activation.8 Subsequently, phosphorylation of pol II’s C-terminal domain by cyclin-dependent kinase 9 (Cdk9) enhances promoter-proximal clearance of any paused pol II.9

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We have recently reported the detection of 2 mutually exclusive modes of transcriptional regulation during cardiac hypertrophy.10 One involves an incremental increase (30%–50%) in the elongational activity of preassembled, promoter-paused, pol II that involved ≈25% of expressed genes, which are predominantly essential/housekeeping genes (eg, RNA synthesis and splicing). Another involved a more robust activation, via de novo pol II recruitment, encompassing ≈4% of specialized genes (eg, contractile, immune reaction, and extracellular matrix genes). These results were the first to demonstrate that promoter-paused pol II plays a key role in incrementally and synchronously increasing housekeeping genes, proportionate to the increase in heart size. Although we know that recruitment of pol II to any promoter requires TFIIB, it is still unclear how TFIIB is regulated, whether it remains attached to pol II-paused promoters, and if the release of paused pol II is dependent on TFIIB availability. To dissect further the transcriptional regulation during cardiac hypertrophy, we performed genome-wide TFIIB chromatin immunoprecipitation-deep sequencing (ChIP-Seq). In this study, we describe the dichotomy in TFIIB dynamics between housekeeping, pol II-paused, genes, and hypertrophy-induced

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genes, and how it can be exploited to selectively and dose dependently inhibit the pathological hypertrophy-induced genes, which include cardiomyopathy, extracellular matrix, and immune reaction genes, in the heart.

**Methods**

**Animals**

C57Bl/6 mice and Sprague Dawley rats were used in this study in accordance with US National Institute of Health Guidelines for the Care and Use of Laboratory Animals (No. 85-23). All animal protocols were approved by the Institutional Animal Care and Use Committee at the Rutgers-New Jersey Medical School.

**Cell Cultures and Treatments**

Cardiac myocytes were prepared as previously described. Briefly, hearts were isolated from 1 to 2 day old Sprague-Dawley rats. After dissociation, the cells were subjected to Percoll gradient centrifugation followed by differential preplating to enrich for cardiac myocytes and deplete nonmyocytes. Myocytes were then plated in DMEM-F12 with 10% fetal bovine serum without antibiotics. Twenty-four hours after plating, the medium was changed, and the cells were infected with recombinant adenoviruses at a multiplicity of infection of 10 to 20 particles/cell.

**Construction of Adenoviruses**

Recombinant adenoviruses were constructed, propagated, and titrated, as previously described by Dr Frank Graham. Briefly, pBHGloxΔE1.3Cre (Microbix), including the ΔE1 adenoviral genome, is cotransfected with the pDC shuttle vector containing the gene of interest, into 293 cells using Lipofectamine (Invitrogen). Through homologous recombination, the test genes integrate into the E1-deleted adenoviral genome. The viruses were propagated on 293 cells and purified using CsCl2 banding followed by dialysis against 20 mmol/L Tris buffered saline with 2% glycerol. Titering is performed on 293 cells overlaid with DMEM plus 5% equine serum and 0.5% agarose.

**DNA Constructs Cloned Into Recombinant Adenovirus**

MicroRNA 1 (MiR-1): the stem-loop of miR-1 to 2 was cloned into recombinant adenovirus under control of cytomegalovirus (CMV) promoter. MiR-1 eraser (anti-miR-1): 2 antisense repeats of the mature miR-1 sequence was synthesized as a double stranded oligonucleotide and cloned into recombinant adenovirus under the control of a U6 promoter. MiR-133a-1: the stem-loop of miR-133a-1 was cloned into recombinant adenovirus under control of CMV and U6 promoter. MiR-SC (scrambled control): the stem-loop expressing a scrambled microRNA (GAACCGAGCCCACCAGCGAGC) was cloned into recombinant adenovirus under the control of CMV and used as control for all adenoviral microRNA constructs. TFIIB accession no. NM_001261.2 (Origene) human CDK9 (Origene) and MSCV-internal ribosomal entry sequence-Luciferase plasmid was purchased from Addgene (plasmid 18760). The plasmid was digested with EcoRI/SalI and the fragment containing internal ribosomal entry sequence-Luciferase sequence was cloned into 3′ region of pDC316-GFP. The construct was cloned into adenovirus.

**Transverse Aortic Constriction**

Twelve-week-old C57Bl/6 mice are anesthetized intraperitoneally with a mixture of ketamine (65 mg/kg), xylazine (13 mg/kg), and acepromazine (2 mg/kg). The adequacy of the anesthetic is confirmed by the loss of tongue retraction reflex. The transverse thoracic aorta between the innominate artery and the left common carotid artery was dissected free, and a 7-0 braided polyester suture was tied around the aorta against a 28-gauge needle with the aid of an operating microscope. The needle was removed, the chest closed, and the mice were extubated and allowed to recover in a Thermocare unit (temperature 88°F or 31°C; humidity 30%–50%; oxygen 1–2 mL/min, low flow range). Postoperative Buprenorphine (0.01–0.05 mg/kg) was administered subcutaneously every 12 hours, as needed. The sham operation involved the same procedure, except the aorta was not constricted.

**ChIP-Seq of Sham, Transverse Aortic Constriction–Induced Hypertrophy and Neonatal Hearts**

The hearts were isolated from 12 to 13 weeks old C57Bl/6 mice 4 days after transverse aortic constriction (TAC, n=3) or a sham operation (n=2), or from normal mice (n=1), and from 2 litters of 1-day-old C57Bl/6 neonate pups. The hearts from the TAC, sham, normal, and neonatal hearts were pooled and subjected to GFP-MPl (Active Motif, Inc.). Immunoprecipitation was performed using anti-GFP antibody (Santa Cruz Biotechnology, sc-225), followed by high throughput Illumina sequencing using Illumina HiSeq. Sequencing of Input DNA taken before immunoprecipitation served as control for normalization and eliminating background noise.

**ChIP-Seq Analysis (Active Motif Inc)**

**Sequence Analysis**

Fifty nucleotides of the sequencing reads (tags, >10 million) with no more than 2 mismatches were aligned to the genome using BWA (Burrows-Wheeler Aligner) algorithm.

**Determination of Fragment Density**

Tags are extended (150–250 bp) at their 3′ end in silico. The genome is divided into 32-n bins and the density of the fragments (extended Tags) in each bin is determined. The results of this is stored in a binary analysis results file.

**Peak Finding (Intervals)**

Intervals are defined genomic regions that represent the fragment density peaks. It must have 3 consecutive bins with fragment densities greater than the threshold (usually 10–20).

**Active Regions**

For the purpose of comparison between samples, regions with overlapping intervals are grouped into active regions.

**Annotation**

The locations and proximities to gene annotations of intervals and active regions are defined and compiled in Excel spreadsheets, which include average and peak fragment densities.

**ChIP-Seq of Cultured Neonatal Myocytes**

Rat neonatal cardiac myocytes were cultured as described above. Sixteen hours after plating, the cells were treated with recombinant adenoviruses expressing with the precursor of miR-1 or a scrambled control for 24 hours. The myocytes were the fixed in freshly prepared 1% formaldehyde solution (11% of 37% formaldehyde, 0.1 mol/L NaCl, 1 mmol/L EDTA pH 8, 50 mmol/L HEPES pH 7.9) for 15 minutes at room temperature. The reaction was stopped with 1/20 volume of 2.5 mol/L Glycine (5 minutes at room temperature), the cells collected and washed with 10 mL of chilled PBS-Igepal solution (1X PBS, 0.5% Igepal), pelleted (800g), and resuspended in PBS-Igepal plus 1 mmol/L PMSF (phenylmethylsulfonyl fluoride). The DNA was analyzed by anti-pol II ChIP-Seq as described above (Active Motif Inc).

**Normalization of the ChIP-Seq Data (Active Motif Inc)**

**Tag Normalization**

The tag number of all samples is reduced (by random sampling) to the number of tags present in the smallest sample
Input File Analysis
The signal map of the Input/IgG control file is essentially analyzed as an additional sample. In this case, the strongest Input/IgG control peaks (which must represent false positives) are determined by simple thresholding of the binary analysis results file, and only Input/IgG control peaks that overlap with Intervals in the ChIP/IP data are used in the analysis. By doing so, the output Active Region table (see Section IV.4, below) will show for each region the corresponding fragment density in the Input/IgG control sample, thus allowing for the identification of possible false-positive ChIP/IP peaks.

Quantitative Polymerase Chain Reaction
Total RNA was reversed transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit or TaqMan MicroRNA Reverse Transcription Kit for microRNAs (applied Biosciences) as per manufacturers protocol. Quantitative polymerase chain reaction was performed using TaqMan gene expression assays (primer/probe sets) on Applied Biosystems 7500 Real-Time PCR system for the following genes; 18s (Mm03928990_g1), GAPDH (Mm99999915_g1), CDK9 (Mm01731275_m1), TFIIB primer 1 (exon boundary 3–4, assay location 283) (Mm01323559_g1), TFIIB primer 2 (exon boundary 5–6, assay location 566) (Mm03047554_g1), ANF (Mm01255747_g1), ANKR1D1 (Mm01204056_g1), VDAC1 (Mm00834272_m1), Pparg1a (Mm01208835_m1), Pnm (Mm00447098_m1), Calfm1 (Mm01336281_g1), Mapk1 (Mm00442479_m1), Acta1 (Mm0080218_g1), Sec23a (Mm00846004_m1), Trappcb6 (Mm01605498_g1), Col1al1 (Mm00801666_g1), Alb (Mm00802090_m1), Akt (Mm01331626_m1), U6 (001973), miR-1 (002222) and miR-21 (000397).

In Vivo Locked Nucleic Acid Anti-TFIIB Oligo Injections
In vivo locked nucleic acid (LNA)–modified anti-TFIIB and scrambled oligonucleotides were purchased from Exiqon. The antisense TFIIB oligo is a 16mer complementary to bp 567 to 582 (NM_145546), 5′- +A+ + T+ + T+ + C+ + G+ + A+ + G+ + A+ + C+ + A+ + C+ + A+ + G+ + C+ + C+ + A+ + C+ + C+ + A+ + C+ + A+ + C+ + C+ + A+ + C+ + C+ + C+ + A+ + C+ + C+ + A+ + C+ + C+ + A+ + C+ + C+ + A+ + C+ + C+ - phosphorothioate bond, + = LNA-modified. Mice (C57/blk, 12-week-old male, 24–26 gm) were subjected to TAC or sham operations, 1 day post-TAC mice were randomly divided into 2 groups, one group received in vivo LNA anti-TFIIB oligo (15 mg/kg, bolus, intravenous via tail-vein), while the other received in vivo LNA Scrambled oligo in similar doses and route. After the indicated time periods, the mice underwent echocardiography to measure cardiac function after which were saged, and heart and liver extracted for analysis.

Western Blotting
Ten microgram of protein samples, each, were subjected to electrophoresis on 4% to 15% SDS-PAGE (Criteron gels; Bio-Rad). The antibodies used for Western blot analysis included anti-ANKRD1 (Santa Cruz), anti-CDK9 (Santa Cruz), anti-Myosin, slow (Sigma), anti-TFIIB (Millipore), anti-GAPDH (Chemicon), anti-VDAC1 (Genscript), Anti-GFP (BD Biosciences), Anti-Luciferase (Novus Biologicals), anti-Nelf-A (Santa Cruz). The signal was detected and quantified by the Odyssey Imaging System (LI-COR).

Immunohistochemistry
Hearts extracted were immediately fixed in 10% neutral buffered formalin for 24 hours before paraffin embedding and sectioning (6 μm). Hematoxylin and acid Sirius red staining was performed to measure Interstitial Fibrosis, for cell size the sections were stained with Wheat germ agglutinin.

Statistics
Calculation of significance between 2 groups was performed using an unpaired, 2-tailed Student t test (excel software). P<0.05 was considered significant.

Results
TFIIB and Cdk9 Are Posttranscriptionally Upregulated During Cardiac Growth
The mechanisms underlying the incremental increase in gene expression that parallels the increase in cardiac myocyte size and mass and its distinction from robust increases in the expression of specific genes during cardiac hypertrophy are not fully elucidated. We have recently reported that these 2 distinct transcriptional changes are regulated by the incremental release of promoter-paused RNA pol II and de novo recruitment of pol II, respectively.10 TFIIB is a key limiting protein that is required for the recruitment of RNA pol II to promoters, while Cdk9 is a component of the positive transcription elongation factor b that enhances promoter clearance of this polymerase and has been shown to play a key role in hypertrophic growth.13 We predicted that, in addition to Cdk9, TFIIB plays a major role in regulating transcription during hypertrophy via variations in its protein levels. Therefore, we determined the expression pattern of both genes by measuring both the mRNA and protein levels of these genes in 1-day-old neonatal and 12-week-old adult hearts before and after TAC. The results show that the mRNA levels of both genes were marginally increased in the TAC hearts, whereas in the neonatal heart they were 2- to 3-folds higher (Figure 1A). However, the proteins of both genes were 2- to 3-fold higher in both the neonatal and TAC hearts (Figure 1B and 1C). Thus, both TFIIB and Cdk9 proteins are higher during physiological and pathological cardiac growth.

To determine whether these increases were a result of transcriptional versus posttranscriptional mechanisms, we explored the data set generated by our anti-pol II and antihistone H3 K9-acetyl (H3K9ac) ChIP-Seq analysis in the neonatal, adult, and hypertrophied hearts (accession: GSE50637). Pol II and H3K9ac density distributions for the TFIIB gene (Gggf2b) reveal reduced transcription start site (TSS) pol II pausing and increased H3K9ac in the growing hearts, with a modest (1.25- and 1.8-fold) increase in in-gene pol II density in the TAC and neonatal versus adult heart, respectively (Figure 1D and 1E). Although Cdk9 exhibited a similar pattern in the TAC heart, both pol II and in-gene H3K9ac densities appeared to be reduced in the neonatal heart, signifying reduced transcription (Figure 1D and 1E). These data, compared with the increase in the mRNA and protein levels, support a role for both enhanced promoter clearance of pol II (ie, reduced pausing) and potentially posttranscriptional regulation of TFIIB and Cdk9 during cardiac hypertrophy.

MiR-1 Targets TFIIB and Cdk9 mRNA
We then examined whether TFIIB and Cdk9 are predicted targets of any microRNA. Noteworthy, we found that both TargetScan14,16 and PicTar17,18 microRNA target prediction software, predict miR-1 as the only, broadly conserved among vertebrates, targeting microRNA of both TFIIB and Cdk9 genes (Figure 2A). In concordance with a role in regulating these genes during hypertrophic growth, mature miR-1 is significantly higher in the adult versus neonatal heart and is downregulated within 24 hours of TAC.19 To validate these targets, we overexpressed pre-miR-1 in cardiac myocytes before adding a CMV promoter-driven green florescence protein

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(GFP)-internal ribosomal entry sequence-luciferase (CMV-GFP-Luc) reporter vector used to monitor basic transcription from a constitutive promoter (no miR-1 targeting site is included in this vector). As seen in Figure 2B and quantified in 2D, both TFIIB and Cdk9 proteins were reduced by exogenous miR-1 but not by miR-133, and, conversely, both were increased by an anti-miR-1 eraser (Figure 2B and 2D). The change in TFIIB and Cdk9 expression was paralleled by a similar change in the expression of the GFP and luciferase reporter genes. A time course analysis, in which miR-1 and CMV-GFP-Luc vectors were added simultaneously, revealed that the downregulation of endogenous TFIIB and Cdk9 preceded the downregulation of reporter genes (Figure 2C and 2E). These results suggest that miR-1 has an effect on basal (constitutive) gene expression, plausibly, through inhibition of TFIIB and Cdk9.

To determine whether the predicted miR-1 target site within the 3′ untranslated regions of Cdk9 or TFIIB affects their translation levels, we engineered expression vectors of TFIIB and Cdk9 cDNA that either encompassed (full-length) or lacked (∆miR-1) the miR-1 target site within the 3′ untranslated region. These vectors were delivered to myocytes cultured in serum-free conditions that promote higher miR-1 expression (Figure I in the Data Supplement); no exogenous miR-1 was added. A dose response shows that the constructs lacking miR-1 recognition site expressed ≈2.5-fold higher protein levels (Figure 2F and 2G).

This difference diminished with increasing concentrations of the constructs, plausibly because of a sponging effect. The data suggest that miR-1 directly suppresses TFIIB and Cdk9 and as a consequence also inhibits constitutive gene expression.

MiR-1 is downregulated during cardiac hypertrophy. Therefore, it remained necessary to determine the relevance of this reduction on the levels of TFIIB and Cdk9 in vivo. The results show that intravenous delivery of LNA-modified anti-sense miR-1 oligonucleotides resulted in ≈70% knockdown of endogenous miR-1 in the heart accompanied by an increase in both TFIIB and Cdk9 proteins within one week (Figure 2H and 2I), confirming that downregulation of miR-1 is indeed sufficient for increasing the expression of these targets. This, however, was not sufficient for inducing fulminant hypertrophy, although, it did induce significant increases in miR-21, ANF, and Ankrd1, albeit to a much lower extent than observed with TAC (Figure 2H and 2I). This shows that downregulation of miR-1 is sufficient for inducing upregulation of Cdk9 and TFIIB, which, however, is not sufficient for inducing cardiac hypertrophy.

TFIIB and pol II Are Constitutively Bound to Paused Promoters But Recruited De Novo to Hypertrophy-Induced Promoters, Which Inhibited by miR-1

Because miR-1 proved to be a direct inhibitor of TFIIB and Cdk9, we predicted that it would dampen general transcription

Figure 1. Transcription factor IIB (TFIIB) and cyclin-dependent kinase 9 (Cdk9) are posttranscriptionally upregulated during cardiac growth. A, RNA was extracted from the neonatal, adult, and transverse aortic constriction (TAC) hearts and subjected to quantitative polymerase chain reaction for the indicated genes. The results were averaged and plotted as relative mRNA change (n=3). Error bars represent SEM and * is P<0.05 vs sham-operated or normal heart. B, Protein was extracted from the same heart tissue as in (A) and analyzed by Western blots for the indicated antibodies. C, The Western blot signals for the different proteins were quantified, averaged, and plotted. Error bars represent SEM and * is P<0.05 vs sham-operated or normal adult (n=3, 2/3 samples shown in the blot). D, Integrated Genome Browser images showing the RNA pol II density (y axis) aligned across the Gtf2b (TFIIB) and Cdk9 genes (x axis) in the neonatal, adult, and TAC hearts. Arrows positioned at the gene’s start site point to direction of transcription. E, Pol II (P) and H3K9ac (A) densities for the TFIIB and Cdk9 genes were plotted as the Log2 values of TAC/adult (T/A) and neonatal/adult (N/A) of the pol II in promoter proximal (PrP, −300 to +300), the in-gene (IP, +300 to end), and the downstream (DP, end to +5000), and of the H3K9ac in promoter proximal (PrA, −1000 to +1000), the in-gene (IA, +1000 to end) region, were plotted.
by inhibiting pol II recruitment and induce its pausing. To test this, we supplied cultured neonatal rat myocytes with exogenous miR-1 for 24 hours before extracting the DNA for pol II ChIP-Seq analysis. The results show 2 major patterns of miR-1–induced changes in pol II densities. In one group, the average pol II density increased with miR-1, while the number of bins (32 nt) was reduced and total pol II density (average density × bins) remained unchanged (Figure 3A). The aligned sequence data of representative genes (Ncl and Calm1) are displayed in Figure 3B. As reflected in those graphs, pol II peaks were generally higher, in particular at the TSS, reflecting an increase in pol II pausing. Importantly, total bound pol II was unchanged, suggesting that pol II recruitment is unaffected in this case. This group includes 72% of genes that are enriched in housekeeping (eg, ribosomal and proteasomal) and signaling pathway genes (Database for Annotation, Visualization and Integrated Discovery [DAVID] v6.7; Table 1).20–22 Notably, we have previously reported that a corresponding set of genes exhibited promoter-proximal pol II pausing in the adult versus neonatal heart that was incrementally released during hypertrophy and was, likewise, independent of an increase in de novo pol II recruitment (Figure 3C).10 The second major group of genes that is regulated by miR-1 is one that characterized by reduced average density, bin numbers, and total pol II density (average density × bins) remained unchanged (Figure 3A). The aligned sequence data of representative genes (Ncl and Calm1) are displayed in Figure 3B. As reflected in those graphs, pol II peaks were generally higher, in particular at the TSS, reflecting an increase in pol II pausing. Importantly, total bound pol II was unchanged, suggesting that pol II recruitment is unaffected in this case. This group includes 72% of genes that are enriched in housekeeping (eg, ribosomal and proteasomal) and signaling pathway genes (Database for Annotation, Visualization and Integrated Discovery [DAVID] v6.7; Table 1).20–22 Notably, we have previously reported that a corresponding set of genes exhibited promoter-proximal pol II pausing in the adult versus neonatal heart that was incrementally released during hypertrophy and was, likewise, independent of an increase in de novo pol II recruitment (Figure 3C).10 The second major group of genes that is regulated by miR-1 is one that characterized by reduced average density, bin numbers, and total pol II densities, reflecting reduced pol II recruitment to genes (Figure 3E). Shown, as examples of this group, are Ankrd1, Nppa, and Nppb genes, exhibiting reduced pol II density across the gene, however, pol II promoter clearance and advancement were not impacted (Figure 3F). This group includes 1.6% of genes that encompass those that are involved in hypertrophic and dilated cardiomyopathy pathways (DAVID v6.7; Table 2).20–22 These represent a similar set of genes that are induced during hypertrophy by de novo recruitment of pol II and does not exhibit promoter-proximal pol II pausing (Figure 3G).10

TFIIB is required for the recruitment of pol II to all promoters. However, whether TFIIB remains bound to pol II–paused promoters, or whether it is released with the release of pausing, remains unknown. Therefore, to determine the dynamics of TFIIB on active promoters, we performed a TFIIB
ChIP-Seq analysis on neonatal, adult, and TAC-induced hearts and aligned the results with our previous pol II and H3K9ac data sets (Figure 3C and 3G). The results show that TFIIB is constitutively bound to promoters that have paused pol II, and undergoes incremental changes (eg, Ncl and Calm1) in inverse relation with changes in pol II pausing, as total gene pol II density remains unchanged (Figure 3D). This was in stark contrast to genes requiring de novo pol II recruitment, which were associated with an equivalent 5- to 15-fold increase in TFIIB recruitment (Figure 3G and 3H). The data confirm that all expressed promoters require TFIIB, albeit with varying binding dynamics - constitutive versus induced.
Table 1. Genes That Exhibit pol II Pausing in the miR-1–Treated Neonatal Myocytes Were Analyzed for Functional Categories Using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7, NIAID/NIH

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<td>2.1E-2</td>
</tr>
<tr>
<td>GnRH signaling pathway</td>
<td>16</td>
<td>3.3E-2</td>
</tr>
<tr>
<td>Gap junction</td>
<td>14</td>
<td>4.4E-2</td>
</tr>
</tbody>
</table>

Table 2. Genes That Exhibit Reduced pol II Recruitment in the miR-1–Treated Neonatal Myocytes Were Analyzed for Functional Categories Using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7, NIAID/NIH

<table>
<thead>
<tr>
<th>Functional Pathway</th>
<th>No. of Genes</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilated cardiomyopathy</td>
<td>11</td>
<td>3.9E-13</td>
</tr>
<tr>
<td>Hypertrophic cardiomyopathy</td>
<td>10</td>
<td>1.1E-11</td>
</tr>
<tr>
<td>Cardiac muscle contraction</td>
<td>6</td>
<td>1.0E-5</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>7</td>
<td>7.8E-5</td>
</tr>
<tr>
<td>Arrhythmogenic right ventricular cardiomyopathy</td>
<td>5</td>
<td>1.7E-4</td>
</tr>
<tr>
<td>Tight junction</td>
<td>5</td>
<td>1.5E-3</td>
</tr>
<tr>
<td>Viral myocarditis</td>
<td>4</td>
<td>4.5E-3</td>
</tr>
<tr>
<td>Adherens junction</td>
<td>3</td>
<td>3.4E-2</td>
</tr>
<tr>
<td>Leukocyte transendothelial migration</td>
<td>3</td>
<td>7.4E-2</td>
</tr>
</tbody>
</table>

Genes exhibiting reduced pol II recruitment are characterized by reduced average density, bin numbers, and total pol II density (average density×bins). miR indicates micro RNA; NIAID, National Institute of Allergy and Infectious Diseases; NIH, National Institutes of Health; and pol II, polymerase II.

Knockdown of TFIIIB Differentially Inhibits Genes Requiring De Novo Recruitment of pol II Versus Promoter-Paused pol II

To confirm the transcriptional data, we measured the mRNA output of select genes. In concordance, there was no significant reduction in Calm1, Trappc6b, or Vdac1 mRNA versus 45% and 55% reductions in Nppa and Myh7 mRNA, respectively, in the presence of excess miR-1 (Figure 4A). Similarly, there was no detectable change in Vdac1 protein, in contrast to an 80% reduction of Ankrd1 protein. Knockdown of TFIIIB mimicked the effect of miR-1 overexpression in downregulating Ankrd1 protein and mRNA by 50%, and Nppa mRNA by = 70%, and, likewise, had little or no effect on Cdk9, Vdac1, or Mapk1 mRNA levels (Figure 4C). Replenishing TFIIIB could only marginally rescue inhibition of Ankrd1 and Nppa by miR-1 overexpression (Figure 4B and 4D), indicating that miR-1 effects are mediated through multiple targets. In contrast, ChIP-Quantitative Polymerase Chain Reaction shows that overexpression of Cdk9 was sufficient for the rescue of miR-1–induced promoter pausing of calm1, Ncl, Slc25a3, and Gapdh (Figure 4E). Either overexpression of miR-1 or TFIIIB knockdown partly inhibited endothelin-induced myocyte hypertrophy (Figure 4F), suggesting that the increase in cell size is not only dependent on both the increase in housekeeping genes but also the increase in specialized genes (eg, growth factors and extracellular matrix genes [Figure II in the Data Supplement]).

Acute, Systemic, Delivery of LNA-Modified Anti-TFIIIB Oligonucleotide Restricts Cardiac Hypertrophy

The above results led us to speculate that acute targeting of TFIIIB would suppress the expression of cardiomyopathy-induced genes while sparing the housekeeping genes, thereby, reducing pathological aspects of hypertrophy. To test this, we systemically delivered LNA-modified antisense TFIIIB oligo 24 hours post-TAC in mice. After 3 weeks, 7 of 7 injected mice exhibited 80% to 90% suppression by occupancy versus degradation of TFIIIB mRNA in the heart (Figure 5F; Figure III in the Data Supplement), in addition to other organs (Fig ure 5F; Figure III in the Data Supplement), whereas only 6 of 7 mice exhibited complete inhibition of hypertrophy-induced increase in TFIIIB protein (Figure 5A; Figure IV in the Data Supplement). The latter also correlated with a significant reduction in heart weight (Figure 5B), myocyte cross-sectional area (Figure 5C) and collagen deposition (Figure 5E), and improved ejection fraction.
In addition, gene expression showed a significant reduction in Myh7 and Ankrd1 proteins (Figure 5A; Figure III in the Data Supplement), and Col1a1, Acta1, and Nppa mRNA (Figure 5F). This is in contrast to the housekeeping genes, Calm1, Pnn, Sec23a, and Trappc6b, which exhibited no significant change during hypertrophy (Figure 5G). Similarly, anti-TFIIB had a differential effect on liver genes (Figure III in the Data Supplement). Thus, acute anti-TFIIB treatment reduces the extent of hypertrophy plausibly by reducing, but not completely inhibiting, the extent of pressure overload hypertrophy-induced genes that majorly contribute to pathological hypertrophy. Importantly, this treatment does not affect housekeeping genes.

**Discussion**

Our previous data show 2 distinct, mutually exclusive, modes of transcription during cardiac hypertrophy with regards to pol II dynamics. One involved promoter-proximal pausing of pol II that was incrementally released on induction of pressure overload, while the other required de novo recruitment (Figure 5D). In addition, gene expression showed a significant reduction in Myh7 and Ankrd1 proteins (Figure 5A; Figure III in the Data Supplement), and Col1a1, Acta1, and Nppa mRNA (Figure 5F). This is in contrast to the housekeeping genes, Calm1, Pnn, Sec23a, and Trappc6b, which exhibited no significant change during hypertrophy (Figure 5G). Similarly, anti-TFIIB had a differential effect on liver genes (Figure III in the Data Supplement). Thus, acute anti-TFIIB treatment reduces the extent of hypertrophy plausibly by reducing, but not completely inhibiting, the extent of pressure overload hypertrophy-induced genes that majorly contribute to pathological hypertrophy. Importantly, this treatment does not affect housekeeping genes.
Xipr2, etc), extracellular matrix (eg, collagen and fibronectin), and immune response genes (eg, chemokines and interleukins). Our previous study also reveals that reduced pol II pausing at housekeeping genes is a common feature of physiological, postnatal, cardiac growth, as well as pathological pressure overload cardiac hypertrophy. In contrast, most of the specialized genes are either not expressed during postnatal growth (eg, Acta1, xipr2, chemokines), or are expressed at significantly lower levels than seen in pathological growth (eg, ankrd1, Nppa, Col1a1, Ctgf). For the complete genome please see our Gene Expression Omnibus ChIP-Seq data set (accession: GSE50637). Although some of these genes promote adaptation to pressure overload and may counteract cardiac hypertrophy, their excessive upregulation is what may contribute to the pathology.23,24 Thus, for the first time, our data revealed distinct basic transcriptional mechanisms that

**Figure 5.** Acute antisense inhibition of transcription factor IIB (TFIIB) reduces cardiac hypertrophy-induced gene expression and the increase in heart weight. Twelve-week old, male, mice were subjected to a sham or transverse aortic constriction (TAC) operation. After 1 day, the mice were randomly selected for injection with saline or 15 mg/kg Locked nucleic acid–modified control or antisense TFIIB (anti-TFIIB) oligo, as indicated (n=7 each). A, After 3 weeks the hearts were isolated, protein was extracted and subjected to Western blotting for the specified genes. One experimental set is shown here; the second set is shown in the online data (set=same day surgery for all included mice). B, Box-plot of the heart weights of the mice, adjusted to tibial length. C, Similarly treated heart were isolated, sectioned and stained with wheat germ agglutinin for delineating cross sectional area. Panels 1 and 2 represent sections from 2 different hearts. Cross-sectional area of 10 myocyte/section/heart was measured and plotted (graph, right). * is P<0.05 vs sham, # is P<0.5 vs TAC+anti-TFIIB. D, All mice were analyzed by echocardiography before euthanasia, and the ejection fraction calculated and plotted. * is P<0.05 vs sham, # is P<0.5 vs TAC+anti-TFIIB. E, Sectioned hearts were stained with Sirius red for estimating collagen content (n=2). The red-stained collaged was quantified, averaged, and plotted (graph, right). F, Total mRNA was extracted from all hearts and select hypertrophy-related genes were quantified by quantitative polymerase chain reaction (qPCR). * is P<0.05 vs sham, # is P<0.5 vs TAC+anti-TFIIB. G, Total mRNA was extracted from all hearts and select housekeeping genes were quantified by qPCR. * is P<0.05 vs sham, # is P<0.5 vs TAC+anti-TFIIB.
regulate housekeeping and specialized genes that could be exploited for selective inhibition of the latter group during hypertrophy.

Our objective was to inhibit collectively the upregulation of genes induced by pressure overload in an acute, dose-dependent, and reversible fashion. Therefore, we directed our attention to general transcription factors. TFIIH is a limiting general transcription factor that is required for the recruitment of pol II to promoter start sites, and, accordingly, the transcription of all promoters. Thus, the dynamics of TFIIH binding to pol II-paused versus de novo pol II-recruited promoters has not been determined yet. As demonstrated by our previous ChIP-Seq data, a comparison between the neonatal, adult, and pressure-overloaded hearts afforded us a unique and ideal model for addressing this question. Thus, we performed a TFIIH ChIP-Seq and aligned it with the pol II results. As expected, all pol II-bound promoters were also occupied by TFIIH. However, in contrast to promoters that required robust de novo recruitment of pol II and TFIIH, those that exhibited pol II pause-release displayed constitutive TFIIH binding, with only incremental, changes during hypertrophy. These results led us to speculate that acute and transient inhibition of TFIIH-induced expression during hypertrophy would selectively inhibit the former group of genes. Short hairpin targeting of TFIIH in neonatal myocytes decreased expression of Myh7 (≈55%) and Nppa mRNA (≈45%) while having no apparent effect of Vdac or mitogen-activated protein kinase (MAPK). This would suggest that the turnover of pol II on the latter promoters is much slower than that the immediate downstream-accumulated pol II is recycled to the promoter without detaching (gene looping).25

One of the promising targeting tools that are designed for specific mRNA inhibition/knockdown are modified antisense oligonucleotides, which are widely utilized in cancer clinical trials.26–28 In particular, we chose the LNA-modified antisense oligo for its superior affinity to complimentary mRNA and, thereby, its high efficiency in inhibiting its target, either by occupancy or by inducing degradation. At a dose of 15 mg/kg, the oligo inhibited TFIIH by ≈80% via mRNA occupancy for up to 3 weeks. Notably, hypertrophy-induced upregulation of TFIIH protein was completely inhibited. It should be emphasized here that TFIIH is required for the transcription of all genes, as confirmed by our ChIP-Seq data (Gene Expression Omnibus, GSE56813), however, because the paused promoters have both preassembled TFIIH and pol II, they were not immediately affected by knockdown of TFIIH. One of the drawbacks of this method, as is the case with most therapies, although, is the systemic delivery of the oligo, which would impact other organs. In our study, we tested the effect on liver gene expression, as in the heart, the oligo had no effect on the housekeeping genes (AKT or MAPK), but inhibited the expression of albumin, which is an inducible gene, by ≈50% (Figure II in the Data Supplement).

Although the involvement of RNA pol II in cardiac hypertrophy has been established in earlier studies, including a 1-adrenergic-induced hypertrophy, the details of its involvement and how, in particular, it regulates the expression of housekeeping genes, and their incremental increase in parallel with cell size, has not been determined. We expect that the mechanism presented in our pressure-induced hypertrophy model, with respect to the dichotomous effect of pol II and TFIIH on transcriptional dynamics, to be similar in other forms of hypertrophy. In support, the set of housekeeping genes that exhibit decreased pausing in hypertrophy, are similar to those exhibiting reduced pausing during postnatal growth. Although the epigenetics that potentially regulates pol II assembly on paused versus inducible promoters has not been delineated, the regulation of pol II pausing by phosphorylation of its c-terminal domain with Cdk9 is well established and has previously shown to play a major role in the development of cardiac hypertrophy. In addition, recently pol II c-terminal domain has been shown to be modified by the p300 acetylase, which regulates its elongational activity in inducible genes, but has no role on housekeeping genes in human fibroblasts. Thus, in conclusion, our study provides new mechanistic insights into general transcriptional regulation during cardiac hypertrophy, which offers a potentially new therapeutic target that promises to reduce pathological aspects of cardiac hypertrophy.

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We want to thank Dr Junichi Sadoshima, Chairman of the Department of Cell Biology and Molecular Medicine, for his support.

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Disclosures
None.

References
we demonstrate and the potential therapeutic advantages offered by locked nucleic acid–modified antisense oligonucleotides. The study uncovers novel transcriptional mechanisms during cardiac hypertrophy and novel therapeutic targets. In addition, cardiomyopathy genes, while sparing housekeeping genes, thus, reducing the extent of hypertrophy and cardiac dysfunction. hypertrophy-induced transcription factor IIB expression. This resulted in collective suppression of hypertrophy-induced and is assembled at promoters in corresponding patterns. We exploited this difference by acutely and specifically inhibiting respectively. The binding of polymerase II is mediated by transcription factor IIB, which is upregulated during hypertrophy, the 2 groups is the preassembly of RNA polymerase II versus de novo recruitment to transcription start sites of promoters, actin, cardiomyopathy gene, and collagen. We found that the main transcriptional mechanism that distinguishes between identified different modes of transcription that distinguish between the regulation of housekeeping genes, such as those involved in respiration, protein and RNA, synthesis/degradation, and those involved in specialized functions such as contractile, calcium handling, extracellular matrix, and immune reaction genes. The former category of genes is that which increases incrementally and underlies the increase in cell and heart size. However, the latter group is exemplified by some of the genes that are known to be robustly induced or downregulated during the pathogenesis of cardiac failure, for example, α-skeletal actin, cardiomyopathy gene, and collagen. We found that the main transcriptional mechanism that distinguishes between the 2 groups is the preassembly of RNA polymerase II versus de novo recruitment to transcription start sites of promoters, respectively. The binding of polymerase II is mediated by transcription factor IIB, which is upregulated during hypertrophy, and is assembled at promoters in corresponding patterns. We exploited this difference by acutely and specifically inhibiting hypertrophy-induced transcription factor IIB expression. This resulted in collective suppression of hypertrophy-induced cardiomyopathy genes, while sparing housekeeping genes, thus, reducing the extent of hypertrophy and cardiac dysfunction. The study uncovers novel transcriptional mechanisms during cardiac hypertrophy and novel therapeutic targets. In addition, we demonstrate and the potential therapeutic advantages offered by locked nucleic acid–modified antisense oligonucleotides.
Acute Targeting of General Transcription Factor IIB Restricts Cardiac Hypertrophy via Selective Inhibition of Gene Transcription
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Figure 1S. MiR-1 is downregulated by fetal bovine serum or, conversely, upregulated upon deprivation of serum. a. Neonatal cardiac myocytes were maintained in either serum free (SF, lanes 1-3, upper panel) or in 10% fetal bovine serum (FBS, lanes 4-6, upper panel) for 24 h, before either adding FBS for 24 h (lane 2) or 48 h (lane 3), or depriving them of FBS for 24 h (lane 5) or 48 h (lane 6), as indicated. RNA was then extracted and analyzed by Northern blotting for miR-1. The lower panel shows 5S, as for loading control. b. The data points for the control and 48 h time point were quantitated and graphed as values relative to the control adjusted to 1.
Figure 2S. Growth factors and extracellular matrix protein are regulated by de novo TFIIB and pol II recruitment during cardiac hypertrophy and are depressed by miR-1. a.-b. PolII, H3K9ac, and TFIIB ChIP-Seq data for neonatal, adult, and TAC-induced hearts are aligned and the data for Ctgf and Col1a1 are viewed with IGB. c.-d. Pol II ChIP-Seq data for miR-1-treated vs. control neonatal myocytes are aligned with the rat genome and the data for Ctgf and Col1a1 are viewed with IGB.
TFIIB
Akt
Alb
Sham
TAC
TAC+anti-TFIIB
3 wk-liver

Figure 3S. LNA-modified anti-TFIIB suppresses endogenous TFIIB mRNA by occupancy in both the heart and liver. Twelve-week old, male, mice were subjected to a sham or transverse aortic constriction (TAC) operation. After 1d later the mice were randomly selected for injection with saline or 15 mg/Kg LNA-modified control or antisense TFIIB (anti-TFIIB) oligo, as indicated. a.-b. After 1 wk the mice were sacrificed and total RNA was extracted from both the heart and liver and analyzed by qPCR for TFIIB. Two different sets of primers were used. Primer 1, for detecting mRNA degradation, was distant from the site targeted by anti-TFIIB, while the Primer 2, for detecting mRNA occupancy by anti-TFIIB, encompassed the targeted site (see ‘methods’ for details). a. Result were averaged and plotted (n=4). b. qPCR results for each liver are plotted separately (n=2). c. Mice were sacrificed 3 wk post-TAC and treatment. Total RNA was extracted from the liver and analyzed by qPCR for TFIIB (primer 2), Akt, and albumin (Alb).
Figure 4S. Acute antisense inhibition of TFIIB reduces cardiac hypertrophy-induced gene expression and the increase in heart weight. Twelve-week old, male, mice were subjected to a sham or transverse aortic constriction (TAC) operation. After 1d the mice were randomly selected for injection with saline or 15 mg/Kg LNA-modified control or antisense TFIIB (anti-TFIIB) oligo, as indicated (n=3 each). After 3 weeks the hearts were isolated, protein was extracted and subjected to Western blotting for the specified genes. One experimental set is shown here; the second set is shown in Fig. 5 (set = same day surgery for all included mice).