Extraocular Muscle Morphogenesis and Gene Expression Are Regulated by *Pitx2* Gene Dose

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Purpose. *PITX2* gene dose plays a central role in Axenfeld-Rieger syndrome. The purpose of this study was to test the hypothesis that the effects of *Pitx2* gene dose on eye development can be molecularly dissected in available *Pitx2* mutant mice.

Methods. A panel of mice with *Pitx2* gene dose ranging from wild-type (+/+) to none (−/−) was generated. Eye morphogenesis was assessed in animals with each *Pitx2* gene dose. We also compared global gene expression in eye primordia taken from e12.5 $Pitx2^{+/+}$, $Pitx2^{+/-}$, $Pitx2^{-/-}$ embryos using gene microarrays. The validity of microarray results was confirmed by qRT-PCR.

Results. Morphogenesis of all extraocular muscle bundles correlated highly with *Pitx2* gene dose, but there were some differences in sensitivity among muscle groups. Superior and inferior oblique muscles were most sensitive and disappeared before the four rectus muscles. Expression of muscle-specific genes was globally sensitive to *Pitx2* gene dose, including the muscle-specific transcription factor genes *Myf5*, *Myog*, *Myod1*, *Smyd1*, *Msc*, and *Csrp3*.

Conclusions. *Pitx2* gene dose regulates both morphogenesis and gene expression in developing extraocular muscles. The expression of key muscle-specific transcription factor genes is regulated by *Pitx2* gene dose, suggesting that sufficient levels of PITX2 protein are essential for early initiation of the myogenic regulatory cascade in extraocular muscles. These results document the first ocular tissue affected by *Pitx2* gene dose in a model organism, where the underlying mechanisms can be analyzed, and provide a paradigm for future experiments designed to elucidate additional effects of *Pitx2* gene dose during eye development. (*Invest Ophthalmol Vis Sci.* 2006;47: 1785–1793) DOI:10.1167/iovs.05-1424

Coordinated movement of the extraocular muscles is necessary to direct gaze and maintain binocular vision. Defects in metabolism, morphology, and innervation of even a single extraocular muscle result in a range of visual disorders,[1–17] some of which have strong genetic bases.[11,18,19] Extraocular muscle is also significant, because it is usually not affected in congenital muscular dystrophies.[20] Therefore, identifying the regulatory networks controlling extraocular muscle development will provide important new knowledge that is likely to impact our understanding of both normal vision and muscular dystrophy.

Extraocular muscle and somitomeric trunk muscle share many similarities but the two muscle types are likely to be specified by distinct regulatory cascades.[21,22] Transplanted somitic mesoderm is unable to respond to local cues within the head and differentiate into extraocular muscles.[22] Conversely, transplanted cranial mesoderm is unable to respond to local cues within the trunk and form skeletal muscles.[22] *Pax3* is an essential early activator of the myogenic regulatory cascade in the somites, but is not expressed in the cranial mesoderm from which extraocular muscles are derived.[22] *Pax7* is expressed in the cranial mesoderm precursors to extraocular and other head muscles and has been proposed as a functional substitute for *Pax3*, but no supporting genetic evidence has been reported.[22] *Myf5*, *Myog*, and *Myod1*, which are essential for differentiation of somitomeric muscle, are all expressed in extraocular muscle primordia, but their functional significance has not been established.[23–26] Taken together, these observations indicate that the regulatory cascades required for development of extraocular muscles remain poorly understood.

Myofibers in extraocular muscles are derived from mesoderm, whereas muscle connective tissue cells arise from neural crest.[27] *Pitx2* encodes a homeodomain transcription factor expressed in both neural crest and mesoderm during eye development, including extraocular muscle primordia.[27] $Pitx2^{-/-}$ embryos exhibit complete agenesis of extraocular muscles, providing direct evidence of essential function in their early specification.[27–31] The response of individual cell types and organs to variations in *Pitx2* gene dose plays a significant role in normal and abnormal organ development. This mechanism was first suggested by the demonstration that heterozygous mutations for gain- or loss-of-function mutations in human *PITX2* contribute to Axenfeld-Rieger syndrome (ARS), a human haploinsufficiency disorder including ocular anterior segment defects and a significant risk of glaucoma.[32–35] Subsequently, an essential role for *Pitx2* gene dose in regulating pituitary, heart, and craniofacial development has been established.[36,37] Extraocular muscle defects are sometimes associated with ARS, although the underlying genetic defects have not been identified.[38] Based on these observations, we hypothesized that *Pitx2* gene dose may play a significant mechanistic role in specifying extraocular muscles during development.

To test this hypothesis directly, we generated an allelic series of mice expressing different levels of *Pitx2*.[37] We examined muscle morphology, the expression of muscle-specific proteins, and global gene expression profiles, to determine the gross and molecular effects of various *Pitx2* doses on extraocular muscle development. The results confirm our hypothesis that the response to *Pitx2* gene dose is an essential mechanism

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in the regulation of extraocular muscle development. Furthermore, *Pitx2* is likely to be a very early initiator of extraocular muscle development by activating a regulatory cascade including *Myf5*, *Myog*, *Myod1*, and, potentially, other muscle-specific transcription factor genes. The results are also significant because they document the first experimental link between *Pitx2* gene dose and an ocular tissue.

Materials and Methods

Animal Husbandry

Generating mice carrying the $Pitx2^{neo}$ and $Pitx2^{-}$ gene-targeted alleles has been described previously.[39] For these experiments, each allele had been backcrossed ($N = 7$) onto the inbred C57BL/6J background, making each animal essentially identical genetically except at the *Pitx2* locus. All breeding was performed at the University of Michigan. Genotyping by polymerase chain reaction was performed as previously described.[39] Animals were housed and handled in accordance with NIH guidelines for animal care. All procedures involving mice were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA). All experiments were conducted in accordance with the principles and procedures established by the National Institutes of Health (NIH) and the Association for Assessment of Laboratory Animal Care (AALAC), and in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Timed-Pregnant Matings

Three types of timed-pregnant matings were performed to produce embryos representing all levels of *Pitx2* expression within the allelic series described herein. Breedings between $Pitx2^{+/-}$ male and female mice were used to generate $Pitx2^{+/+}$, $Pitx2^{+/-}$, and $Pitx2^{-/-}$ embryos. Likewise, $Pitx2^{+/neo}$ mice were bred to produce $Pitx2^{+/+}$, $Pitx2^{+/neo}$, and $Pitx2^{neo/neo}$ embryos. $Pitx2^{neo/-}$ compound-heterozygotes were produced by breeding $Pitx2^{+/neo}$ males to $Pitx2^{+/-}$ females. The morning after mating was designated as embryonic day (E)0.5. Pregnant mice were killed by cervical dislocation at either E12.5 or E14.5 and embryonic tissue was either processed for histology or stored in RNA stabilization reagent (RNAlater; Ambion, Austin, TX) at −20°C for subsequent RNA isolation. DNA was isolated from extraembryonic membranes and genotyping was performed by polymerase chain reaction.[39]

Tissue Preparation and Histology

Embryos intended for histology were fixed in 4% paraformaldehyde in PBS for 2 to 4 hours at room temperature. Fixed embryos were washed three times in PBS, dehydrated through graded ethanol, washed three times in methyl salicylate, and embedded in paraffin (Paraplast Plus; Fisher Scientific, Pittsburgh, PA). Sections were cut at 7 μm and mounted on slides (Superfrost Plus; Fisher Scientific) using poly-L lysine solution (Sigma-Aldrich, St. Louis, MO) diluted 1:10 in DEPC-treated water. Dewaxing and rehydration was performed according to standard methods.

Immunohistochemistry and Photography

Immunohistochemistry was performed with a mouse monoclonal antibody to developmental myosin heavy-chain (Vector Laboratories, Burlingame, CA). Antigen retrieval was performed by boiling in 10 mM citrate buffer (pH 6.0), for 10 or 20 minutes, followed by rinsing in water for 10 minutes to cool. Endogenous peroxidase activity was quenched for 10 minutes in 3% hydrogen peroxide in water, followed by a 30-minute block in signal enhancer (ImageiT; Invitrogen, Carlsbad, CA). Antibody blocking was performed with a kit (Mouse On Mouse [MOM]; Vector Laboratories) according to the manufacturer's recommendations. Primary antibody was applied at a 1:5 dilution in MOM diluent (Vector Laboratories) and incubated overnight at 4°C. Biotin-labeled anti-mouse IgG supplied in the kit was used as a secondary antibody. A tyramide signal-amplification (TSA) kit (Invitrogen) was used to visualize sites of myosin heavy-chain expression. Photography was performed on a fluorescence microscope (Eclipse 800; Nikon, Tokyo, Japan) using matched aperture and exposure settings to hold signal detection invariant between genotypes.

Gene Expression Analysis

Eye primordia were microdissected from E12.5 $Pitx2^{+/+}$, $Pitx2^{+/-}$, and $Pitx2^{-/-}$ embryos. Total RNA was isolated from the eyes of individual embryos (RNAqueous Micro Kits; Ambion). cDNA and biotin-labeled cRNA was generated from 1 μg of RNA from each embryo (MessageAMP kits; Ambion). The in vitro–labeling reactions were extended overnight to increase yield. Labeled cRNA was hybridized to mouse gene microarrays (Mouse Genome 430 ver. 2.0 GeneChip; Affymetrix, Santa Clara, CA) and processed by the standard protocol. Gene expression profiles were generated from four animals from each of the three *Pitx2* genotypes using a total of 12 arrays. Initial data preparation was then performed with a methodology that performed background correction, quantile normalization, and summarization of expression scores (Microarray Suite ver. 5.0. Robust Multichip Average [RMA]; Affymetrix).[40]

A two-stage, direct-screening procedure based on the Benjamini and Yekutieli[41] reconstruction of the false-discovery rate confidence interval (FDR-CI) was used to assign probabilities to the *x*-fold changes of gene responses.[42–44] This method is distinct from approaches based on the conventional *t*-test, because it allows the experimenter to control statistical significance and biological significance in determining positive differential responses.[42] In the first stage, a set of genes with putative expression changes was identified with an FDR-α test. In the second stage, this set of genes was further screened to establish biological and statistical significance. A minimum *x*-fold change (fc-min) of 1.5-fold was established as the threshold for establishing biological significance and all probesets with $P < 1$ were reported. For hierarchical analysis, the top 200 FDR-CI constrained gene profiles were standardized to have mean of 0 and SD of 1 across all groups and were clustered using hierarchical clustering implemented in Cluster and TreeView.[45] Samples were grouped by expression profiles rather than genotype and Euclidean distance was chosen for clustering as the measure of expression profile similarity. Muscle-specific array hits were identified by automated and manual inspection of the National Center for Biotechnology Information (NCBI, Bethesda, MD) Entrez Gene records. Original.cel data files will be deposited with the Gene Expression Omnibus (www.ncbi.nih.gov/geo/).

Real-Time PCR Confirmation

Real-time RT-PCR was performed (*Taq*Man Gene Expression Arrays) on custom-designed microfluidics cards (Affymetrix). E12.5 $Pitx2^{+/+}$, $Pitx2^{+/-}$, and $Pitx2^{-/-}$ embryos were obtained from *Pitx2*− heterozygous matings. The eyes and surrounding periocular mesenchyme were microdissected and stored (RNAlater; Ambion). Total RNA was extracted from homogenized tissue (RNAqueous-micro kit; Ambion). cDNA was reverse transcribed from 1 to 5 μg total RNA by (SuperScript III with random primers; Invitrogen-Life Technologies, Gaithersrburg, MD) according to the manufacturer's protocol. cDNA was diluted as 1 μg total RNA/100 μL final volume (10 ng/μL). Gene expression assays (*Taq*Man; Applied Biosystems, Inc., Foster City, CA) were performed with master mix (RealTime Ready; Q.BIOgene, Carlsbad, CA) in a thermal cycler (iCycler; Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Relative changes (*x*-fold) compared with wild type were calculated by using the $2^{-D\Delta Ct}$ method and normalized to each of four independent "housekeeping" genes (*Hprt*, *Arl10c*, *Hsd17b12*, and 18S rRNA) whose expression has been independently confirmed as unaffected by the *Pitx2* genotype. Standard error was computed from four samples of each genotype. Relative expression levels obtained after normalization to *Hprt* are depicted in Figure 4, but normalization to the other three housekeeping gene yielded analogous results.

RESULTS

Generation of *Pitx2* Allelic Series

To evaluate the effects of *Pitx2* gene dose in the developing eye, we used $Pitx2^{+}$, $Pitx2^{neo}$, and $Pitx2^{-}$, to generate an allelic series of mice expressing graded levels of *Pitx2* expression (Fig. 1A).[39] By definition, each $Pitx2^{+}$ allele contributes 50% of PITX2 protein expression in wild-type mice. Conversely, the $Pitx2^{-}$ allele is a complete null allele and expresses no functional PITX2 protein. $Pitx2^{-}$ homozygotes die by E15.5 of cardiovascular defects. $Pitx2^{neo}$ is a hypomorphic (reduced-function) allele, because the PGK-neo cassette between exons 3 and 4 interferes with normal splicing of the *Pitx2* transcript, leading to reduced levels of *Pitx2* mRNA and accordingly reduced protein expression. $Pitx2^{neo}$ homozygotes also die of cardiovascular defects but not until postnatal day 1, confirming that this is a milder genetic lesion.[39] By interbreeding these three alleles, we were able to generate an allelic series with estimated protein expression levels ranging from 100% ($Pitx2^{+/+}$) down to 0% ($Pitx2^{-/-}$; Fig. 1B).

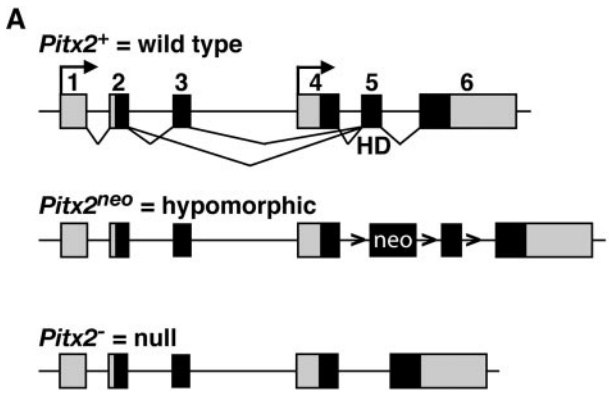
Dose Effect of *Pitx2* on Extraocular Muscles

Embryos were harvested at E14.5 and examined by standard histologic techniques to determine whether eye structures were affected by *Pitx2* gene dose. Changes in the extraocular muscle were the most readily apparent. As expected, $Pitx2^{+/+}$ embryos had discrete, well-formed, and easily identified extraocular muscle condensations (Fig 2A), whereas $Pitx2^{-/-}$ embryos lacked extraocular muscles altogether (data not shown).[39] Consistent with our hypothesis, extraocular muscle development in embryos with intermediate *Pitx2* genotypes (*+/neo*, *+/−*, *neo/neo*, and *neo/−*) had phenotypes that paralleled *Pitx2* gene dose closely. The superior oblique muscle was uniformly absent in all $Pitx2^{+/-}$ embryos examined ($n = 5$) and therefore appeared to be the most sensitive to *Pitx2* gene dose. The inferior oblique muscle was missing in some but not all $Pitx2^{+/-}$ embryos (Fig. 2C). Both oblique muscles were consistently absent in $Pitx2^{neo/neo}$ embryos (Fig. 2D). Evidence of all four rectus muscles was still present in $Pitx2^{+/-}$ and $Pitx2^{neo/neo}$ embryos, but these muscles became increasingly smaller and more disorganized as *Pitx2* gene dose was reduced (Figs. 2C−E). The most common defect observed was an abnormal continuity between the lateral and inferior rectus muscles. These muscles were consistently distinct in wild-type embryos (Fig. 2A) The abnormalities were observed in both $Pitx2^{+/-}$ (Fig. 2C) and $Pitx2^{neo/neo}$ (Fig. 2D) embryos, but were more pronounced in $Pitx2^{neo/neo}$ embryos. Muscle condensations are undetectable in $Pitx2^{neo/-}$ embryos (Fig. 2E), analogous to what is observed in $Pitx2^{-/-}$ embryos (Ref. 39 and data not shown). By examining sections taken from older embryos, we have established that these phenotypes do not result simply from delayed growth of extraocular muscles. Taken together, these results suggest that morphologic development of extraocular muscles corresponds strongly with *Pitx2* gene dose.

To be certain that extraocular muscles were really reduced or missing, rather than simply unrecognizable due to unexpected morphologic changes, we performed immunofluorescence to detect developmental myosin heavy-chain (dMyh) protein (Figs. 2A′–E′), which was expressed in all differentiated muscle fibers. All slides were stained at the same time, and sections were photographed with matched camera settings, to compare relative levels of protein expression between genotypes. Our results confirmed our initial conclusions from H&E-stained sections, that overall morphogenesis of extraocular muscles correspond closely with *Pitx2* gene dose and that normal development of the superior and inferior oblique muscles requires somewhat higher levels of *Pitx2* function than normal development of the four rectus muscles. Furthermore, we observed a marked reduction in dMyh staining intensity beginning at the $Pitx2^{+/-}$ level, which was even more pronounced in $Pitx2^{neo/neo}$ embryos. Although immunofluorescence is not a quantitative assay as performed, these results provide the first experimental evidence that muscle-specific gene expression levels may also correlate with *Pitx2* gene dose.

Gene Expression Profiles

To gain further insight into the mechanisms underlying *Pitx2* gene dose effects on extraocular muscle development, we initiated a comparison of global gene expression in eye primordia isolated from E12.5 $Pitx2^{+/+}$, $Pitx2^{+/-}$, and $Pitx2^{-/-}$ embryos ($n = 4$ per genotype). These genotypes correspond to 100%, 50%, and 0% of the normal PITX2 function levels, respectively. RMA, a robust and well-established methodology, was used to quantify signal intensity and normalize signals.[40] Normalized data were then analyzed using the robust two-step FDR-CI method to identify statistically significant ($P < 1$) dif-



B

Genotype	Estimated % Expression
+/+	100%
+/neo	70%
+/-	50%
neo/neo	40%
neo/-	20%
-/-	0%

FIGURE 1. *Pitx2* gene-targeted alleles and the allelic series. (**A**) Genomic organization of the *Pitx2* locus and *Pitx2* alleles. *Arrows* above exons indicate transcriptional initiation sites. mRNA isoforms *Pitx2a* and *Pitx2b* are transcribed from the 5′ promoter and differ by alternative splicing of exon 3. *Pitx2c* utilizes an alternative promoter and lacks exons 1, 2, and 3. $Pitx2^{neo}$ is a hypomorphic allele containing a neo resistance gene within the intron between exons 4 and 5. $Pitx2^{-}$ lacks exon 4, which encodes the essential homeodomain (HD). (■) Coding sequences; (▨) noncoding sequences. (**B**) Genotypes within the allelic series and estimated Pitx2 expression levels.

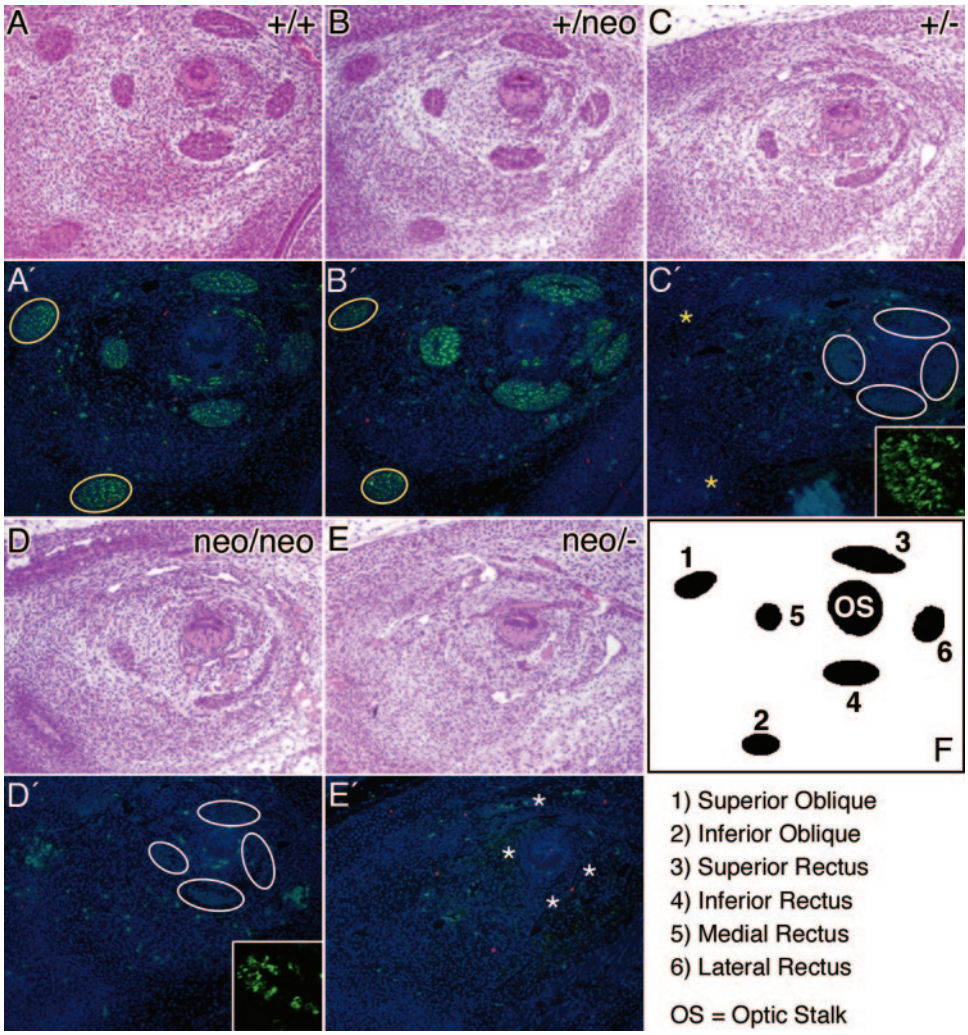


FIGURE 2. Extraocular muscle morphogenesis correlates strongly with *Pitx2* gene dose. Sagittal sections immediately medial to the optic cup taken from E14.5 embryos of the indicated genotypes. Sections were stained by hematoxylin and eosin (**A**–**E**) or developmental myosin heavy-chain (dMyh) immunofluorescence (**A′**–**E′**). Sections in (**A′**–**E′**) were photographed with invariant camera settings and exposure times, to facilitate comparison of relative dMyh expression levels. *Insets*: medial rectus muscles. Superior and inferior oblique muscles (*yellow* ovals in A′ and B′) are more sensitive to *Pitx2* gene dose than rectus muscles (*white* ovals in C′ and D′). All extraocular muscles, including rectus muscles (E′, ✶), are absent in $Pitx2^{neo/-}$ embryos. (**F**) Schematic key for position of extraocular muscles. Because of their significance to human ocular health, only rectus and oblique muscles are shown in the key; the retractor bulbi muscle has been omitted. Magnification, ×20; *insets*, ×40.

ferentially expressed genes.[41,42] This approach is now well-established.[43] Hierarchical clustering of the top 200 differentially expressed genes revealed striking patterns. Three distinct patterns of gene expression were observed based on their response to *Pitx2* gene dose (Fig. 3). Expression of genes in set A decreased in parallel with *Pitx2* gene dose, indicating that these genes are dose sensitive and activation correlates positively with PITX2 protein levels. Expression of genes in set B was reduced in eyes of $Pitx2^{-/-}$ but not $Pitx2^{+/-}$ embryos, indicating that these genes are not affected by *Pitx2* gene dose. Finally, expression of genes in set C increased with decreasing *Pitx2* gene dose, indicating that these genes are dose sensitive, but that activation correlates negatively with increasing PITX2 protein levels. Because whole eye primordia were used as the RNA source for these experiments, differentially expressed genes represent both myogenic and nonmyogenic functions. We next evaluated each gene in sets A, B, and C to identify those that could be confirmed as muscle-specific based on previously published results or publicly available expression data. In all, 50 muscle-specific, dosage-sensitive genes were identified (Table 1). Consistent with the decrease of extraocular muscle morphogenesis in response to *Pitx2* gene dose, expression of all the muscle-specific genes decreased in parallel with *Pitx2* gene dose (Fig. 3; set A). No muscle-specific genes were identified in set C, whose expression increases with decreasing *Pitx2* gene dose.

Real-Time PCR Validation of Microarray Results

To determine the overall validity of our differential gene expression data, we selected 15 muscle-specific genes representing a range of change (*x*-fold) and statistical confidence levels to re-evaluate by quantitative real-time PCR (TaqMan; Applied Biosystems). All 15 genes were confirmed as sensitive to *Pitx2* gene dose. In all cases, the reported change and confidence interval were equal to or greater than those predicted by the microarray experiment (Fig. 4). Taken together, we conclude that our gene-expression profiles accurately identified the muscle-specific genes that are sensitive to *Pitx2* gene dose.

Pitx2 Dose Dependence of Muscle-Specific bHLH Transcription Factor Genes

To gain insight into potential underlying mechanisms that might account for the dependence of extraocular muscle development on *Pitx2* gene dose, we analyzed the biological functions of the muscle-specific dose-dependent genes. As would be predicted, genes with a variety of biological functions were represented (Fig. 5). Prominent members of the list are *Myf5*, *Myog*, *Myod1*, *Smyd1*, *Msc*, and *Csrp3*, all of which encode transcription factors or nuclear-associated proteins that have been established as essential for skeletal and/or cardiac muscle differentiation and function[46–50] (Fig. 5, Table 1). Numerous genes encoding components of the muscle structural–

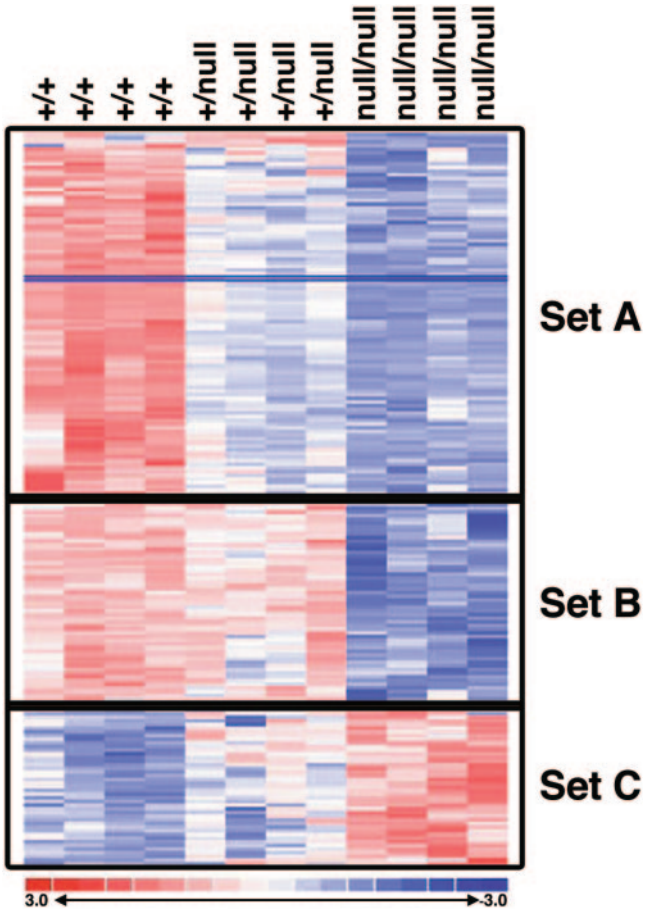


FIGURE 3. Heat map of hierarchical clustering analysis. Three patterns were identified after unsupervised clustering. Genes in set A are dose sensitive, and expression decreases in parallel with *Pitx2* gene dose. In set B, gene expression is reduced or absent in $Pitx2^{-/-}$ eyes, but is unaffected in $Pitx2^{+/-}$ eyes. Genes in Set C are dose sensitive, and expression increases with decreasing *Pitx2* gene dose. *Red*: increased gene expression; *blue*, reduced gene expression.

contractile apparatus or proteins required for muscle-specific metabolism were also represented (Table 1, Fig. 5).

DISCUSSION

The possibility that alterations in PITX2 protein function levels play a significant role in development and disease was first indicated by the heterozygous gain- or loss-of-function mutations in human *PITX2*, which are a significant cause of Axenfeld-Rieger Syndrome.[32–35] Subsequently, the effects of altered PITX2 function on the expression of downstream target genes have been established in cell culture, and the central roles of *Pitx2* gene dose on pituitary gland, craniofacial, and heart development were demonstrated using gene-targeted mice.[36,37] We now show that morphogenesis and gene expression in extraocular muscle are also sensitive to alterations in *Pitx2* gene dose. Our results suggest important mechanisms regulating extraocular muscle development, which is poorly understood. More generally, these findings are significant, because abnormal development of the ocular anterior segment and a high risk for glaucoma are seminal features of Axenfeld-Rieger syndrome, and extraocular muscle represents the first opportunity to determine the effects of *Pitx2* gene dose on an ocular tissue.

There are seven extraocular muscles in the mouse (Fig. 2F): the superior and inferior oblique muscles, the four rectus muscles, and the retractor bulbi muscle. Humans lack the retractor bulbi muscle, and so we focused our attention on understanding the effects of *Pitx2* gene dose on the other six. We demonstrate that morphogenesis of extraocular muscles was highly sensitive to *Pitx2* gene dose effects. The superior and inferior oblique muscles were consistently more sensitive than the four rectus muscles. This is strikingly similar to the function of *Pitx2* in the developing pituitary gland, where there is a strong correlation between overall morphogenesis of Rathke's pouch, the early pituitary primordium, and the level of *Pitx2* gene dose.[37] In addition, the five neuroendocrine cell lineages of the mature anterior pituitary gland are differentially sensitive to the effects of *Pitx2* gene dose, with gonadotropes being the most sensitive and lactotropes and corticotropes the least sensitive.[37] Similar to the pituitary, the extraocular muscles are composed of a mixture of different fiber types, distinguished in mature muscles by their diameter, innervation pattern, oxidative potential (number and size of mitochondria, pigmentation, extent of vascularization) and ability to transmit an action potential (extent of sarcoplasmic reticulum).[51,52] We predict that, as seen in the pituitary, the different fiber types may be differentially sensitive to *Pitx2* dosage and that the overall reductions in extraocular muscle size seen in our mutants may be related to preferential loss of specific fiber types. Unfortunately, most mice in our allelic series do not survive beyond birth, and suitable molecular markers for distinguishing between the different muscle lineages or their precursors at early time points have not yet been identified.

Previously established functions of PITX2 in other organ systems suggest molecular mechanism(s) that may underlie the effects of *Pitx2* gene dose on the development of extraocular muscle. Decreased proliferation of muscle precursors may be a contributing factor, since *Pitx2* has been implicated as playing a role in cellular proliferation in skeletal and cardiac muscles.[53] However, we find no evidence of proliferative changes in the extraocular muscle primordia of our allelic series (data not shown). Alternatively, increasing levels of apoptosis could also explain the progressive reduction of extraocular muscle size in our mutants. Apoptosis has been implicated in the loss of pituitary cells in *Pitx2* mutant mice.[54] However, we find no evidence of increased apoptosis in the mutant extraocular muscle primordia by TUNEL assay (data not shown). Therefore, it seems likely that precursors fated to become extraocular muscle cells are present in animals of all *Pitx2* genotypes but fail to initiate or sustain their muscle differentiation program efficiently in the case of animals with reduced PITX2 function and never initiate the program in animals with no PITX2 function. Some muscle-specific genes affected by *Pitx2* gene dose may be direct targets of PITX2 regulation, as suggested by the observation that a subset contain $\geq$1 predictes PITX2 binding sites within 2 kb of the transcription-initiation site (data not shown). Others may be indirect PITX2 targets or simply markers of muscle the muscle phenotype. Identification of the regulatory elements required for endogenous expression of each gene and testing of these elements for responsiveness to PITX2 will be necessary, to distinguish between these possibilities.

An important insight into the underlying mechanism(s) is offered by our gene expression analysis, which demonstrates that the genes for *Myf5*, *Myog*, and *Myod1* all require PITX2 for their expression and are sensitive to *Pitx2* gene dose effects. These genes encode bHLH class transcription factors that are well established individually and in combination for specification and differentiation of skeletal and other muscle types.[55–58] Expression of these genes in the primordia of extraocular muscles has been noted previously, but the functional signifi-

TABLE 1. Muscle-specific Gene Expression Correlates Strongly with *Pitx2* Gene Dosage

Affy Probeset*	UID†	Gene Name‡	Gene Symbol‡	Biological Function‡	WT vs. Hom. AFC§	WT vs. Hom. *P*‖	WT vs. Het. AFC§	WT vs. Het. *P*‖
1415927_at	11464	Actin, alpha, cardiac	*Actc1*	Structural/motor	−5.55	0.00	−2.44	0.81
1422580_at	17896	Myosin, light polypeptide 4	*Myl4*	Structural/motor	−4.38	0.00	−2.81	0.17
1427115_at	17883	Myosin, heavy polypeptide 3, skeletal muscle, embryonic	*Myh3*	Structural/motor	−4.28	0.00	−2.44	0.52
1418370_at	21924	Troponin C, cardiac/slow skeletal	*Tnnc1*	Structural/motor	−4.09	0.00	−2.16	0.24
1417464_at	21925	Troponin C2, fast	*Tnnc2*	Structural/motor	−3.90	0.00	−2.45	0.14
1452651_a_at	17901	Myosin, light polypeptide 1	*Myl1*	Structural/motor	−3.40	0.00	−1.91	0.54
1450813_a_at	21952	Troponin 1, skeletal, slow 1	*Tnni1*	Structural/motor	−2.98	0.00	−1.84	0.38
1419391_at	17928	Myogenin	*Myog*	Transcription factor	−3.83	0.01	−2.07	1.00
1420757_at	17877	Myogenic factor 5	*Myf5*	Transcription factor	−2.81	0.01	−2.22	0.16
1436939_at	217012	Cardiomyopathy-associated 4	*Cmya4*	Unknown EST	−0.93	0.01	−0.97	0.20
1419606_a_at	21955	Troponin T1, skeletal, slow	*Tnnt1*	Structural/motor	−3.84	0.01	−2.20	0.24
1448327_at	11472	Actinin alpha 2	*Actn2*	Structural/motor	−2.37	0.01	−1.82	0.17
1416454_s_at	11475	Actin, alpha 2, smooth muscle, aorta	*Acta2*	Structural/motor	−2.25	0.01	−1.58	1.00
1448371_at	17907	Myosin light chain, phosphorylatable, fast skeletal muscle	*Mylpf*	Structural/motor	−3.34	0.02	−1.94	0.38
1418726_a_at	21956	Troponin T2, cardiac	*Tnnt2*	Structural/motor	−4.26	0.03	−3.23	0.42
1418420_at	17927	Myogenic differentiation 1	*Myod1*	Transcription factor	−1.65	0.05	−1.28	1.00
1419487_at	53311	Myosin binding protein H	*Mybph*	Structural/motor	−2.09	0.06	−1.64	0.37
1420682_at	11443	Cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)	*Chrnb1*	Ion trafficking/signaling	−1.12	0.07	−0.83	0.94
1427306_at	20190	Ryanodine receptor 1, skeletal muscle	*Ryr1*	Ion trafficking	−1.26	0.08	−1.15	0.56
1427735_a_at	11459	Actin, alpha 1, skeletal muscle	*Acta1*	Structural/motor	−3.23	0.09	−1.70	1.00
1427445_a_at	22138	Titin	*Ttn*	Structural/motor	−2.81	0.11	−1.85	1.00
1441667_s_at	12180	SET and MYND domain containing 1	*Smyd1*	Transcription factor	−1.67	0.12	−1.20	0.60
1422529_s_at	12373	Calsequestrin 2	*Casq2*	Ion trafficking	−1.27	0.14	−0.87	0.94
1418417_at	17681	Musculin	*Msc*	Transcription factor	−1.86	0.19	−1.06	1.00
1418095_at	66106	Small muscle protein, X-linked	*Smpx*	Structural/motor	−1.53	0.20	−1.25	0.85
1418798_s_at	56504	Serine/threonine kinase 23	*Stk23*	Metabolism	−1.35	0.21	−0.33	1.00
1418373_at	56012	Phosphoglycerate mutase 2	*Pgam2*	Metabolism	−1.25	0.40	−0.85	1.00
1426650_at	17885	Myosin, heavy polypeptide 8, skeletal muscle, perinatal	*Myh8*	Structural/motor	−1.33	0.55	−0.99	1.00
1418852_at	11435	Cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)	*Chrna1*	Ion trafficking/signaling	−0.68	0.58	−0.53	1.00
1426731_at	13346	Desmin	*Des*	Structural/motor	−0.57	0.63	−0.39	1.00
1444139_at	73284	DNA-damage-inducible transcript 4-like	*Ddit4l*	Unknown EST	−0.80	0.64	−0.37	1.00
1418155_at	58916	Titin immunoglobulin domain protein (myotilin)	*Ttid*	Unknown EST	−0.85	0.67	−0.72	1.00
1422943_a_at	15507	Heat shock protein 1	*Hspb1*	Stress	−0.78	0.68	−0.36	1.00
1416889_at	21953	Troponin 1, skeletal, fast 2	*Tnni2*	Structural/motor	−0.88	0.69	−0.63	1.00
1460318_at	13009	Cysteine and glycine-rich protein 3	*Csrp3*	Transcription factor	−0.81	0.74	−0.52	1.00
1417101_at	69253	Heat shock protein 2	*Hspb2*	Stress	−0.87	0.76	−0.68	1.00
1448553_at	140781	Myosin, heavy polypeptide 7, cardiac muscle, beta	*Myh7*	Structural/motor	−0.86	0.89	−0.56	1.00
1429223_a_at	69585	Hemochromatosis type 2 (juvenile) (human homolog)	*Hfe2*	Signaling	−0.54	0.89	−0.46	1.00
1419312_at	11937	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	*Atp2a1*	Ion trafficking	−0.51	1.00	−0.43	1.00
1450917_at	17930	Myomesin 2	*Myom2*	Structural/motor	−0.51	1.00	−1.49	1.00
1418413_at	12391	Caveolin 3	*Cav3*	Metabolism	−0.49	1.00	−0.50	1.00
1450118_a_at	21957	Troponin T3, skeletal, fast	*Tnnt3*	Structural/motor	−0.49	1.00	−0.45	1.00
1420693_at	17929	Myomesin 1	*Myom1*	Structural/motor	−0.47	1.00	−0.40	1.00
1448826_at	17888	Myosin, heavy polypeptide 6, cardiac muscle, alpha	*Myh6*	Structural/motor	−0.45	1.00	−0.37	1.00
1426043_a_at	12335	Calpain 3	*Capn3*	Metabolism	−1.27	1.00	−0.39	1.00
1421984_at	20855	Stanniocalcin 1	*Stc1*	Signaling	−0.53	1.00	−0.38	1.00
1429783_at	56376	PDZ and LIM domain 5	*Pdlim5*	Structural/motor	−0.41	1.00	−0.38	1.00
1436867_at	106393	Sarcalumenin	*Sr1*	Ion trafficking	−0.41	1.00	−0.43	1.00
1427520_a_at	17879	Myosin, heavy polypeptide 1, skeletal muscle, adult	*Myh1*	Structural/motor	−0.40	1.00	−0.35	1.00
1419738_a_at	22004	Tropomyosin 2, beta	*Tpm2*	Structural/motor	−0.38	1.00	−0.66	1.00

Hom., homogenous; Het., heterogenous.

* Affymetrix Mouse 430 ver. 2.0 chip probeset ID.

† Entrez Gene accession numbers.

‡ As reported in Entrez Gene records.

§ Average fold change, expressed in log base 2.

‖ *P* incorporates a 20% false-discovery rate, so that any non-1 value is considered statistically significant.[43]

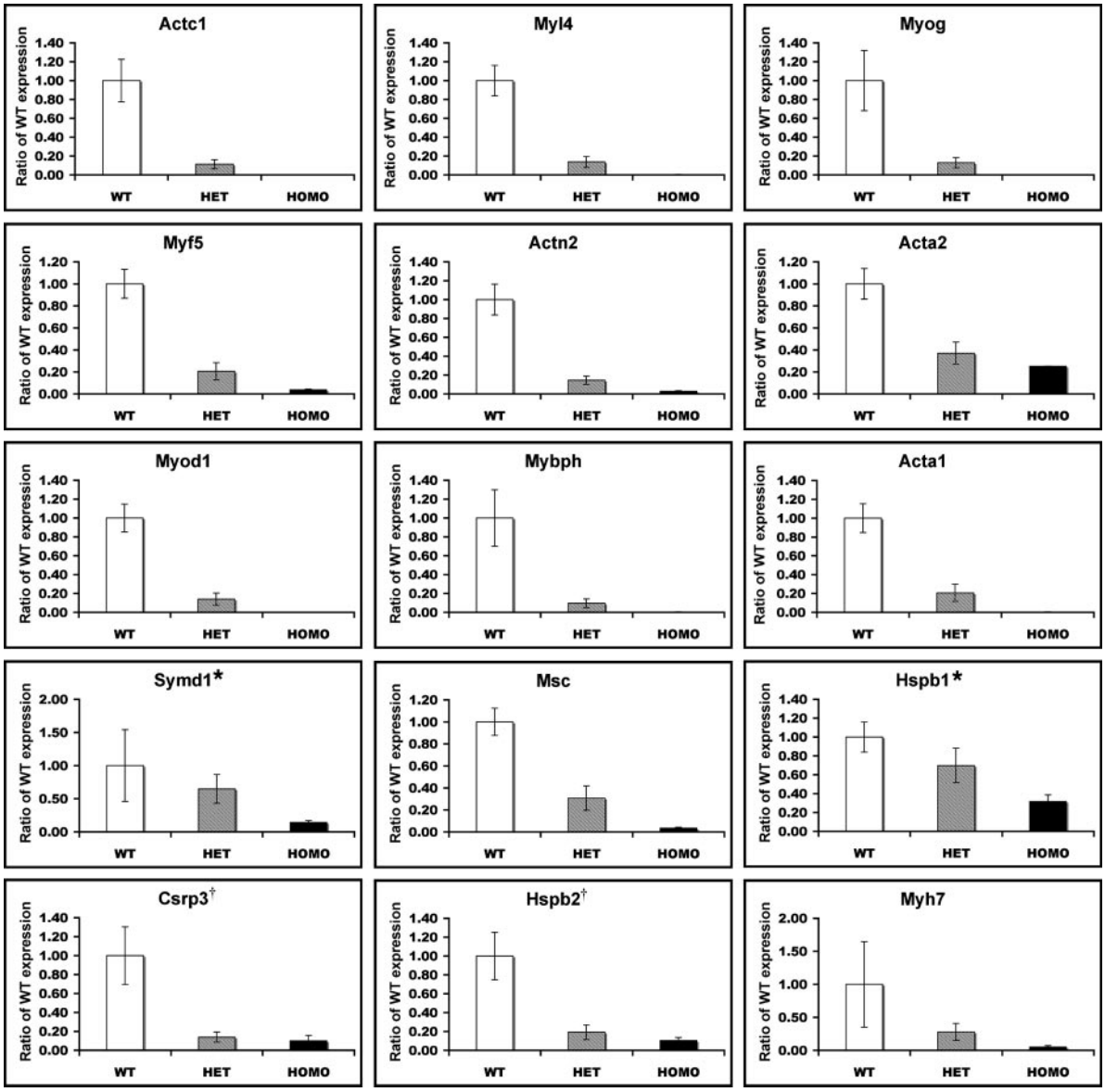


FIGURE 4. Quantitative real-time PCR validation of microarray results. Histograms illustrate observed expression levels for selected dose-sensitive genes. Expression levels are calculated as the ratio of wild-type expression. (□) $Pitx2^{+/+}$ (WT) expression level; (▨) represent $Pitx2^{+/-}$ (HET) expression levels; (■) $Pitx2^{-}$ (HOMO) ratio levels. Histograms illustrate average expression values ($n = 3$ embryos per genotype). Error bars, SEM for each genotype. *Overlapping confidence intervals for WT versus HET; †overlapping confidence intervals for HET versus HOMO.

cance of this expression has not been clear.[26,55] We propose that expression of these muscle-regulatory genes is required singly, or in combination, for specification and/or differentiation of extraocular muscles, similar to what has been demonstrated previously in other muscle groups. Future testing of this hypothesis requires careful analysis of the effects of individual and combinatorial knockouts of these genes on extraocular muscle development.

Among the key differences between extraocular muscles and other muscle groups is that *Pax3* and other very early key regulators of trunk myogenesis that are essential for activation of the muscle-regulatory genes are not expressed in the extraocular muscles.[21,22] *Pax7*, which is expressed, has been proposed as a functional replacement for *Pax3* in extraocular and other head muscles.[22] However, there is to date no genetic evidence to support this hypothesis. Our results provide compelling genetic evidence that PITX2 is essential to initiate muscle differentiation in extraocular muscles through a mechanism that includes activation of a set of muscle-regulatory genes. Although our results establish that *Pitx2* is genetically required for activation of *Myf5*, *Myog*, and *Myod1*, it is not possible to determine from our current data whether these genes are direct or indirect downstream targets of PITX2. However, it is interesting to note that the *cis*-acting regulatory elements required to recapitulate expression from these genes in transgenic mice have been identified, and each contains one or more potential PITX2 binding sites (Refs. 55,59,60 and data not shown). Therefore, it is feasible that each of these key regulatory genes are direct targets of PITX2 in extraocular muscles. Future testing of this hypothesis will require introduction of each relevant transgene into *Pitx2* mutant mice, as well as definitive proof of the PITX2-binding sites in cell culture and biochemical assays.

Although our current results are exciting because they identify the first model system for understanding the role of *Pitx2* gene dose effects in an ocular tissue, defects in extraocular

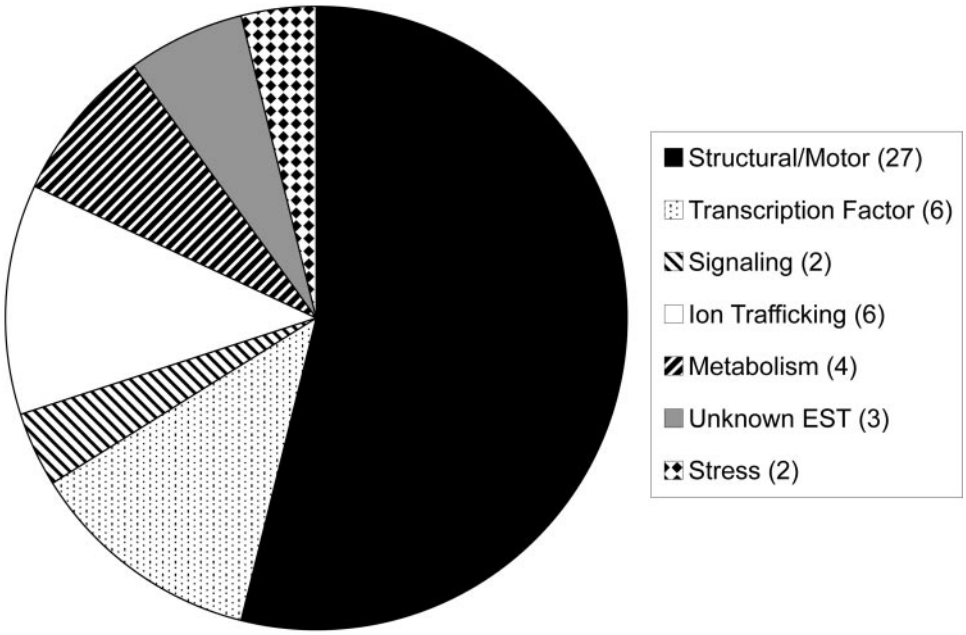


FIGURE 5. Biological functions of muscle-specific array hits. The number of genes in each class is indicated in parentheses.

muscle development and function are unlikely to account for the anterior segment changes and glaucoma observed in patients with Axenfeld-Rieger syndrome. However, we hypothesize that other ocular structures, in addition to extraocular muscles, are sensitive to *Pitx2* gene dose, and our current results establish that our *Pitx2* allelic series combined with gene expression analysis are likely to be a powerful approach for testing this hypothesis.

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