Analysis of Mutations of the *PITX2* Transcription Factor Found in Patients with Axenfeld-Rieger Syndrome

Tim Footz,[1] *Faisal Idrees,*[2] *Moulinath Acharya,*[1] *Kathy Kozlowski,*[3] *and Michael A. Walter*[1]

Purpose. To assess the effects of previously uncharacterized *PITX2* missense mutations found in patients with Axenfeld-Rieger syndrome and to determine the functional roles of the C-terminal region of PITX2.

Methods. Recombinant PITX2 proteins were analyzed with the use of cellular immunofluorescence, electrophoretic mobility shift, reporter transactivation, and protein half-life assays in human trabecular meshwork cells.

Results. Two homeobox mutations, R43W and R90C, resulted in severely reduced DNA-binding and transcriptional activation despite normal nuclear localization. L105V, located C-terminal to the homeodomain, resulted in normal localization, reporter gene transactivation, and protein half-life, but with an altered mobility shift pattern of protein-DNA complexes. N108T, also located C-terminal to the homeodomain, resulted in an altered mobility shift pattern and with slightly increased reporter transactivation and shortened protein half-life. The PITX2 C-terminal region contains at least three domains, each with distinct modulating effects on reporter transactivation.

Conclusions. PITX2 homeobox mutations predictably resulted in decreased function of the protein. However, the two C-terminal mutations exhibited only subtle defects on PITX2 transactivation and protein-DNA binding, suggesting that ocular development is sensitive to even slight alterations of PITX2 function. The C-terminal mutations L105V and N108T lie in a domain that inhibits PITX2 transcriptional activation. These two mutations produce electrophoretic mobility shift assay patterns representing altered protein-DNA interactions that may be important for accurate target gene selection. Additionally, N108T resulted in a less stable PITX2 mutant protein with elevated activity that may result in stochastic dysregulation during critical stages of development. Together, the results clearly indicate that stringent control of PITX2 is required for normal ocular development and function. (*Invest Ophthalmol Vis Sci.* 2009;50:2599–2606) DOI:10.1167/iovs.08-3251

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Axenfeld-Rieger syndrome (ARS) encompasses a spectrum of ocular anterior chamber defects, such as iris hypoplasia, corectopia, polycoria, iridocorneal adhesions, and prominent Schwalbe line, with a high incidence of early-onset glaucoma.[1] The occurrence of these and other congenital anterior chamber defects, in conjunction with variably expressed systemic malformations such as dental and umbilicus anomalies, are known to be the result of mutations in the transcription factors *PITX2,*[2] *FOXC1,*[3,4] and *PAX6.*[5]

Various genetic defects that can cause ARS have been identified in *PITX2*, located on chromosome 4q25. Missense mutations are usually restricted to the DNA-binding homeodomain[2,6–15] (HD; Fig. 1), whereas splice-site,[2,8,16,17] frameshift, and nonsense[2,8,9,13,18–21] mutations may occur throughout the gene, leading to truncated protein products. Microdeletions of 4q25 have also been reported.[20,22,23]

In vitro assays are often used to study the effects of mutations on a protein's physical interactions and reporter transcriptional activation abilities and on subcellular distribution. HD mutations can result in a change from a normally nuclear-restrictive immunostaining pattern.[24] Mislocalized PITX2 protein would be unable to fulfill its roles as a direct[25–31] and indirect[32,33] regulator of target gene transcription. Specifically in ocular development, PITX2 has been implicated as a key effector of anterior segment patterning by integrating retinoic acid and Wnt signaling.[34] HD mutations can hamper the ability of the protein to bind cognate DNA target sequences, resulting in abnormal regulation of target genes; this has been directly measured in reporter assays.[9,24,31,35–38]

Protein-truncating mutations lead to unexpected effects on PITX2 activity. In one report, recombinant PITX2A, designed to test a frameshift mutation that removes the C-terminal 46 amino acids, exhibited reduced activation of a *Dlx2* reporter construct in Chinese hamster ovary (CHO) cells.[36] In another report, recombinant PITX2A proteins based on mutations that remove the C-terminal 139 or 150 amino acids demonstrated increased activation of a *prolactin* reporter construct in various cell types, including CHO cells.[21] These results suggest that the in vivo context of PITX2 binding is critically important to its specific activity as a transcription factor. For further evidence of the complex nature of PITX2 function, the C-terminal region of PITX2 as a whole was shown to facilitate intramolecular interactions that modulate DNA binding and transactivation,[37,38] and the multiple isoforms produced from alternative promoter use and alternative splicing[39,40] exhibit subtle differences in transactivation ability and tissue-specific and developmentally specific expression patterns.[27,41–43] Additionally, the phosphorylation of PITX2 modulates its activity.[36,44]

In this study, we assayed the effects of previously uncharacterized *PITX2* missense mutations from patients with ARS (Table 1), including two in the C-terminus, and further dissected the functional roles of the C-terminal region of PITX2.

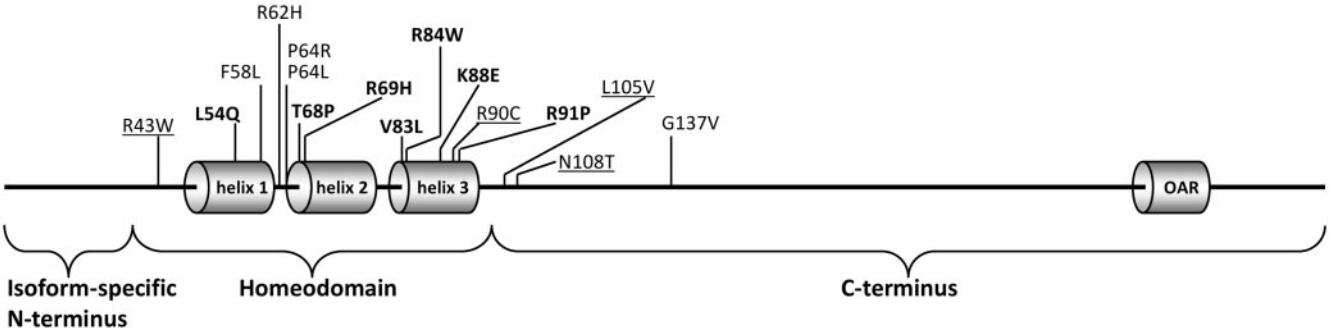

FIGURE 1. Schematic of missense mutations in PITX2. A representation of the linear sequence of PITX2 is shown, with confirmed or suspected α-helical regions depicted as cylinders. Expressed isoforms differ only in the sequence N-terminal to the conserved 60-amino acid homeodomain. Missense mutations are labeled with their positions drawn relative to their order in the primary amino acid sequence of PITX2A. Mutations that have been previously characterized by functional studies are in *bold*, whereas mutations studied in this report are *underlined* (and, because of their locations, would be present in all PITX2 isoforms).

MATERIALS AND METHODS

PITX2A cDNA Constructs

The wild-type Xpress-tagged PITX2A protein isoform was expressed from a cDNA carried in the pcDNA4/HisMaxA plasmid (Invitrogen, Burlington, ON, Canada), whose construction was previously described.[24] R43W, R90C, L105V, and N108T variants of the *PITX2A* cDNA were generated with the use of a mutagenesis kit (QuickChange Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA) according to the manufacturer's instructions. Forward mutagenesis primers of each complementary pair were as follows: R43W, 5′-GCA AAG GCG GCA GTG GAC TCA CTT TAC C-3′; R90C, 5′-GGT TTG GTT CAA GAA TTG TCG GGC CAA ATG G-3′; L105V, 5′-CCA GCA GGC CGA GGT ATG CAA GAA TGG C-3′; and N108T, 5′-CGA GCT ATG CAA GAC TGG CTT CGG GCC-3′. *Nar*I/*Sac*II fragments of the mutant constructs were then subcloned into a pCI-HA-PITX2A vector.[32] HA-PITX2A deletion constructs have been described.[32] Plasmid DNA was isolated (Maxiprep or QIAprep Spin Miniprep kits; Qiagen Inc., Mississauga, ON, Canada), and open-reading frames were fully sequenced.

Protein Expression and Western Blot Analysis

COS-7 cells and human trabecular meshwork (HTM) cells were cultured in Dulbecco's modified Eagle medium (4.5 g/L and 1 g/L glucose, respectively) + 10% fetal bovine serum + 1% penicillin-streptomycin at 37°C with 5% CO_2. Then 10^6 cells on 100-mm–diameter plates were transfected with 4 μg plasmid DNA and 12 μL reagent (FuGENE 6; Roche Diagnostics, Laval, QC, Canada) or 800 ng DNA + 2.4 μL reagent (FuGENE 6; Roche Diagnostics) with 2×10^5 cells in 35-mm–diameter wells. Cells were harvested by scraping 48 hours after transfection. Protein extracts were prepared on ice by lysis in cationic buffer (20% glycerol, 20 mM HEPES, pH 7.6, 500 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Triton X-100) supplemented with 1 mM phenylmethylsulfonyl fluoride and 0.5% protease inhibitor cocktail (P8340; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), as described.[24] Whole-cell protein extracts were resolved by SDS-PAGE (10% or 12% gels) and detected by immunoblotting onto nitrocellulose and hybridizing with anti-Xpress (Invitrogen) or HA probe (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies to detect epitope-tagged recombinant proteins, as previously described.[24] Blots were also immunoprobed with αTFIID (TBP; Santa Cruz Biotechnology, Inc.) antibodies to detect an unrelated, endogenously expressed protein to serve as a loading control.

Immunofluorescence

As described, 10^5 HTM cells were plated onto glass coverslips in 35-mm–diameter wells and transfected with plasmid DNA. Immunofluorescence was performed as described previously[45] except that fixation was performed for 20 minutes. Antibodies (αXpress and αMouse-Cy3; Jackson ImmunoResearch, West Grove, PA) were used at 1:500.

Electrophoretic Mobility Shift Assay

Whole-cell protein extracts from transfected COS-7 cells were equalized to 1× wild-type PITX2A protein levels by Western blot analysis. ^{32}P-Labeled double-stranded DNA probe containing the *bicoid* binding site (bicoidBS, underlined) was prepared by Klenow fragment-mediated end-filling of overlapping oligonucleotides (forward, 5′-GAT CCA AAT AAT CCC AAC AGA-3′; reverse, 5′-GAT CTC TGT TGG GAT TAT TTG-3′) with ^{32}P-dCTP. Increasing amounts of cell extracts were incubated for 15 minutes in 10-μL reactions containing 5% glycerol, 1 mM dithiothreitol, 0.5 μg poly(dI-dC), 1 μg single-stranded salmon sperm DNA, and 20 ng FOXC1BS oligomers[46] as nonspecific blockers before

TABLE 1. Summary of Phenotypes Associated with PITX2 Mutations

PITX2 Mutation	Reference	Ocular Findings	Other Findings
R43W	Idrees et al.[11]	Posterior embryotoxon, iris hypoplasia, peripheral anterior synechiae, corectopia, glaucoma	Dental, umbilical, and brain abnormalities
R90C	Perveen et al.[8]	Rieger anomaly	Dental and umbilical abnormalities, cleft palate, learning difficulties
L105V	Phillips[10]	Iris hypoplasia, angle defect, posterior embryotoxon	Dental and umbilical abnormalities
N108T	Phillips[10]	Iris hypoplasia, angle defect, posterior embryotoxon	Dental and umbilical abnormalities

the addition of 100,000 cpm bicoidBS probe and incubation for 15 minutes. Samples were electrophoresed by nondenaturing 6% PAGE in 1× Tris-Glycine-EDTA buffer at 15 V/cm at room temperature, and the gel was subsequently dried to filter paper before exposure to x-ray film (SuperRX; Fuji Film, Tokyo, Japan).

Reporter Transactivation Assay

A bicoidBS luciferase reporter plasmid was constructed by subcloning the bicoidBS oligonucleotide into the *Bgl*II restriction site of the pGL3-promoter vector (Promega, Madison, WI) in the 5′-TAATCC-3′ orientation and then replacing the SV40 promoter with the TK promoter.[46] *PITX2A*-containing plasmid (160 ng) or the appropriate empty expression vector was cotransfected (in triplicate) with 60 ng pGL3-bicoidBS-TK reporter and 60 ng pCMVβ transfection control vector into HTM cells (4×10^4 cells/15-mm well, 24-well plate) with 0.28 μL reagent (FuGENE 6; Roche Diagnostics). Cells were harvested with 100 μL buffer (Passive Lysis Buffer; Promega) 48 hours after transfection. Firefly luciferase activity was measured by luminometry (Turner Designs, Sunnyvale, CA) from 10 μL protein lysate mixed with 100 μL reagent (Luciferase Assay Reagent; Promega) and was standardized to the β-galactosidase (internal control) activity quantitated by the β-galactosidase enzyme assay system (Promega) from 75 μL protein lysate.

Protein Stability

Cycloheximide (CHX; 50 μg/mL) was added to HA-PITX2A–transfected HTM cells 24 hours after transfection to inhibit protein synthesis, and lysates were harvested at various times. Bradford assay-equalized samples were subjected to immunoblot analysis and to probing with HA-probe and αTFIID antibodies. Band intensities were determined by densitometric scanning (Image Station 4000MM and Molecular Imaging Software version 4.0.5; Eastman Kodak, Rochester, NY). Rates of decay of PITX2A proteins were determined from two independent experiments.

Results

Expression of Mutant Proteins

Idrees et al.[11] discovered that the PITX2 missense mutation R43W segregated with typical ARS phenotypes (variable expression of iris hypoplasia, posterior embryotoxon, corneal adhesions, corectopia, glaucoma, redundant periumbilical skin, craniofacial dysmorphism, microdontia, and hypodontia) in a multigenerational pedigree. This was the first report of a PITX2 mutation that was also associated with brain abnormalities (variable expression of small sella turcica or enlarged cisterna magna).[11] Perveen et al.[8] reported an uncharacterized HD mutation, R90C, that also associated with typical ARS findings. The first report of PITX2 missense mutations in the C-terminal domain was by Phillips,[10] who discovered that L105V and N108T were associated with iris hypoplasia, angle defects, posterior embryotoxon, oligodontia, microdontia, maxillary hypoplasia, and redundant umbilical skin. These four nonsynonymous substitutions (summarized in Table 1) were introduced into Xpress-tagged PITX2A constructs and into HA-tagged constructs to test their expression and function in mammalian cells. As shown in Figure 2a, these amino acid substitutions did not have any effects on the apparent molecular weight of PITX2A in HTM cells.

Subcellular Localization

To investigate the subcellular localization of the Xpress-PITX2A mutant constructs, transiently transfected HTM cells grown on coverslips were fixed and immunoprobed with fluorescently labeled antibodies (Fig. 2b). As expected, wild-type PITX2A localizes almost exclusively to the nuclei of transfected cells. PITX2A harboring the R43W, R90C, L105V, or N108T mutations exhibited the same distribution as wild type, though transfection with R90C was routinely inefficient and resulted in few fluorescing cells per slide with no obvious effect on the viability of the culture (data not shown).

Electrophoretic Mobility Shift Assay

In an electrophoretic mobility shift assay (EMSA), lysates from COS-7 cells transfected with wild-type Xpress-PITX2A displayed a complex migration pattern of bands representing PITX2A protein bound to the bicoidBS (TAATCC) DNA element (Fig. 2c). The R43W and R90C mutations appeared to completely abolish the formation of any detectable complexes because no band shifts were observed in the EMSA. The L105V mutation resulted in an overall visually reduced amount of shifted probe, with a relative reduction of higher mobility bands. The N108T mutation caused an altered pattern of shifted bands, with an increased abundance of high mobility bands. It is unknown whether the results of this assay reflected altered in vivo binding specificity or selectivity of PITX2 or whether the different shifted complexes represent monomeric versus dimeric PITX2 binding. However, the unusual patterns demonstrated that the L105V- and N108T-bearing proteins retained the ability to bind a cognate target sequence, though in ways that were qualitatively different from the pattern of wild-type PITX2.

Transcriptional Activation Assay for PITX2A Mutations

Luciferase reporter assay was performed to compare the effects of the ARS mutations on the transactivation potential of Xpress-PITX2A. Wild-type PITX2A caused an approximately 10-fold increase in activation (compared with empty expression vector) of a reporter driven by a TK promoter containing the bicoidBS upstream (Fig. 2d). R43W and R90C samples exhibited only 23% and 37% of the activity of wild type, respectively, whereas L105V was not statistically different from that of wild type (Fig. 2d). N108T activity was slightly higher than wild type (118%), with statistical significance.

Protein Stability

HA-PITX2A–transfected HTM cells were subjected to cycloheximide treatment to halt protein synthesis as a means of studying protein turnover in vivo. The levels of wild-type HA-PITX2A started declining after 1 hour of CHX treatment, and a small amount was detected after 6 hours (Fig. 3). R90C was chosen as a representative of HD mutations; hence, R43W was not included in this experiment. The R90C and L105V constructs did not show any deviation from wild type with respect to the slope of HA-PITX2A decay over the time-course. However, the N108T construct exhibited a statistically significant steeper negative slope, suggesting that this mutant protein is less stable and has a reduced intracellular half-life.

Dissection of PITX2A Functional Domains

To further assess the significance of mutations in a region outside the PITX2 HD, a series of protein truncations[32] (Fig. 4) was subjected to the same transcriptional activation assay as described to assign functionality to the different domains. For clarity, we have subdivided the protein into five distinct regions (Fig. 4b), as follows: I (residues 1–38) is the N-terminus specific to isoform A; II (residues 39–98) comprises the homeodomain; III (residues 99–159) has no distinct structural or

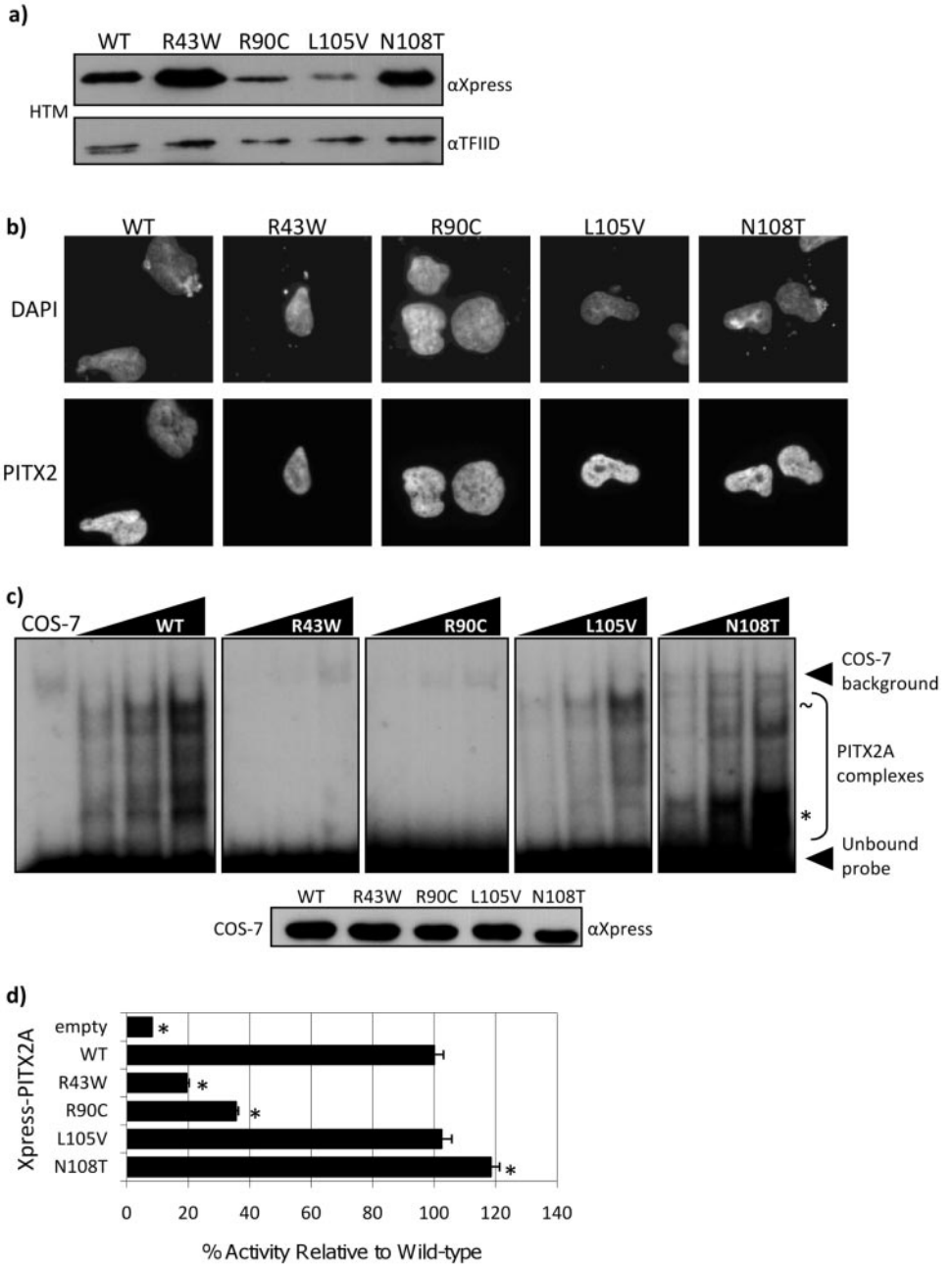

FIGURE 2. Analysis of ARS-causing mutations in Xpress-PITX2A constructs. (**a**) Western blot analysis of 20 μg transfected HTM whole-cell lysates, probed with αXpress antibody to reveal the 36-kDa recombinant PITX2 protein and subsequently probed with αTFIID to serve as a loading control. (**b**) Fluorescence microscopy of transfected HTM cells probed sequentially with αXpress and αMouse-Cy3 antibodies (*bottom*). Nuclei were counterstained with DAPI (*top*). (**c**) Electrophoretic mobility shift assay of increasing amounts of whole-cell lysates from transfected COS-7 cells (equalized by Western blot analysis of adjusted volumes of lysates, shown below the autoradiograms). Untransfected COS-7 lysate is shown as a negative control exhibiting a single low-mobility shifted background band. Complexes of radiolabeled bicoidBS probe bound to PITX2A are shifted to various bands in the middle of the autoradiograph, with unbound probe migrating to the bottom. High-mobility (*) and low-mobility (~) bands altered in the L105V and N108T samples, respectively, are indicated. (**d**) Transactivation assay in cotransfected HTM cells. Luciferase values (from activation of the bicoidBS-reporter plasmid) were normalized to β-galactosidase (expressed from the transfection control plasmid), averaged for three separate transfections, and expressed relative to the ratio for wild-type Xpress-PITX2A. Error bars represent SEM compared with wild-type. *Asterisks*: samples with a significant difference ($P < 0.05$; *t*-test) calculated from comparison with wild-type.

functional properties; IV (residues 160–232) is a low-complexity region with a preponderance of serine, proline and asparagine residues; and V (residues 233–271) includes the OAR domain (14 amino acids homologous to *o*tp, *a*ristaless, and *r*ax; PROSITE PS50803) and the remainder of the C-terminus.

First, the expression of the HA epitope-tagged constructs in HTM cells was confirmed through Western blot analysis (Fig. 4a). All HA-PITX2A proteins migrated with the expected apparent molecular weight, except that Δ233–271 presented as a doublet band. A similar doublet can also be observed in PITX2 C-terminal truncations, studied by Saadi et al.[21] We sought, but did not find, evidence that the doublet resulted from differential phosphorylation or O-GlcNAc glycosylation (data not shown).

Next, the truncation-construct plasmids were cotransfected in HTM cells with the bicoidBS-luciferase plasmid in a reporter assay (Fig. 4b). The level of reporter activation by the wild-type HA-PITX2A construct was similar to that of the Xpress-tagged construct (data not shown). Deleting the HD (Δ39–98) from PITX2A severely compromised reporter activation as expected because the DNA-binding domain was absent. Activity of the Δ1–38 construct was significantly reduced, to 32%. This indicated that the isoform-specific N-terminus of PITX2A is necessary for most of the transactivation potential of the protein. At a significance threshold of $P < 0.05$, the constructs Δ160–190 and Δ191–232 are not significantly different from wild type, but they showed a tendency to produce less reporter activation than wild type (60%–70%), suggesting that region IV may constitute an activation domain to further enhance the transactivity conferred onto the PITX2 protein by the N-terminus. The constructs Δ99–159 and Δ233–271 each produce reporter activation at nearly 300% of wild-type PITX2, indicating that regions III and V serve to inhibit the transactivity of PITX2A.

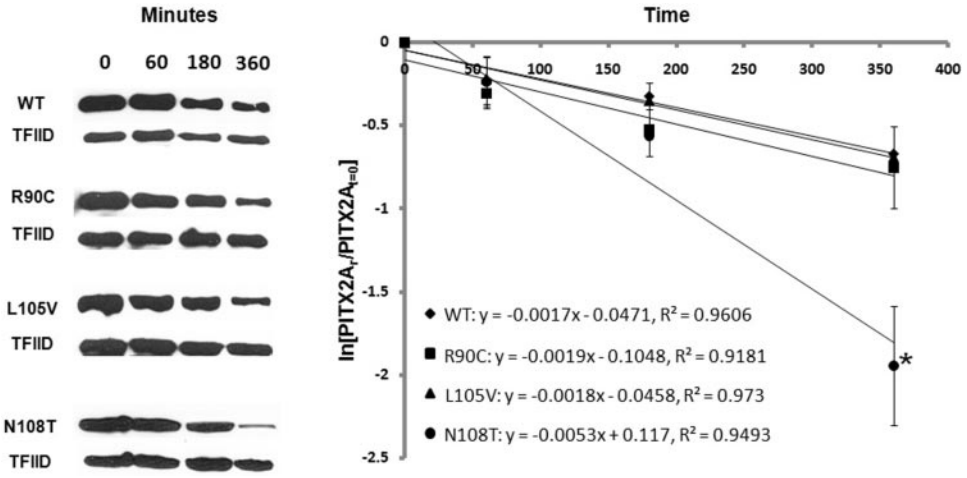

FIGURE 3. Stability of PITX2A constructs. Transfected HTM cells were treated with CHX for the indicated times to inhibit protein synthesis, and 15 μg cell extracts were probed sequentially, by Western blot analysis, with the antibodies HA-probe (to detect recombinant PITX2) and αTFIID (to serve as a loading control). Immunoblots were scanned, and net pixel intensities of the bands were measured with imaging software. PITX2A values were normalized to TFIID, averaged for two separate transfections, and expressed compared with wild-type HA-PITX2A. *Asterisk*: the slope of N108T decay was significantly different ($P = 0.05$, *t*-test) from that of wild-type.

DISCUSSION

The functional outcomes of a mutation in PITX2 can often be reliably predicted because of the characteristic genotype/phenotype correlation established from molecular studies.[24] Based on structural modeling of the PITX2-DNA interface,[47] it is clear that mutations in HD residues that make direct contact with a DNA target should reduce affinity for a DNA probe in EMSA experiments. The ARS mutations R43W and R90C followed this prediction accordingly (Fig. 2c). Given that R5 in the HD (equivalent to R43 in PITX2A) makes direct contact with DNA,[47] the R43W mutation would likely prevent binding of the mutant PITX2A protein to the target sequence because the W43 side chain is bulky and would sterically hinder incorporation into the protein-DNA interface. Similarly, R52 (equivalent to R90 in PITX2A) is extremely well conserved (Fig. 5)[48] and contacts DNA.[47] It is, therefore, unlikely to tolerate substitution. PITX2A constructs bearing these two mutations resulted in no detectable binding to the bicoidBS probe. Consequently, it is not surprising that both mutant constructs also failed to result in transactivation of a bicoidBS-containing reporter plasmid at wild-type levels (Fig. 2d). Activation of R90C was greatly reduced, and that of R43W was almost at empty vector values. Transient trace level binding may occur in vivo that in vitro mobility shift assays are unable to detect, which may explain why these mutant constructs have residual activity.

It has been shown that a construct encompassing region III (specifically, residues 99–193) is involved in PITX2 dimerization.[37] Therefore, we hypothesized that the L105V and N108T mutations might affect intermolecular interactions. Our EMSA experiment revealed differences in the quality of DNA binding of these mutant Xpress-PITX2A constructs. The L105V-transfected sample appears to have a reduced number of high-mobility DNA-protein complexes that, in contrast, are enriched in the N108T-transfected sample. It is possible that our EMSA results indicate PITX2 binding to DNA probes in monomer (high-mobility) or dimer (low-mobility) forms[38] in which L105V favors, but N108T discourages, dimerization. Regard-

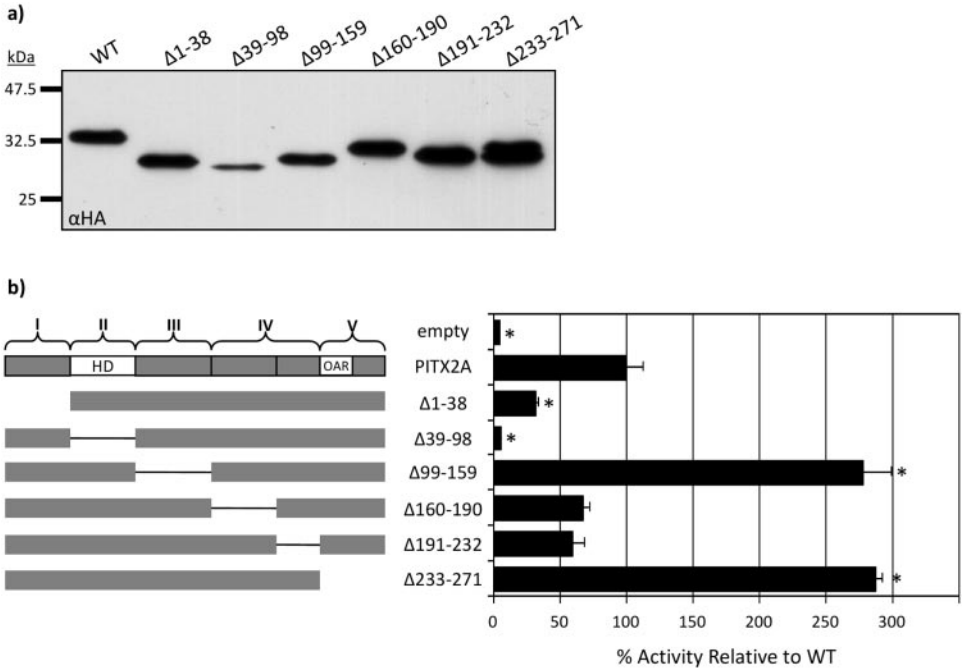

FIGURE 4. Functional dissection of PITX2A. (**a**) Western blot analysis of 20 μg transfected HTM whole-cell lysates (80 μg for Δ39–98) probed with HA-probe antibody to detect recombinant PITX2A. (**b**) Transactivation assay of cotransfected HTM cells. Luciferase values (from activation of the bicoidBS-reporter plasmid) were normalized to β-galactosidase (expressed from the transfection control plasmid), averaged for three separate transfections, and expressed compared with the ratio for wild-type HA-PITX2A. Error bars represent SEM, relative to wild type. *Asterisks*: samples with a significant difference ($P < 0.05$; *t*-test) calculated from comparison with wild-type.

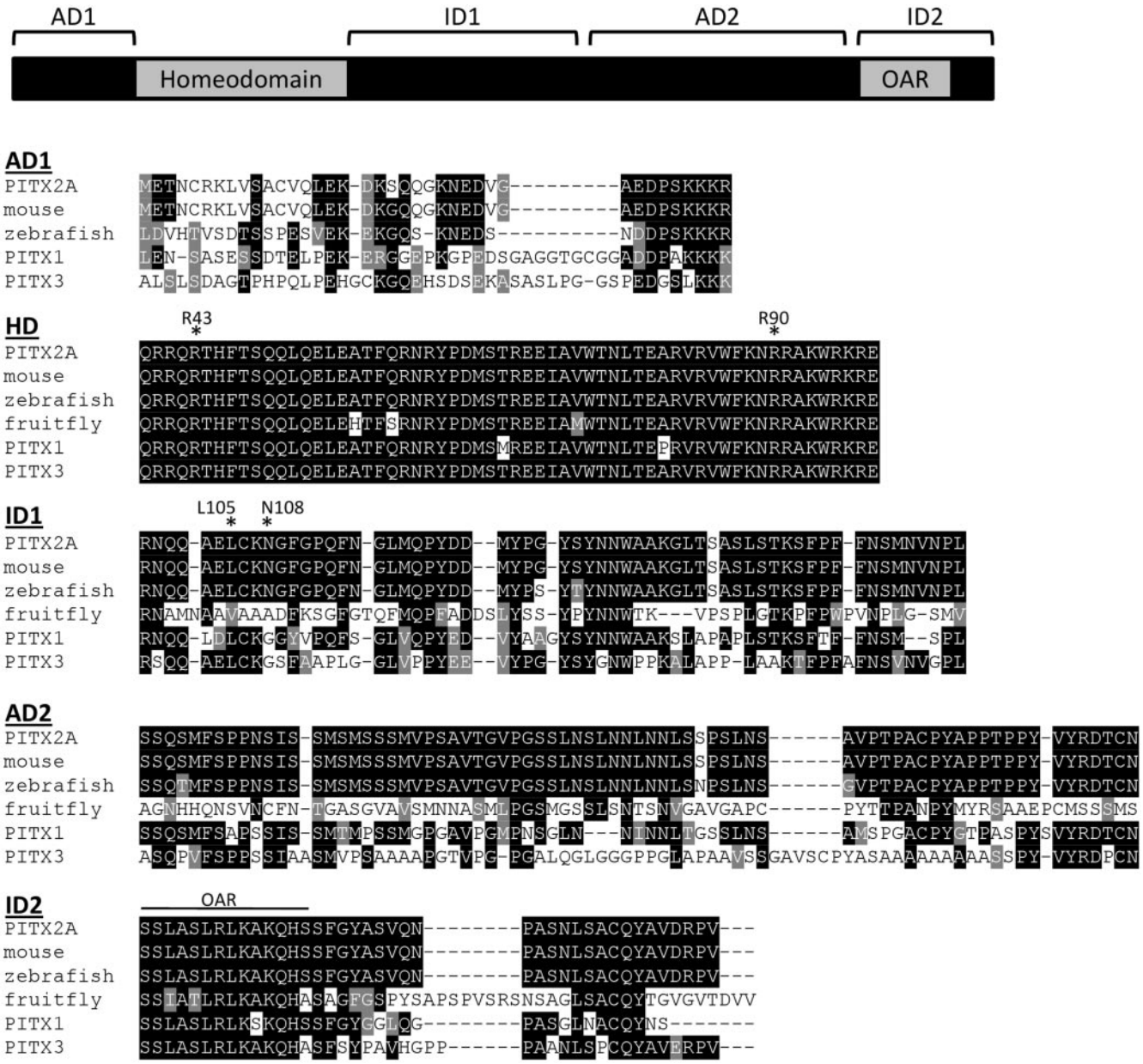

FIGURE 5. Evolutionary conservation of PITX2 domains. AD1, specific to isoform A of PITX2, is located at residues 1–38. AD2 is located at residues 160–232. The HD is located at residues 39–98. Transcriptional inhibitory domains are located at residues 99–159 (ID1) and 233–271 (ID2). ID2 contains the OAR motif. Sequences used in the domain alignments are as follows: human PITX2A (NP_700476.1), mouse Pitx2A (NP_001035969.1), zebrafish PITX2A (NP_571050.1), fruit fly Ptx1A (NP_733410.2), human PITX1 (NP_002644.4) and human PITX3 (NP_005020.1). Alignments were generated with commercial software (ClustalW2; http://www.ebi.ac.uk/Tools/clustalw2/index.html). *Black*: identical residues. *Gray*: similar residues. Mutations analyzed in this report are indicated by *asterisks*.

less, our EMSA results show that L105V and N108T can still bind the bicoidBS probe at levels similar to those of wild type. Luciferase assay revealed that L105V has no significant effect on transactivation of the bicoidBS-containing reporter. N108T exhibits slight hyperactivity on this synthetic target promoter suggesting that, like the previously studied PITX2 HD mutation V83L, anterior ocular development is sensitive to even subtle gain-of-function mutations in PITX2.[9] These mutations may elicit more dramatic responses on other native target sequences that are sensitive to their effects on monomeric versus dimeric protein-DNA binding.

Any alteration to a primary amino acid sequence can influence a protein's secondary and tertiary structure, which can manifest as a change in its cellular abundance. The longevity of maintaining detectable levels of a given protein in cells disrupted for protein synthesis is a measure of the molecule's stability. To investigate the possibility of the C-terminal PITX2 mutations having effects on protein stability, the levels of recombinant PITX2 proteins in cells treated with CHX were determined. The L108T mutation did not lead to a measurably altered protein half-life. Interestingly, however, the N108T mutation affected protein stability by causing the protein to decay faster than wild-type HA-PITX2A. The consequence of the combination of qualitatively altered DNA-binding with elevated transactivity and protein instability in a patient harboring the N108T mutation would likely be dysregulation of PITX2

targets and is strongly consistent with the suggestion that PITX2 activity is under stringent control for normal ocular development and function.

The C-terminus of PITX2 has been shown to have inhibitory[38] and stimulatory[21] effects on transactivation, depending on the specific deletion construct and promoter context examined. Our PITX2A deletion constructs were used to further delineate discrete domains that confer inhibitory or stimulatory effects on PITX2 transactivation. Our results with constructs Δ1–38 and Δ39–98 (Fig. 4b) are consistent with a previous study[38] demonstrating that the N-terminus of PITX2A (region I) enhances transcriptional activation of a reporter and that the HD (region II) is essential for function as a transcription factor. Removal of residues 99–159 from PITX2A resulted in a hyperactive effect revealing that region III inhibits PITX2 activity. The slightly increased activity observed with the N108T construct (Fig. 2d) may be attributable to disruption of the region III inhibitory domain. However, L105V had no effect on transactivity in our assay, suggesting that localized disruption of the intermolecular or intramolecular interactions of region III is not a commonality in these "neighboring" mutations. Instead, there remains the possibility that these C-terminal mutations exerted their disease-associated effects in specific cell or binding target contexts that our reporter assay did not test. We propose that residues 160–232 encompass a single stimulatory module (region IV) based on the apparent hypoactive effects of the separate Δ160–190 and Δ191–232 constructs and the characteristic low-complexity in amino acid sequence spanning these adjacent segments (Fig. 5). The separate constructs did not result in a statistically significant difference in transactivation when considered at a stringent value ($P < 0.05$), but we predict they would show additive effects if tested in a combined Δ160–232 construct. Finally, the extreme C-terminus (region V) containing the OAR domain encodes a distinct inhibitory domain. Construct Δ233–271 results in increased transactivation to a degree similar to deletion of region III. In summary, Figure 5 depicts PITX2 as a protein composed of a mosaic of functional modules. Activation domain (AD) 1 is poorly conserved in zebrafish, fruit flies (not shown), and paralogous human PITX proteins, which is an indication of the specialization of PITX2 encoded by the alternative isoforms that differ only at the N-terminus. The HD is very well conserved throughout evolution, reflecting its functional sensitivity to mutation. Inhibitory domain (ID) 1 and AD2 are moderately well conserved in various homologous and paralogous proteins. By virtue of their location in ID1, the mutations L105V and N108T could perturb the transactivation ability of PITX2 independently of its ability to bind DNA. Finally, inhibitory ID2 encompasses the OAR and, like the HD, its functional significance is highlighted by the high degree of evolutionary conservation.

Although our C-terminal deletions are not exact re-creations of previously studied expression constructs[21,38,44] and we did not use the same cell types or reporter plasmids, it is interesting to analyze the published results of molecular dissections of PITX2 in the context of these new findings. Deletion of ID2 (Fig. 5), or most of the region, had the effect of slightly decreasing PITX2 transactivation of various reporters in COS-7 or CHO cells (with the CΔ39 and ΔT1261 constructs).[38,44] This region was also demonstrated to be required for binding by PIT1, which acts to promote synergistic transactivation with PITX2A.[38] Those authors proposed a model by which self-regulating intramolecular interactions involving the PITX2 C-terminal tail are relieved by intermolecular interactions, such as homodimerization or binding to other transcription factors, such as PIT1, but are not relieved when ID2 is absent.[38] In our assay with HTM cells, however, we observed that deletion of ID2 results in greatly increased transactivation (Fig. 4b). We interpret this result as evidence that cell-specific factors may alleviate PITX2-intrinsic inhibition despite the absence of this regulatory domain that is important in other cellular contexts. Saadi et al.[21] reported that larger C-terminal deletions of PITX2A (into ID1) led to increased transactivation in COS-7 and CHO cells. Presumably, these constructs disrupt the intramolecular interactions that otherwise suppress PITX2A activity. This scenario is consistent with the behavior of our Δ99–159 construct, which helps to delineate the regions of the PITX2 C-terminus required for the inhibition of activity (ID1 and ID2).

This is the first report of PITX2 functional analyses performed in an ocular cell line (HTM) relevant to ARS and glaucoma. The R43W and R90C mutations, which lie within the HD, alter residues structurally known to be important for protein-DNA contacts and, as expected, severely reduce the ability of PITX2 to bind and transactivate the bicoidBS target. These findings are consistent with the unusually severe clinical presentations of patients with R43W mutations. The L105V mutation is enigmatic in that it had no effect on any quantitative assayed properties of PITX2A in vivo. Because the original report of the L105V and N108T mutations studied the sequence of only *PITX2* and did not provide details of the affected pedigrees,[10] it remains a possibility that the associated ARS features were caused by defects in genes other than *PITX2*. In this manner, L105V and N108T may be rare variants with no or only mild effects on patient phenotypes. However, the unique EMSA bandshift patterns for the L105V and N108T samples suggests these mutations alter PITX2 homodimerization, which may be important for the precise regulation of specific target genes. The N108T mutation exhibits elevated reporter activation but also causes destabilization of PITX2. The combination of these altered PITX2 properties, which result from the N108T mutation, could manifest as stochastic effects on protein function and target gene regulation during ocular development. We identified three discrete functional domains in the C-terminal tail of PITX2. Two of these domains are inhibitory to reporter transactivation and are separated by a domain responsible for increasing transactivity. The L105V and N108T mutations are located in the HD-proximal inhibitory domain (ID1) and may exert their ARS-associated effects through the involvement of molecular interactions of this domain in a target gene-specific, cell-specific, or isoform-specific context. These analyses establish that prediction of the effects of PITX2 mutations outside the HD is not straightforward. In this context, the characterization of distinct functional regions in the PITX2 C-terminal domain will be a useful tool for further studies on the complexities of PITX2 regulation.

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