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Structural and Functional Analysis of Single Nucleotide Polymorphic Variants of Purine-Rich Element Binding Protein B

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Abstract

Purine-rich element-binding protein B (Pur β) inhibits myofibroblast differentiation by repressing the expression of the smooth muscle α -actin gene (*Acta2*). Several reports have identified the structural domains in Pur β that enable its characteristic interaction with purine-rich single-stranded DNA (ssDNA) sequences in the *Acta2* promoter. However, little is known about the physical and functional effects of single nucleotide polymorphisms that alter individual amino acid residues in Pur β . This study evaluated seven rare single amino acid variants of human *PURB* engineered into the homologous mouse Pur β protein. Mapping the location of variant residues on a homology model of the Pur β homodimer suggested that most of the altered residues are remote from the predicted ssDNA-binding regions of the protein. The repressor activity of each Pur β variant was assessed in transfected fibroblasts and smooth muscle cells via *Acta2* promoter-reporter assays. A Q64* nonsense variant was completely inactive while missense variants exhibited repressor activity that ranged from ~1.5-fold greater to ~2-fold less than wild-type Pur β . Lower activity variants P223L and R297Q were expressed in bacteria and purified to homogeneity. Each variant was physically indistinguishable from wild-type Pur β in terms of quaternary structure and thermostability. Results of DNA and protein binding assays indicated that the P223L and R297Q variants retained high affinity and specificity for purine-rich ssDNA sequences but differed in their interaction with other *Acta2* regulatory proteins. These findings suggest that the presence of certain variant residues affects the *Acta2* repressor activity of Pur β by altering its interaction with other transcription factors but not with ssDNA.

Keywords

Pur β ; myofibroblast; smooth muscle α -actin; single-stranded DNA; YB1

Wound healing is a complex process involving a network of injury-activated cell types and regulatory factors that participate in the systematic repair of tissue damage. A key feature of this process is the formation and contraction of granulation tissue that effectively brings wound edges together.[Gurtner et al., 2008; Martin, 1997; Tomasek et al., 2002] Once the

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wound is closed, certain cell types in the granulation tissue typically undergo apoptosis. [Desmouliere et al., 1995] However, in fibrocontractive diseases, granulation tissue remains active resulting in excessive accumulation of extracellular matrix (ECM) and additional tissue contraction, which collectively disfigure and impair the affected organ. [Gabbiani, 2003; Rockey et al., 2015; Schurch, 1997; Tomasek et al., 2002] Myofibroblasts are a distinctive cell type in granulation tissue and directly contribute to wound contraction and stability by transducing force from internal contractile proteins to the local ECM and by producing ECM components such as collagen. [Dugina et al., 2001; Gabbiani, 2003; Gabbiani et al., 1976; Tomasek et al., 2002] The primary driver in the pathogenesis of fibrosis is the persistent activation of resident connective fibroblasts to form differentiated myofibroblasts. [Darby and Hewitson, 2007; Hinz et al., 2007] Therefore, a better understanding of the molecular mechanisms that control myofibroblast differentiation may provide insight into potential regulatory targets to limit fibrosis and tissue damage.

One of the early cytological characteristics of fibroblast to myofibroblast differentiation is the appearance of cytoskeletal stress fibers, which form the force-generating contractile apparatus. [Gabbiani, 2003; Hinz, 2007; Hinz et al., 2001b] While a number of components have been implicated in stress fiber formation, the expression of the smooth muscle α -actin isoform has been shown to be a distinctive biochemical marker of the myofibroblast phenotype. [Darby et al., 1990] The presence of smooth muscle α -actin in stress fibers also enhances myofibroblast contractile activity making the regulation of smooth muscle α -actin expression relevant to both the healthy and pathological states of wound repair. [Hinz et al., 2001a] A number of studies have revealed that the mouse smooth muscle α -actin gene (*Acta2*) is subject to both positive and negative transcriptional regulation in fibroblasts by virtue of the interaction of certain trans-activators and trans-repressors with cognate cis-acting elements in the 5'-flanking region of the gene. [Carlini et al., 2002; Cogan et al., 2002; Strauch and Hariharan, 2013; Subramanian et al., 2004] The repertoire of transcription factors involved in *Acta2* gene activation include a combination of ubiquitous, growth factor-responsive, and muscle-associated factors. For example, the Smad activator proteins have been reported to induce *Acta2* expression by binding to CAGA elements following the exposure of fibroblasts to transforming growth factor β 1 (TGF- β 1). [Strauch and Hariharan, 2013; Subramanian et al., 2004] Specificity proteins 1 and 3 (Sp1, Sp3), serum response factor (SRF), and transcription enhancer factor 1 (TEF1) are also components of a multi-protein *Acta2* activation complex formed by their interaction with G/C-rich, CARG, and muscle-CAT (MCAT) recognition motifs, respectively. [Carlini et al., 2002; Cogan et al., 2002; Subramanian et al., 2004] Conversely, repression of *Acta2* in fibroblasts appears to be governed by the purine-rich element-binding proteins A and B (Pur α and Pur β) and Y-box binding protein 1 (YB1), which bind in a strand-specific manner to DNA sequences that overlap the G/C-rich and MCAT elements. [Carlini et al., 2002; Hariharan et al., 2014; Kelm et al., 1999a] Pur α and Pur β proteins may also mediate gene repression by binding directly to other transcription factors associated with positive and negative regulation of *Acta2* including TEF1, Smad3, and YB1. [Carlini et al., 2002; Hariharan et al., 2014]

Insights into the specific molecular mechanism by which Pur α and Pur β repress *Acta2* have been provided by studies that have uncovered the unique structural features of each protein. The primary sequences of Pur α and Pur β each contain three regions of internal homology

known as Pur repeats I, II, and III.[Graebisch et al., 2010; Rumora et al., 2013] The solved x-ray crystal structures of the truncated repeat I-II region of *Drosophila melanogaster* (*Dm*) Pura and full-length *Borrelia burgdorferi* (*Bb*) Pura showed that certain β -strand and α -helix forming sequences located in each repeat associate to form a so-called PUR domain. Importantly, this domain adopts a whirly-like fold and is capable of binding to ssDNA. [Graebisch et al., 2010; Graebisch et al., 2009] The more recently reported crystal structure of repeat III from *Dm* Pura confirmed that another PUR domain is formed by the intermolecular association of two repeat III sequences in a manner which mediates Pura dimerization.[Weber et al., 2016] This result is consistent with other biochemical data indicating that repeat III also corresponds to the dimerization domain in Pur β . [Rumora et al., 2013] Yet despite sharing similar structural features, the isolated repeat III domain from Pur β binds with high affinity and specificity to ssDNA, while the isolated repeat III domain from Pura is apparently deficient in binding to ssDNA.[Rumora et al., 2013; Weber et al., 2016] This distinction along with other structural differences may explain why Pur β functions as a dominant repressor of *Acta2* in comparison to Pura when evaluated using both gain-of-function and loss-of-function approaches.[Kelm et al., 2003; Knapp et al., 2006]

The National Heart Lung and Blood Institute (NHLBI) Grand Opportunity Exome Sequencing Project has analyzed the exomes of over 200,000 individuals enrolled in a number of well-known epidemiological studies to identify variants within the population. To date, a total of 13 rare, single nucleotide polymorphisms (SNPs) have been identified in the open reading frame of human *PURB* of which six encode missense substitutions and one a pre-mature stop codon. [Exome Variant Server, NHLBI GO Exome Sequencing Project] Curiously, five of the six missense substitutions are located within or close to the repeat III dimerization domain. Because of the conspicuous positional and chemical features of these substitutions, we chose to evaluate whether the presence of variant residues would affect the structure and/or function of Pur β in relation to repression of *Acta2* in fibroblasts. Our findings reveal that some amino acid substitutions produce relatively modest but significant changes in the repressor activity of Pur β toward the *Acta2* promoter. Results of biochemical assays indicate that these changes are likely dictated by how the Pur β variants physically interact with other transcriptional regulatory proteins rather than with ssDNA.

MATERIAL AND METHODS

Materials

Coomassie PlusTM Protein Assay Reagent, PageRuler Prestained Protein Ladder, PierceTM Enhanced Chemiluminescence Reagent, SYPRO[®] Orange, and 6 $\times$ His tag antibody HIS.H8 were obtained from Thermo Fisher Scientific, Grand Isle, NY. Cell culture media were procured from Mediatech, Inc., Manassas, VA. Fetal bovine serum (FBS) was purchased from Sigma-Aldrich, St. Louis, MO. Costar[®] EIA/RIA plates were acquired from Corning Inc., Corning, NY. Polyplus-transfection SA jetPEI[®] and jetPRIME[®] transfection reagents were purchased from VWR International, Radnor, PA. Luciferase Assay System and Reporter Lysis Buffer were obtained from Promega Corp., Madison, WI. Immobilon[®]-P polyvinylidene difluoride membrane (PVDF) transfer membrane, 2, 2'-AZINO-bis [3-

ethylbenziazoline-6-sulfonic acid] (ABTS) peroxidase substrate solution, and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody clone 6C5 were obtained from EMD Millipore, Billerica, MA. StreptaWell strips were purchased from Roche Diagnostics, Indianapolis, IN. The QuikChange II XL Site-Directed Mutagenesis Kit was acquired from Agilent Technologies, Santa Clara, CA. Horseradish peroxidase (HRP)-conjugates of goat anti-rat IgG, goat anti-rabbit IgG, goat anti-mouse IgG, and mouse IgG kappa binding protein were procured from Santa Cruz Biotechnology, Inc. Dallas, TX.

Computational Modeling of Pur β Protein Structure

Updated computational homology models of the *Mus musculus* (*Mm*) Pur β monomer and homodimer were generated based on *Dm* Pura crystal structures: 3K44 (Pura repeats I-II, no DNA) and 5FGO (two Pura repeats III, no DNA).[Graebisch et al., 2009; Weber et al., 2016] A combination of web-based modeling servers, bioinformatics, and simulated annealing (CNS version 1.3)[Rice and Brunger, 1994] were used to create models using approaches similar to those previously described.[Brunger et al., 1998; Graebisch et al., 2010; Rumora et al., 2013] Briefly, amino acid sequences corresponding to *Mm* Pur β residues 1–236 and 180–324 were separately submitted to the web-based modeling server I-TASSER [Roy et al., 2010; Zhang, 2008] using 3K44 and 5FGO, respectively, as templates. The structural predictions generated by I-TASSER were checked for consistency using several other web-based modeling programs including PSIPred, LOMETS and Phyre2. [Kelley et al., 2015; McGuffin et al., 2000; Wu and Zhang, 2007] Both resulting homology models were examined in PyMOL and selected glycine residues in the linking region between repeats II and III were aligned.[Schrodinger, 2010] Using PyMOL editing features, glycine 218 or 225 was used as an axis to rotate the repeat III subdomain away from repeats I-II and then any regions of overlap were resolved to create a composite model of the 324 residue monomer. Several different models of the Pur β monomer were generated by systematically adjusting the angle of rotation around glycine 218 or 225. To form a homodimer, the repeat III sequences from two Pur β monomers were aligned to the crystal structure of the intermolecular domain of Pura, 5FGO, using Lsqman.[Kleywegt, 1996] Models of the homodimer were optimized by simulated annealing, which effectively relaxed close contacts and regularized local bond angle geometries. A final model was selected as it best fit with the dimensions of the Pur β homodimer based on hydrodynamic analyses of the protein.[Ramsey et al., 2007]

Construction of Expression Vectors Encoding Pur β Variants

The mammalian expression plasmid encoding N-terminal hexahistidine-tagged murine Pur β (pCI-NHis-Pur β) has been previously described.[Kelm et al., 1999a] Plasmids encoding six different full-length Pur β variants (L46V, G215S, P223L, F274L, G282S, R297Q) and one nonsense variant (Q64*) were generated in pCI-NHis-Pur β backbone via site-directed mutagenesis. Primers were selected using the QuikChange Primer Design Program (Table S1) and mutated plasmids were amplified using procedures specified by the manufacturer of the QuikChange II XL kit. Correctly mutated plasmids were validated by automated DNA sequencing performed by the Vermont Cancer Center DNA Analysis Facility. Mutated plasmids were isolated from bacterial host cells via double cesium chloride gradient centrifugation. The purity of isolated plasmids was assessed by electrophoresis of intact and

restriction enzyme-digested plasmid on 1% w/v agarose gels. The cDNA sequences encoding Pur β variants P223L and R297Q were released from their respective pCI-NHis-Pur β plasmids by treatment with *EcoRI* and *SaII*. Isolated cDNAs were then subcloned into the bacterial expression vector pQE30 linearized with the same restriction enzymes. The resultant pQE30-NHis-Pur β P223L or R297Q plasmids were transformed into *Escherichia coli* JM109 cells. Plasmids were purified and sequenced as described above.

Acta2 Promoter-Luciferase Reporter Assays

AKR-2B mouse embryo-derived fibroblasts (MEFs) and A7r5 rat aortic smooth muscle cells (SMCs) were cultured as previously described.[Wang et al., 2005] For transfections, AKR-2B MEFs were seeded into 6 well plates at a density of 4×10^4 cells/well in McCoy's 5A medium with 5% v/v heat-inactivated FBS. A7r5 SMCs were seeded at a density of 6×10^4 cells/well in Dulbecco's modification of Eagle's medium with 10% v/v FBS. The cells were cultured for 24 h at 37°C in a humidified 5% CO₂ incubator. Adherent cells were then transiently transfected with a mixture of expression and reporter plasmids at a ratio of 2 μ L jetPEI® or jetPrime® reagent per 1 μ g of DNA as instructed by the manufacturer. Mixtures contained 1 μ g of empty pCI plasmid or pCI-NHis-Pur β plasmid plus 0.9 μ g of *Acta2* promoter-luciferase reporter plasmid (pVSMP8-Luc) [Rumora et al., 2013] and 0.1 μ g of pSV40- β Gal control reporter plasmid. Transfected AKR-2B cells or A7r5 cells were cultured for 48 h or 72 h, respectively. Cells were washed twice with phosphate-buffered saline and then extracted with 1 $\times$ Reporter Lysis Buffer supplemented with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μ g/mL each of pepstatin A, leupeptin, and aprotinin. Cell lysates were frozen at -20°C. Prior to use in endpoint assays, lysates were thawed and subjected to centrifugation at $15,800 \times g$ for 30 min at 4°C. Total protein concentration in the cleared supernatants was determined by Bradford assay using bovine serum albumin (BSA) as a standard. Reporter enzyme activity was assessed in a Turner Designs TD-20/20 luminometer as directed by the manufacturer of the Luciferase Assay System reagents. Luciferase activity measured in each cell lysate was normalized for total protein content. Numerical datasets were analyzed via one-way analysis of variance in conjunction with Dunnett's multiple comparison test with significance set at $p < 0.05$. Statistical analyses were conducted with the use of Prism 7.01 software (Graphpad Software, Inc., La Jolla, CA).

Immunoblotting of Cell Extracts

Total protein at amounts ranging from 15–30 μ g from AKR-2B cells and 30–50 μ g from A7r5 cells was precipitated using five volumes of ice cold ethanol to one volume of cleared cell lysate. Mixtures were incubated at -20°C for at least 1 h before centrifugation at $15,800 \times g$ for 30 min at 4°C. Protein-containing pellets were air dried briefly and then dissolved in 2 $\times$ sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample preparation buffer composed of 40 mM Tris-Cl pH 6.8, 1% w/v SDS, 10% v/v glycerol, 0.01% w/v bromophenol blue, 5% v/v β -mercaptoethanol. After heating protein samples for 5 min at 96°C, the samples were subjected to electrophoresis on 8% acrylamide/bisacrylamide (29:1) mini-gels. Separated proteins were then transferred to PVDF membrane in transfer buffer consisting of 25 mM Tris base, 192 mM glycine, 20% v/v methanol for 90 min at 125 volts. Blots were incubated overnight in blocking buffer

composed of 5% w/v nonfat dry milk powder dissolved in wash buffer composed of 20 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1 % v/v Tween 20. Blots were probed sequentially for 1–2 h with primary mouse anti-6×His tag antibody followed by HRP-conjugated goat anti-mouse IgG or HRP-conjugated mouse IgG kappa binding protein. Primary antibody and secondary HRP-conjugated reagents were used at a working dilution of 1:2000 in blocking buffer. Blots were rinsed 3–4 times with wash buffer after the primary and secondary incubations. Immunoreactive bands were revealed with the use of chemiluminescent detection reagents and visualized on a Fujifilm LAS-4000 imaging system or blue autoradiography film. Blots were routinely reprobed with mouse anti-GAPDH monoclonal antibody as a loading control.

Recombinant Protein Purification

NHis-Purβ wild type (WT) or variant (R297Q, P223L) proteins were expressed in and purified from *E. coli* JM109 cells as previously described.[Rumora et al., 2016] Some modifications to the process were necessary to optimize the production and purity of the Purβ variants as outlined in Supporting Information. All recombinant protein preparations were subjected to calibrated size exclusion chromatography as a final purification step. [Ramsey et al., 2007] Final protein concentrations were determined by dye-binding assay using Coomassie Plus™ Protein Assay Reagent and NHis-Purβ WT as a calibration standard. The concentration of NHis-Purβ WT stock solutions was ascertained by absorbance measurement at 280 nm and calculated using a theoretical molar extinction coefficient for Purβ.[Rumora et al., 2013] The purification of NHis-tagged mouse YB1 (MSY1) from *E. coli* was performed as previously described.[Knapp et al., 2007]

Thermal Shift Assay

The thermostability of purified NHis-Purβ variants was assessed by differential scanning fluorimetry as previously described.[Rumora et al., 2016] Briefly, each protein was diluted to concentrations ranging from 75 to 300 µg/mL in buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0 with 10 mM β-mercaptoethanol and 5× SYPRO Orange. Solutions were aliquoted into the wells of a 96-well polymerase chain reaction (PCR) plate at 40 µL/well. Plates were covered with optical sealing film and centrifuged briefly. Plates were heated in increments of 1°C per min starting at 25°C and ending at 94°C with the use of an Applied Biosystems 7500 Fast Sequence Detection System real-time PCR instrument. Fluorescence signal emitted at 568 nm was detected and recorded over time. The minimum baseline fluorescence ($F_{\min}$) measured in each sample well was subtracted from all data points collected and the difference was divided by the maximum fluorescence ($F_{\max}$) measured in each sample well. Data points collected between 40°C and 71°C were analyzed via non-linear fitting to the Boltzmann equation with the use of Prism 7.01 software to determine the melting temperature (T_m) of each Purβ variant.

Quantitative Purβ-DNA Interaction Assay

An enzyme linked immunosorbent assay (ELISA) system was used to evaluate nucleoprotein complex formation between purified recombinant Purβ and immobilized DNA as previously described.[Rumora et al., 2013] Oligonucleotides tagged with 3' biotin were diluted to a concentration of 0.5 nM in coating buffer composed of 20 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂-6H₂O. Aliquots of 100 µL were applied to streptavidin-

coated microtiter wells, which were incubated for 2 h at room temperature. In all subsequent steps, solutions were applied to wells in volumes of 100 μ L except for the wash and blocking buffers, which were used at 300 μ L. Wells were rinsed with wash buffer consisting of 20 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05% v/v Tween 20 and then incubated for 1 h with blocking buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 2% w/v BSA. Wells were emptied and NHis-Pur β WT or variant proteins were then applied at concentrations ranging from 9.0 nM to 0.074 nM in binding buffer supplemented with 0.5 mM dithiothreitol (DTT) and 1 μ g/mL polydeoxythymidylate (dT32). The binding buffer was composed of 20 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2% w/v BSA and 0.05% v/v Tween 20. Wells were incubated for ~17 h at room temperature and then rinsed three times with wash buffer. Primary rabbit anti-mouse Pur β 302–324 antibody [Kelm et al., 1999a] diluted to a concentration of 1 μ g/mL in binding buffer was next applied to the wells. After a 1 h incubation with primary antibody, wells were washed three times and then incubated for 1 h with goat anti-rabbit IgG-HRP diluted 1:4000 in binding buffer. Wells were washed three times followed by the addition of ABTS peroxidase substrate solution. Colorimetric reactions were quenched by adding an equal volume of 1% w/v SDS. After a 5 min stabilization period, endpoint absorbance readings at 405 nm were acquired using a 96 well microplate reader. To correct for background signal, absorbance values produced in wells with NHis-Pur β but no DNA were subtracted from A405 values obtained in wells with NHis-Pur β plus DNA. Apparent K_d or EC50 values were determined by non-linear least squares fits of data points to equations for either a hyperbola or a four parameter dose response with the use of Prism 7.01 software.

Quantitative Pur β -MSY1 Interaction Assay

The relative affinity of Pur β for MSY1 was assessed using an ELISA strategy as previously described.[Rumora et al., 2016] The compositions of the coating, blocking, binding, and wash buffers were the same as those used in the solid-phase protein-DNA interaction assay except where noted. Purified MSY1 diluted to 100 nM was initially adsorbed to the wells of an EIA/RIA plate in coating buffer composed of 20 mM HEPES, 150 mM NaCl, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH 7.5 with 5.0 μ g/mL BSA for 3 h at room temperature. MSY1-coated wells were washed and blocked prior to the application of varying concentrations of purified NHis-Pur β WT or variant protein. Wells were then incubated overnight at 4°C for 17 h. Wells were washed and then incubated with primary rabbit anti-Pur β 302–324 antibody at 1 μ g/mL for 1 h followed by secondary goat anti-rabbit IgG-HRP at a 1:4000 dilution for 1 h. Wells were washed and ABTS substrate was added. Color development was quenched by the addition of an equal volume of 1% w/v SDS. Following a stabilization period of 5 min, endpoint absorbance values at 405 nm were obtained using a 96 well microplate reader. Background absorbance values generated in MSY1-or BSA-coated wells incubated with binding buffer alone were subtracted from absorbance values obtained in wells incubated with fluid-phase Pur β . The contribution of non-specific binding was accounted for by subtracting absorbance values obtained in BSA only-coated wells from MSY1-coated wells at identical Pur β protein concentrations. Data points were analyzed using an equation for a hyperbola to determine apparent K_d with the use of Prism 7.01 software.

Qualitative Protein-Protein Interaction Assay

The ability of native mouse or human cell extract-derived proteins to interact with recombinant Pur β WT and variant proteins was screened using an ELISA approach. Nuclear extracts were prepared from AKR-2B fibroblasts or HEK 293FT cells as described in Supporting Information. NHis-Pur β WT, P223L, or R297Q were immobilized on EIA/RIA wells at 300 nM in coating buffer composed of 20 mM HEPES pH 7.5, 150 mM NaCl, 5 μ g/mL BSA, and 0.5 mM DTT for 2 h at room temperature. Pur β -coated wells were rinsed twice with wash buffer consisting of 20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.05% v/v Tween-20 and then blocked in buffer supplemented 2% w/v BSA for 2 h. Nuclear extract was then applied at 300 μ g protein/mL in binding buffer composed of 20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.05% v/v Tween 20, and 0.2% w/v BSA. Wells were incubated at 4°C for 17 h and then washed three times. Wells were then incubated with selected primary antibodies diluted to 1 μ g/mL in binding buffer for 1 h followed by HRP-conjugated secondary antibodies at 1:2000 dilutions for 1 h. The specific primary and secondary antibodies used are listed in Supporting Information. Color development was achieved by addition of ABTS substrate solution and quenched with an equal volume of 1% w/v SDS. After 5 min stabilization period, absorbance values at 405 nm were obtained using a 96 well microplate reader. Values were corrected for background absorbance measured in wells that contained buffer instead of nuclear extract. Numerical datasets were analyzed via one-way analysis of variance and Dunnett's multiple comparison test with significance set at $p < 0.05$ with the use of Prism 7.01 software.

Pur β Antibody Screening Assay

The effect of variant amino acid residues on epitope recognition by Pur β antibodies was tested by ELISA as previously described.[Rumora et al., 2016] Briefly, purified Pur β WT or variant proteins at 100 nM were immobilized on the wells of EIA/RIA plates in coating buffer composed of 20 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂·6H₂O, 0.5 mM DTT and 5.0 μ g/mL BSA for 17 h at 4°C. Wells were then washed and blocked. This was followed by application of varying concentrations of primary rabbit antibodies recognizing Pur β residues 210–229 or 302–324.[Kelm et al., 1999a] After a 1 h incubation, wells were washed and HRP-conjugated goat anti-rabbit IgG diluted 1:4000 was next applied for 1 h. Generation of a colorimetric signal and acquisition of absorbance values were carried out as described above.

RESULTS

Identification of Rare SNP-Encoded Pur β Variants

The NHLBI Grand Opportunity Exome Sequencing Project has identified allelic variants in the exomes of over 200,000 individuals and compiled them in a searchable web-based server. [Exome Variant Server, NHLBI GO Exome Sequencing Project] A query of this database for *PURB* revealed 13 SNPs in the open reading frame of which six produce synonymous codons, six encode missense variants (L34V, G203S, P211L, F262L, G270S, and R285Q), and one (Q52*) generates a premature stop codon (Table S2). Interestingly, five of the six residues changed in the rare missense variants are located in or near the third repeat of Pur β , a region known to enable protein dimerization and high affinity binding to

purine-rich ssDNA.[Rumora et al., 2013] Sequence alignment of Pur β proteins from several species revealed that the wild-type amino acid residues found at the positions of the SNP-generated missense and premature stop codons are highly conserved among mammalian Pur β sequences (Figure 1).

The web-based Polymorphism Phenotyping v2 (PolyPhen2) predictor was used to estimate whether the presence of the variant amino acid residues might affect the structure and function of Pur β . The projected effects ranged from benign to probably damaging, with four of the six variant amino acid substitutions achieving at least a possibly damaging classification (Table S2). However, considering the chemical properties of the amino acids involved in the “benign” substitutions, the PolyPhen2 predictions must be taken *cum grano salis*. Consequently, we chose to empirically determine how these particular amino acid substitutions affect the structure and function of Pur β .

Mapping of Variant Residues on a Homology Model of *Mm* Pur β

An updated model of the higher order structure of full-length Pur β was computationally derived to gain a greater appreciation for the position of the common and variant amino acid residues in relation to the specific subdomains formed by Pur repeats I, II, and III (Figure 2A). Homology models of the *Mm* Pur β monomer and homodimer were generated using a combination of web-based modeling servers, energy minimization, and the known three-dimensional structures of specific subdomains of *Dm* Pura. The crystal structure of the *Dm* Pura repeat I-II domain served as a template for *Mm* Pur β repeat I-II and the crystal structure of the *Dm* Pura repeat III dimerization domain served as a template for *Mm* Pur β repeat III in the modeling program I-TASSER. Composite models of the intact Pur β monomer and homodimer were then derived using PyMOL alignment and editing features in conjunction with other energy minimization and alignment optimization programs. Consistent with the earlier model of the Pur β homodimer [Rumora et al., 2013], the updated model includes three distinct subdomains putatively formed by the intramolecular association of sequences in repeats I and II and the intermolecular association of two repeat III sequences (Figure 2B). However, the new model also features a more compact linker region between repeats II and III and slightly altered positions of α helices and β strands in the intermolecular dimerization domain (Figure S1).

Mouse and human Pur β are ~94% identical in primary amino acid sequence with the core region comprising repeats I, II, and III being 99% identical. With the exception of a longer glycine-rich N-terminus, mouse Pur β contains the same common amino acid residues at variant positions identified in the human protein (Figure 1). The location of these amino acids were mapped on the new computational model of *Mm* Pur β to gain a better understanding of how substitutions at these positions may impact protein structure and function (Figure 2C and D). The L46V (L34V in human) substitution is in an unstructured N-terminal region of repeat I just prior to the first β strand. The Q64* (Q52*) nonsense mutation also resides in repeat I between the second and third β strands and would produce a small truncated N-terminal peptide (Figure 2C and D, inset). All other missense substitutions are associated with the repeat III segment of the protein. P223L (P211L) and G215S (G203S) reside in a linker region connecting the subdomains formed by repeats I-II

and repeat III. G282S (G270S) and R297Q (R285Q) are located within the α helices of the repeat III dimerization domain, while F274L (F262L) is present in a loop preceding each α helix. Depiction of surface area of the Pur β homodimer implies that all but one of the amino acids involved in missense substitutions, namely G282S, would likely be solvent exposed (Figure 2D). Superimposing the new homology model of Pur β on the x-ray diffraction-derived model of *Dm* Pura repeats I-II in complex with ssDNA suggests that most of these variant residues are relatively far away from residues predicted to mediate interaction with ssDNA (Figure S2).

***Acta2* Repressor Activity of Pur β Variants**

The effect of variant residues on the transcriptional repressor function of Pur β was assessed by evaluating the relative activity of each engineered Pur β variant toward an *Acta2* promoter-reporter construct in rodent fibroblast or SMC lines. Mammalian expression plasmids encoding NHis-Pur β WT or variant proteins were transiently co-transfected with an *Acta2* promoter-luciferase reporter construct into mouse AKR-2B fibroblasts. The particular promoter-reporter construct used contains a large ~3.6 kb fragment of the murine *Acta2* gene, which includes DNA sequence beginning in the 5' flanking region and extending through the first intron (–1070 to +2582). Five of the six Pur β missense variants were modestly impaired in their ability to repress the *Acta2* promoter in AKR-2B cells, with the exception of the L46V variant, which appeared to be a slightly more effective repressor than wild-type Pur β (Figure 3A). Missense variants G215S, P223L, and R297Q were the most significantly different compared to wild-type Pur β averaging ~2-fold less activity. These functional differences could not be explained by a reduction in protein synthesis or stability as Western blotting of whole cell lysates was consistent with equivalent expression of NHis-Pur β WT and each missense variant (Figure 3B). Furthermore, biochemical analysis of the nuclear and cytosolic distribution of each Pur β missense variant expressed in fibroblasts indicated that the amino acid substitutions had no effect on the ability of the protein to traffic to the nucleus (Figure S3).

The repressor activity of the Pur β variants was also evaluated in rat A7r5 SMCs to determine if the functional effects conferred by the amino acid substitutions was dependent or independent of cell type. Interestingly, all the missense variants were at least partially deficient in repressor activity toward the transfected *Acta2* promoter-reporter construct in A7r5 SMCs (Figure 3C). The magnitude of the inhibitory effect seen with the G215S, P223L, and R297Q substitutions was similar in both cell types. However, the activity of variants G282S, F274L, and L46V was more significantly impaired in transfected SMCs than in fibroblasts. Once again, these functional differences could not be accounted for by altered expression of the Pur β variants compared to the wild-type protein based on Western blot analysis (Figure 3D). Unlike the missense variants, the stop-gained variant, Q64*, was completely devoid of *Acta2* repressor activity in both cell types (Figure 3A and C). This result was expected since this nonsense mutation would theoretically produce a highly truncated and presumably nonfunctional polypeptide.

Structural Integrity of Purified Pur β Missense Variants

Pur β missense variants P223L and R297Q were selected for further biochemical analysis because of the distinctive location of the amino acid substitutions and the fact that each variant showed significantly reduced *Acta2* repressor activity in both fibroblasts and SMCs. His-tagged Pur β variants were expressed in *E. coli* and purified to homogeneity. Calibrated size exclusion chromatography was employed as the final step in the purification process for the purpose of removing residual contaminants and to determine the quaternary structure of the variants. Column fractions were monitored by a combination of SDS-PAGE to assess protein purity and dye-binding assay to determine protein concentration. NHis-Pur β WT, P223L and R297Q each eluted from the S200 column at a volume consistent with the homodimeric form of the native protein (Figure S4B–D). SDS-PAGE analysis revealed that protein preparations were virtually free of contamination with each purified Pur β variant exhibiting an apparent molecular weight corresponding to the monomeric form of the denatured protein (Figure S4E).

The structural stability of each Pur β variant was next evaluated by thermal shift assay in aqueous buffer to assess the degree to which amino acid substitutions impact protein folding and thermostability. The thermal shift curves generated with the P223L and R297Q variants were very similar to the wild type protein (Figure 4A). The calculated melting temperatures of R297Q, P223L, and Pur β WT were $57.61 \pm 0.89^\circ\text{C}$, $58.64 \pm 0.51^\circ\text{C}$, and $59.04 \pm 1.11^\circ\text{C}$, respectively (Figure 4B). The near identity of the thermal shift profiles and temperatures of unfolding suggest that the P223L and R297Q variants are equally thermostable to the wild-type protein and thus share comparable folded states.

Antibody Recognition of Pur β Variants

Affinity-purified rabbit antibodies directed against amino acid sequences 201–229 or 302–324 of mouse Pur β were initially screened for their ability to detect each epitope in the purified Pur β variants. NHis-Pur β WT, R297Q, and P223L were each passively immobilized on the wells of high-binding ELISA plates and primary antibodies recognizing Pur β epitopes 210–229 or 302–324 were applied at concentrations ranging from 0.0156 to 1.0 $\mu\text{g/mL}$. Since the Pur β 210–229 epitope contains the 223 residue, the rabbit anti-Pur β 210–229 antibody interacted more weakly with the P223L variant compared to Pur β WT and R297Q (Figure S5). Conversely, the rabbit anti-Pur β 302–324 antibody bound to Pur β WT and each variant equivalently on account of the epitope being independent of site of the mutated P223L and R297Q residues. Based on these results, the rabbit-anti-Pur β 302–324 antibody was selected for use in all subsequent solid-phase binding assays used to detect Pur β interaction with DNA or with other proteins.

DNA Binding Properties of Pur β Variants

The interaction of Pur β R297Q and P223L with DNA was assessed using an ELISA system in which multiple single-stranded and double-stranded biotinylated oligonucleotides (Table S3) were immobilized on streptavidin-coated microtiter wells at a fixed concentration of 0.5 nM. The specific DNA sequences initially studied included the individual purine-rich strands of known Pur β recognition elements from the *Acta2* promoter (PE32-bF, SPUR32-bF), as well as the complementary pyrimidine-rich strands (PE32-bR, SPUR32-bR) as negative

controls (Figure 5A). Each *Acta2* cis-element was also evaluated in dsDNA configuration. Additionally, a purine-rich element from the 5'-flanking region of the human *MYC* gene (PUR24-bF) was utilized as an independent positive control for Pur β -ssDNA interaction. [Kelm et al., 2018] Fluid-phase Pur β WT and variant proteins were evaluated at a limiting protein concentration of 1.0 nM in the primary binding step in the ELISA. Subsequent antibody-based detection of solid-phase nucleoprotein complexes revealed that the interaction of Pur β P223L and R297Q with purine-rich ssDNA sequences was indistinguishable from wild-type Pur β (Figure 5B). Furthermore, all Pur β proteins tested showed little or no binding to dsDNA, pyrimidine-rich ssDNA, or wells with no DNA consistent with the preferential interaction of Pur β with purine-rich ssDNA.

The relative binding affinity of Pur β P223L and R297Q for purine-rich ssDNA was next quantitatively determined by generating protein-ssDNA binding curves using the same ELISA system. To do so, solutions of NHis-Pur β protein at concentrations ranging from 9.0 to 0.074 nM were incubated with wells coated with a fixed concentration (0.5 nM) of PE32-bF. Solid-phase nucleoprotein complexes were then detected by sequential incubation of wells with primary and secondary antibodies followed by color development. The resultant binding profiles were essentially superimposable indicating that the relative binding affinity and stoichiometry of the Pur β variants for PE32-bF ssDNA were comparable to wild-type Pur β (Figure 6 A, B). The apparent K_d determined for each variant protein based on hyperbolic fits was 0.160 ± 0.012 nM for P223L and 0.156 ± 0.017 nM for R297Q compared to 0.160 ± 0.012 nM for WT.

Heterotypic Protein Binding Properties of Pur β Variants

Pur β has been implicated in modulating *Acta2* promoter activity through interaction with other transcription factors, including the co-repressor protein mouse YB1 (MSY1). Direct protein-protein interaction assays were performed to determine if Pur β -MSY1 association is impacted by the amino acid substitutions in the Pur β variants. Solutions of fluid-phase NHis-Pur β WT, P223L, and R297Q at differing concentrations were incubated with microplate wells coated with a fixed concentration of MSY1. Solid-phase Pur β -MSY1 complexes were then detected with the use of the same ELISA system employed in the DNA binding assay. The raw binding curves generated indicated that P223L has slightly higher affinity for MSY1 compared to Pur β WT, while R297Q has a slightly lower affinity (Figure 7A). Binding constants were calculated after correcting for non-specific binding of Pur β to wells coated with BSA alone (Figure 7B). The apparent K_d determined for each variant protein was consistent with P223L having a higher binding affinity for MSY1 ($K_d = 24.84 \pm 4.40$ nM) than R297Q ($K_d = 42.75 \pm 5.70$) with the wild-type protein showing an intermediate affinity ($K_d = 31.4 \pm 3.70$).

A screening assay for the formation of multiprotein complexes with Pur β was also conducted to determine if other transcription factor interactions were altered by the amino acid substitutions in the Pur β P223L and R297Q variants. Microtiter wells coated with purified NHis-Pur β WT, P223L, or R297Q were incubated overnight with nuclear extract prepared from AKR-2B MEFs. Solid-phase nuclear protein-Pur β complexes were then detected by ELISA using a number of different primary antibodies directed against

transcription factors implicated in *Acta2* gene regulation (Table S4). The protein-protein interaction profile generated using AKR-2B nuclear extract was consistent with the P223L variant having a somewhat stronger affinity for MSY1 compared to R297Q and wild-type Pur β (Figure 8A). The interaction of Pura and Sp3 was comparable for Pur β WT, P223L, and R297Q. Interestingly, the formation of protein complexes containing Smad 2/3 and Smad4 was apparently inhibited in wells coated with the P223L variant in comparison to Pur β WT or R297Q. However, it is important to note that wells coated with P223L had higher levels of background binding by the Smad2/3 and Smad4 antibodies than Pur β WT or R297Q coated wells. This screening assay was also conducted with nuclear extract from HEK293 cells to assess the reproducibility of these findings with human cell-derived factors. Consistent with the results obtained using MEF-derived nuclear extract, Pur β P223L once again showed a higher binding capacity for human YB1 but was apparently deficient in interaction with Smad4 and Smad2/3 (Figure 8B). Pur β WT, R297Q, and P223L were essentially equivalent in binding human Pura, Sp3, and the retinoblastoma tumor suppressor protein (RB), a factor previously implicated in the cell-cycle dependent regulation of *Acta2* promoter activity.[Wang et al., 2005] A small but significant decrease in human TEF1 binding by Pur β P223L was also detected in extracts from HEK293 cells.

DISCUSSION

The smooth muscle cell-like myofibroblast plays a critical, albeit transient, functional role in promoting wound healing following tissue injury. On the other hand, persistent myofibroblast activation and survival contributes to dysfunctional wound healing and fibrosis due to excessive ECM accumulation and aberrant tissue constriction.[Gabbiani, 2003; Gabbiani et al., 1976; Tomasek et al., 2002] Possible therapeutic strategies to limit destructive tissue fibrosis include the targeting of molecular regulators that promote sustained connective tissue fibroblast to myofibroblast differentiation. Some studies have evaluated components of inflammatory response pathways, but have been met with limited success.[Strauch and Hariharan, 2013] An alternative approach is to target the regulatory factors that control *Acta2* expression since smooth muscle α -actin contributes to myofibroblast contractility and is the classic marker of the myofibroblast phenotype.[Darby et al., 1990; Hinz et al., 2001a] The transcriptional repressor protein, Pur β , is of special interest because of its unique ability to interact with specific ssDNA sequences in the *Acta2* promoter and with other transcription factors that dictate *Acta2* expression in fibroblasts. [Carlini et al., 2002; Hariharan et al., 2014; Kelm et al., 1999a]

A number of recent reports have shed light on the structural features of Pur β that account for its functional activity as an ssDNA-binding protein and repressor of *Acta2* gene expression. Limited tryptic digestion of recombinant *Mm* Pur β revealed that the residues 29–302 are necessary for high affinity binding of the protein to a purine-rich ssDNA sequence from the *Acta2* promoter.[Rumora et al., 2010] This core region of Pur β contains sequence elements termed Pur repeats I, II, and III that are highly conserved in Pura. Homology modeling suggested that *Mm* Pur β repeats I and II together fold to form an intramolecular PUR domain while two repeat IIIs associate to form an intermolecular PUR domain.[Rumora et al., 2013] This original computational analysis was based, in part, on information gleaned from the x-ray crystal structure of the homologous repeat I-II domain of *Dm* Pura.

[Graebisch et al., 2009] Empirical cell-based assays in conjunction with biochemical analyses demonstrated the necessity of the intermolecular and intramolecular PUR domains in mediating both the *Acta2* repressor and ssDNA binding activities of Pur β . [Rumora et al., 2013]

Recently, the x-ray crystal structure of the repeat III intermolecular domain of *Dm* Pur α was solved revealing a similar, but not identical, structure to the repeat I-II intramolecular domain. [Weber et al., 2016] With this new structural information in hand, we generated revised homology models of the *Mm* Pur β intermolecular domain and the intact Pur β homodimer. Like the earlier model, the updated model of the *Mm* Pur β homodimer reported herein depicts the protein in an extended conformation with each PUR domain as a distinct folded entity. This concept is consistent with hydrodynamic studies suggesting that the Pur β homodimer adopts an elongated conformation in solution. [Ramsey et al., 2007] In addition, the glycine-rich linker region between repeats II and repeat III is slightly more condensed (by ~ 5 Å) than in the previous model. [Rumora et al., 2013] This linker region, which is unique to Pur β , may allow for some degree of flexibility in the orientation of repeat I-II intramolecular domains relative to repeat III intermolecular domain particularly in the DNA-bound state.

The repeat III intermolecular domain plays several essential functional roles in the Pur β homodimer. [Rumora et al., 2013] In addition to mediating protein self-association, the repeat III intermolecular domain has a higher affinity for purine-rich ssDNA than the repeat I-II intramolecular domain and accounts for heterotypic binding of Pur β to other transcription factors. A recent report identified several positively charged residues associated with one of the β strands in each Pur repeat of Pur β that contribute to *Acta2* repressor function of the protein. [Rumora et al., 2016] Interestingly, mutation of the R267 in the third repeat had the single most dramatic effect on the interaction of Pur β with ssDNA. In light of this finding, the functional role of other structural elements in repeat III including the α helices and flexible linker region between repeats II and III seemed to merit further investigation.

A perusal of the NHLBI Exome Sequencing Project Exome Variant Server revealed 13 rare, SNP-associated variants in the human *PURB* open reading frame of which six encode missense substitutions and one a premature stop codon. Interestingly, five of the six missense variants are associated with the repeat III domain. Mapping the positions of the missense residues on our updated homology model revealed that most of the variant residues are predicted to reside in either the α helix of repeat III or in the linker region separating repeats II and III. All but one variant residue is likely to be solvent accessible. Additionally, the premature stop variant in repeat I would necessarily produce a highly truncated, and presumably, non-functional peptide. Therefore, we hypothesized that the missense substitutions and premature stop at these highly conserved positions may alter the activity of Pur β , and as such, may provide insight into the functional role(s) of the flexible regions and repeat III α helices. This idea was tested by engineering the specific amino acid substitutions into the backbone of *Mm* Pur β , which is 99% identical to human Pur β in the core region containing repeats I, II, and III. The effects of these amino acid substitutions on

the structure and function of Pur β was then systematically evaluated using a combination of cellular and biochemical approaches.

The functional activity of each of the engineered variants was initially assessed in transiently transfected MEFs and SMCs by way of *Acta2* promoter-reporter assays in conjunction with Western blotting. With the exception of the Q64* stop codon variant, all other missense variants were stably expressed in both cell types, and in the case of MEFs, were capable of trafficking to the nucleus of the cell. Results of luciferase reporter assays showed that the premature stop variant construct, Q64*, was completely deficient of *Acta2* repressor activity while four of the six missense variants (G215S, P223L, F274L, R297Q) were modestly but significantly less active than wild-type Pur β in both cell types. Two other variants, L46V and G282S, exhibited either greater or lesser *Acta2* repressor activity depending on the cell type studied. This result suggests that these particular amino acid substitutions may affect the interaction of Pur β with other regulatory factors, which are unique to each cell type. The R297Q and P223L variants were selected for further biochemical characterization as both missense variants had significantly reduced *Acta2* repressor activity in both MEFs and SMCs. Furthermore, each substitution results in a substantive change in the physicochemical properties of the amino acid residue at that particular position.

NHis-Pur β WT, R297Q, and P223L were each expressed in *E. coli* and purified to homogeneity under non-denaturing conditions. Calibrated SEC analysis of each variant indicated that the single amino acid substitutions had no effect on the ability of the protein to self-associate into a homodimer. Thermal shift assays showed that each variant was equally thermostable to wild-type Pur β suggesting that there were no ill effects of the R297Q and P223L substitutions on protein folding. Qualitative ELISA-based assessment of the interaction of purified Pur β with DNA targets of differing sequence and secondary structure indicated that each variant bound preferentially to purine-rich ssDNA sequences from the *Acta2* promoter. Like the wild-type protein, Pur β R297Q and P223L demonstrated little to no interaction with pyrimidine-rich ssDNA or dsDNA. Moreover, quantitative analysis of Pur β -ssDNA complex formation showed that the relative affinity and specificity of the P223L and R297Q variants for purine-rich ssDNA was the same as Pur β WT. Taken together, these results clearly demonstrate that the R297Q and P223L substitutions have no effect on the DNA binding properties of Pur β . These empirical findings are consistent with computational modeling indicating that P223 and R297 are located in regions of the protein that probably do not make direct contact with ssDNA.

The degree to which the amino acid substitutions affects interaction of Pur β with other *Acta2* regulatory proteins was next investigated. In contrast to results of comparative DNA binding assays, quantitative analysis of the binding of Pur β to co-repressor partner MSY1 indicated that the P223L and R297Q substitutions altered protein-protein interaction. While P223L displayed a slightly higher affinity for MSY1, R297Q exhibited a slightly lower affinity for MSY1 compared to Pur β WT. Interestingly, previous studies have suggested that structural elements residing in both the repeat I-II intramolecular domain and the repeat III dimerization domain mediate the interaction of Pur β with MSY1.[Rumora et al., 2016; Rumora et al., 2013] Hence, a P223L substitution in the linker region may alter the spatial orientation of inter-and intramolecular domains in a manner which enhances MSY1 binding

affinity. An R297Q substitution in the α helix of repeat III may impact ionic interactions in a manner that reduces MSY1 binding affinity.

Pur β P223L and R297Q were also screened for their ability to interact with several other transcription factors implicated in *Acta2* gene regulation to determine if these amino acid substitutions impact Pur β binding to nuclear extract-derived proteins. AKR-2B MEFs and HEK 293 cells were used as a source of mouse and human nuclear proteins. Consistent with the results obtained with purified MSY1, Pur β P223L was more efficient in binding MSY1/YB1 from nuclear extract of both cell types than Pur β WT and R297Q. Increased interaction of the P223L variant with MSY1/YB1 may facilitate more efficient removal of the variant from the nucleus owing to the coordinated transport of Pur β -MSY1/YB1 complexes by *Acta2* mRNA.[Kelm et al., 1999b; Strauch and Hariharan, 2013] In contrast, P223L was defective in binding to transcription factors regulated by TGF- β 1 signaling, namely Smad2/3 and Smad4. It has been established that Smad4 associates with Smad2 or Smad3 to induce smooth muscle α -actin gene expression in response to TGF- β 1 signaling.[Derynck and Zhang, 2003] Furthermore, Pur β has been shown to physically associate with protein complexes containing phosphorylated Smad3 (pSmad3) during the process of myofibroblast differentiation and to inhibit the interaction of pSmad3 activator with the *Acta2* promoter.[Hariharan et al., 2014] Although the ELISA format using nuclear extract as a source of protein binding partners does not allow the ready discrimination of direct vs. indirect interactions, the apparent inability of Pur β P223L to efficiently bind Smad proteins may explain why this variant is partially deficient in *Acta2* repressor activity. While this interpretation is mechanistically plausible, it will require further experimental validation because of some technical concerns about the specificity of the commercial Smad antibodies, which had a higher background signal in P223L-coated wells in the ELISA.

In summary, this study has characterized the functional consequence of rare amino acid substitutions at conserved positions in Pur β based on SNPs identified in human *PURB* by the NHLBI Exome Sequencing Project. The location of the modified residues mapped to regions of the Pur β homodimer that are not predicted to be directly involved in mediating protein-ssDNA interaction. Curiously, while the nonsense variant predictably resulted in a complete loss-of-function, many of the missense variants differed modestly but significantly from wild-type Pur β in *Acta2* repressor activity (~1.5 to 2-fold) and, in some cases, in a cell type-dependent manner. The mechanistic basis for these functional differences was not attributable to altered expression, subcellular localization, polypeptide folding/stability, or ssDNA binding properties but appeared to be due to subtle changes in Pur β variant interaction with other gene regulatory factors. At the present time, we can only speculate as to whether any of the human *PURB* SNP variants are phenotypically significant from the standpoint of fibrosis or any other disease process. Interestingly, a variety of missense, in-frame deletion, and truncation mutations in *PURA* have been linked to profound neurodevelopmental phenotypes.[Hunt et al., 2014; Lalani et al., 2014; Tanaka et al., 2015] As for the potential disease consequence of being a carrier of a *PURB* nonsense allele, at least one study has found that hemizygous deletion of *PURA* or *PURB* is linked to myelodysplastic syndrome that progresses to acute myelogenous leukemia.[Lezon-Geyda et al., 2001] Moreover, our findings with the engineered missense variants do fit with the general concept that rare variants may contribute to disease susceptibility through small

changes in protein-protein association.[Bodmer and Bonilla, 2008] Finally, our results also suggest that the α -helices in the repeat III dimerization domain and less structured linker regions, participate in protein-protein interactions that affect the *Acta2* repressor activity of Pur β .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

Pura	purine-rich element binding protein A
Purβ	purine-rich element binding protein B
SMAA	smooth muscle α -actin
ssDNA	single-stranded DNA
MCAT	muscle-CAT box
Sp1/3	specificity protein 1 and 3
Dm	<i>Drosophila melanogaster</i>
Mm	<i>Mus Musculus</i>
YB1	Y-box binding protein 1
MSY1	mouse YB1
MEF	mouse embryo fibroblast
SMC	smooth muscle cell
ECM	extracellular matrix
TGF-β1	transforming growth factor β 1
SNP	single nucleotide polymorphism
NHLBI	National Heart Lung and Blood Institute
FBS	fetal bovine serum
PCR	polymerase chain reaction
SEC	size exclusion chromatography

WT	wild type
BSA	bovine serum albumin
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
nt	nucleotide
h	hour
min	minute
sec	second

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Figure 2.

Structural features of *Mm* Pur β highlighting the location of variant residues. (A) A linear schematic of the *Mm* Pur β protomer highlights three regions of internal homology known as PUR repeats I, II, and III in blue, red, and green respectively. Numbers displayed above the diagram represent the amino acids encompassing each repeat. (B) An updated homology model of the *Mm* Pur β homodimer depicts two intramolecular PUR domains composed of repeats I and II (red and blue) and an intermolecular PUR domain composed of a repeat III dimer (green). (C) Amino acid positions associated with missense substitutions in human Pur β are highlighted in a cartoon depiction of the *Mm* Pur β homodimer as follows: L46V (blue), G215S (green), P223L (purple), F274L (orange), G282S (yellow), and R297Q (red). (D) A representation of the surface area of the *Mm* Pur β homodimer is shown with the position of variant residues in the same colors as noted above. In the case of the Q64*

nonsense variant, the predicted truncated product is shown in the insets of panels (C) and (D).

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Figure 3.

The effect of amino acid substitutions on the transcriptional repressor activity of Purβ. AKR-2B MEFs (A, B) or A7r5 SMCs (C, D) were transiently co-transfected with expression vectors encoding NHis-Purβ WT or variants with the indicated single amino acid substitutions together with an *Acta2* promoter-luciferase reporter construct. Cells were harvested 48 or 72 h later and cell extracts were assayed for luciferase activity, total protein concentration, and NHis-Purβ expression. (A, C) Total protein adjusted luciferase values from cells transfected with pCI-NHis-Purβ plasmids were normalized to baseline values from cells transfected with empty pCI vector (defined as 1) and the reciprocal was taken to obtain fold repression. Data are presented as a bar graph specifying the group mean and standard error. Asterisks denote statistical differences from Purβ WT based on Dunnett's multiple comparison test as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. (A) Bars show the relative activity of Purβ variants expressed in AKR-2B MEFs (mean and SEM, $n = 12-18$). (B) Immunoblot detection of NHis-Purβ WT and missense variants in AKR-2B cell lysates (top) and GAPDH as a loading control (bottom). (C) Bars show the relative activity of Purβ variants expressed in A7r5 SMCs (mean and SEM, $n = 9$

except for Q64* where $n = 6$). (D) Immunoblot detection of NHis-Pur β WT and missense variants in A7r5 cell lysates (top) and GAPDH as a loading control (bottom).

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Figure 4.

Evaluation of the thermostability of Purβ variants. (A) The thermal unfolding of purified NHis-Purβ WT, R297Q, and P223L was monitored by differential scanning fluorimetry. Solutions of 300 μg/mL protein in aqueous buffer containing SYPRO Orange were heated in 1°C increments per min and changes in fluorescence were monitored over time. The Boltzmann equation was used to fit the fluorescence data points and the resulting thermal shift curves for Purβ WT, R297Q, and P223L are displayed. (B) Additional assays were performed at protein concentrations ranging from 75 to 300 μg/mL. Multiple T_m values for Purβ WT, R297Q, and P223L were determined from the midpoint of thermal shift curves generated at each protein concentration. Bars show the average T_m determined for each protein (mean and SD, n = 6–8).

Figure 5.

Qualitative assessment of the interaction of Purβ variants with different DNA sequences. (A) A schematic of the mouse *Acta2* 5'-flanking region and first intron is shown highlighting various *cis*-elements implicated in gene activation (MCAT, THR, TCE, CArG) and the location of Purβ recognition sequences PE32 and SPUR32. Numbers denote the 5' and 3' ends of each element. CArGi identifies a functional CArG element located in the first intron between exons 1 and 2 (Ex 1 and Ex 2). (B) The binding of NHis-Purβ WT, R297Q, and P223L to purine-rich ssDNA (PE32-bF, SPUR32-bF, PUR24-bF), pyrimidine-rich ssDNA (PE32-bR, SPUR32-bR), or annealed dsDNA (dsPE32-bR, dsSPUR32-bR) was assessed by ELISA. The concentration of fluid-phase Purβ and solid-phase DNA was fixed at 1.0 nM and 0.5 nM, respectively, in the primary binding step of the assay. Solid-phase Purβ-DNA complexes were detected using a primary antibody recognizing a carboxy terminal epitope

in Pur β . Bars show relative A405 values normalized to the signal generated by Pur β WT in complex with PE32-bF defined as 1 (mean and SD, n = 4–12).

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Figure 6.

Quantitative analysis of the interaction of Pur β variants with ssDNA. The binding of NHis-Pur β WT, R297Q, and P223L to a purine-rich, *Acta2*-derived, ssDNA sequence (PE32-bF) and a corresponding mutant (3I5T7) was assessed by ELISA. The concentration of fluid-phase Pur β ranged from 9.0 to 0.074 nM while the concentration of the solid-phase DNA was fixed at 0.5 nM in the primary binding step of the assay. Solid-phase Pur β -DNA complexes were detected using a primary antibody recognizing a carboxy terminal epitope in Pur β . Each variant was evaluated in comparison to the wild-type protein in multiple titration trials conducted with PE32-bF (mean $\pm$ SD, $n = 5-18$) or 3I5T7 (mean $\pm$ SD, $n = 3-5$). Binding curves were generated by fitting normalized absorbance values to an equation for a hyperbola (A) or a four-parameter logistic model (B) for the purpose of determining apparent binding constants, which are noted in the text.

Figure 7.

Quantitative analysis of the interaction of Purβ variants with MSY1. (A) The binding of NHis-Purβ WT, R297Q, and P223L to MSY1 was assessed by ELISA. The concentration of fluid-phase Purβ ranged from 400 to 3.13 nM. MSY1 was immobilized on microtiter wells at a fixed concentration of 100 nM. Solid-phase Purβ-MSY1 complexes were detected using a primary antibody directed against the carboxy terminal tail of Purβ. Each Purβ variant was evaluated in comparison to the wild-type protein in multiple titration trials. Non-specific binding was measured concurrently by assessing Purβ binding to wells coated with BSA alone. Binding curves were generated by fitting data points to the equation for a hyperbola (mean $\pm$ SEM, $n = 10-16$). (B) Absorbance values obtained in wells coated with BSA only were subtracted from absorbance values generated in wells coated with MSY1. Binding curves represent specific Purβ-MSY1 interaction. Apparent binding constants are noted in the text.

Figure 8. Qualitative analysis of the interaction of Purβ variants with selected nuclear extract-derived proteins. (A) The binding of fluid-phase nuclear proteins from AKR-2B MEFs to solid-phase NHis-Purβ WT, R297Q, and P223L immobilized at 300 nM was assessed by ELISA. Solid-phase transcription factor-Purβ complexes formed after an overnight incubation were detected using primary antibodies against MSY1, Purα, Sp3, Smad 4, or Smad 2/3. Absorbance values were normalized to the Purβ WT-MSY1 complex defined as 1.0 and the data are plotted in a bar graph (mean and SEM, n = 6–12). (B) The binding of fluid-phase nuclear proteins from HEK293 cells to solid-phase NHis-Purβ WT, P223L, and R297Q was assessed by ELISA in the same manner as described above (mean and SEM, n = 6–12). Asterisks denote statistical differences from Purβ WT based on one-way ANOVA and Dunnett's multiple comparison test as follows: *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.