NEAT1_2 and DAZAP1, paraspeckle components, interact with PXR to negatively regulate CYP3A4 induction

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Running Title

Paraspeckles traps nuclear PXR to negatively regulate CYP3A4

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Abbreviations: DAZAP1, DAZ-associated protein 1; DMEM, Dulbecco’s modified Eagle’s medium; DMP, dimethyl pimelimidate; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FISH, fluorescence in situ hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LLPS, liquid–liquid phase separation; NEAT, nuclear enriched abundant transcript; NONO, non-POU domain-containing octamer-binding protein; RIP, RNA immunoprecipitation; SFPQ, splicing factor proline and glutamine rich; P450, cytochrome P450; PXR, pregnane X receptor; RIP, RNA immunoprecipitation; siRNA, small interfering RNA
Abstract

Human pregnane X receptor (PXR) is a major nuclear receptor that upregulates the expression of drug-metabolizing enzymes such as CYP3A4. In our recent study, it was revealed that PXR interacts with DAZ-associated protein 1 (DAZAP1), which is an essential component of the paraspeckle, a membraneless nuclear body, and the interaction was disassociated by rifampicin, a ligand of PXR. The purpose of this study was to clarify the roles of paraspeckles in PXR-mediated transcriptional regulation. Immunoprecipitation assays using PXR-overexpressing HepG2 (ShP51) cells revealed that PXR interacts with not only DAZAP1 but also NEAT1_2, a long noncoding RNA included in the paraspeckle, and that the interaction between PXR and NEAT1_2 was disassociated by rifampicin. These results suggest that PXR is trapped in paraspeckles and that the activation of PXR by its ligands facilitates its disassociation from paraspeckles. Induction of CYP3A4 by rifampicin was significantly enhanced by the knockdown of NEAT1_2 or DAZAP1 in ShP51 cells and their parental HepG2 cells. A luciferase assay using a plasmid containing the PXR response elements of CYP3A4 revealed that the increased CYP3A4 induction by siNEAT1_2 or siDAZAP1 was due to the increased transactivation by PXR. These results suggest that paraspeckles play a role in trapping nuclear PXR in the absence of the ligand to negatively regulate transactivation of its downstream gene. Collectively, this is the first study to demonstrate that the paraspeckle components NEAT1_2 and DAZAP1 negatively regulate CYP3A4 induction by PXR.

Significance Statement

This study revealed that PXR interacts with paraspeckle components NEAT1_2 and DAZAP1 to suppress CYP3A4 induction by PXR, and the interaction is dissociated by PXR ligands. This finding provides a novel concept that paraspeckles formed by LLPS potentially affect drug metabolism via negative regulation of PXR function.
Introduction

In eukaryotic cells, various proteins and nucleic acids are orderly arranged in the nucleus and cytoplasm (Handwerger and Gall, 2006). Recently, it was revealed that the ordered localization of proteins and nucleic acids in the membraneless intracellular microenvironment is modulated by liquid–liquid phase separation (LLPS) (Shin and Brangwynne, 2017). Proteins, which contain RNA/DNA binding domain, a low-complexity domain, and intrinsically disordered regions, interact with specific DNA or RNA to aggregate each other, and then, such molecular interactions drive LLPS (Kato et al., 2012; Molliex et al., 2015; Treeck and Parker, 2018). LLPS, which was originally studied in the field of physical chemistry, has been extended to the field of cell biology since the 2010s. Advances in research have recently revealed that membraneless organelles such as Cajal bodies (Hardin et al., 1969) and nuclear speckles (Swift, 1959) are formed via LLPS (Peng et al., 2021). Although knowledge has recently accumulated that membraneless organelles are involved in gene regulation (Sawyer et al., 2016; Chen and Belmont, 2019), the significance of their biological function remains largely unknown.

Paraspeckles are one of the most studied membraneless structures formed via LLPS with a diameter of approximately 360 nm (Fox et al., 2002). They consist of a long noncoding RNA nuclear enriched abundant transcript (NEAT)1 and 40 kinds of proteins and are localized in the nucleus (Naganuma and Hirose, 2013). For NEAT1, there are two isoforms, NEAT1_1 (a short form) and NEAT1_1_2 (a long form), which are generated from a gene by alternative 3’ processing (Sunwoo et al., 2009), and the latter has been reported to be essential for the formation of paraspeckles (Li et al., 2017). Among the 40 proteins, 7 proteins, including non-POU domain-containing octamer-binding protein (NONO) and splicing factor proline and glutamine rich (SFPQ) protein, which facilitate the synthesis and stabilization of NEAT1_2, as well as DAZ-associated protein 1 (DAZAP1), which facilitates paraspeckle formation without affecting NEAT1_2 expression (Naganuma et al., 2012), are also essential for paraspeckle formation (Mao et al., 2011). It has been reported that stress, viral infection, and some diseases, such as cancer and Alzheimer’s disease, increase NEAT1_2 expression in various tissues, promoting the formation of paraspeckle assembly (Adriaens and Marine, 2017; McCluggage
and Fox, 2021; Szafranski et al., 2015). Paraspeckles trap RNA, RNA binding proteins and transcription factors to suppress the transition of RNA into the cytoplasm (Prasanth et al., 2005), to facilitate degradation of RNA (Shen et al., 2021), or to alter target gene expression (Imamura et al., 2014). Thus, paraspeckles work as molecular sponges to regulate gene expression.

Pregnane X receptor (PXR) is one of the major nuclear receptors expressed in the liver and small intestine, playing an important role in the regulation of xenobiotic metabolism and energy metabolism (Kliewer et al., 1998). PXR translocates from the cytoplasm to the nucleus in response to ligands such as rifampicin and ritonavir (Blumberg et al., 1998), forms a heterodimer with RXRα, and activates transcription of its downstream genes, such as cytochrome P450 (CYP) 3A4 (Lehmann et al., 1998; Kliewer et al., 2002). CYP3A4 metabolizes 40% of clinically used drugs, and its induction via PXR is the cause of drug–drug interactions (Sinz, 2013). In addition to the drug-metabolizing enzymes, PXR upregulates the expression of gluconeogenic factors such as glucose-6-phosphatase (G6Pase) and lipogenic factors such as cluster of differentiation 36 (CD36) (Bhalla et al., 2004; Kodama et al., 2004; Kodama et al., 2007). Elucidation of the regulatory mechanism of PXR function may provide a clue to avoid PXR-mediated adverse events, including drug–drug interactions, hyperglycemia, and hepatic steatosis.

Recently, we elucidated proteins interacting with PXR in ShP51 cells, human PXR-overexpressing HepG2 cells treated with rifampicin, by coimmunoprecipitation using an anti-PXR antibody followed by proteomic analysis (Kurosawa et al., 2023). DAZAP1 was found to interact with PXR, and the interaction was decreased by rifampicin treatment. In this study, we aimed to clarify the role of the paraspeckle component in PXR function.
Materials and Methods

Chemicals and reagents

Rifampicin was purchased from Wako (Osaka, Japan). Lipofectamine RNAiMAX, Lipofectamine 3000, Silencer Select siRNAs for human NEAT1_2 (s341849) (siNEAT1_2), human DAZAP1 (s25512) (siDAZAP1), and negative control #1 (siControl) were purchased from Thermo Fisher Scientific (Waltham, MA). Dynabeads protein G was purchased from Veritas (Tokyo, Japan). Dimethyl pimelimidate (DMP) was purchased from Tokyo Chemical Industry (Tokyo, Japan). The Nano-Glo Dual-Luciferase Reporter Assay System was obtained from Promega (Madison, WI). RNAiso and random hexamers were from Takara (Shiga, Japan). ReverTra Ace and ScriptMAX Thermo T7 Transcription Kits were purchased from Toyobo (Osaka, Japan). FITC RNA Labeling Mix was purchased from Roche (Basel, Switzerland). All of the primers were commercially synthesized at Eurofins Genomics (Tokyo, Japan). Mouse anti-human DAZAP1 (sc-373987) and PXR (sc-48340) monoclonal antibodies and mouse IgG (sc-2025) were obtained from Santa Cruz Laboratories (Santa Cruz, CA). Mouse anti-His-tag monoclonal antibody (D291-3) and mouse anti-FLAG-tag monoclonal antibody (F1804) were obtained from MBL (Aichi, Japan) and Sigma Aldrich (St. Louis, MO), respectively. Mouse anti-TATA binding protein (TBP) monoclonal antibody (66166-1-Ig) was obtained from proteintech (Rosemont, IL). Rabbit anti-human GAPDH polyclonal antibody and FITC polyclonal antibody (A-889) were purchased from IMGENIX (San Diego, CA) and Thermo Fisher Scientific, respectively. IRDye 680 goat anti-rabbit IgG and goat anti-mouse IgG were purchased from LI-COR Biosciences (Lincoln, NE). Cy2 goat anti-rabbit IgG (ab6940) was obtained from Abcam (Cambridge, UK). All other chemicals and solvents were of the highest grade commercially available.

Cell cultures

Human hepatocellular carcinoma-derived HepG2 cells and human embryonic kidney-derived HEK293T cells were obtained from Riken Gene Bank (Tsukuba, Japan) and
American Type Culture Collection (Manassas, VA), respectively. ShP51 cells, which are a HepG2 cell line stably expressing human PXR (Kodama and Negishi, 2011), were kindly provided by Dr. Negishi (National Institute of Environmental Health Sciences, NC). HepG2 cells and ShP51 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and nonessential amino acids (NEAAs) (Thermo Fisher Scientific). HEK293T cells were cultured in DMEM containing 10% FBS and 4.5 mg/mL glucose. These cells were cultured at 37 °C under an atmosphere of 5% CO₂ and 95% air.

Construction of the pTarget/DAZAP1-His plasmid

The expression plasmid of DAZAP1 with a His-tag at the C-terminus was constructed as follows. The coding sequence of DAZAP1 was amplified using the DAZAP1 S primer and the DAZAP1+His-tag AS primer (Supplemental Table 1) with cDNA from ShP51 cells as a template and subcloned into the pTarget vector.

Transfection of siRNA or expression plasmid and drug treatment

ShP51 and HepG2 cells were seeded into 12-well plates and transfected with 5 nM siNEAT1_2 or/and siDAZAP1 using Lipofectamine RNAiMAX. HEK293T cells were cotransfected with the pTarget/DAZAP1-His plasmid and the pcDNA3.1/FLAR-PXR plasmid, which was kindly provided by Dr. Negishi (National Institute of Environmental Health Sciences, NC), using Lipofectamine 3000. After incubation for 24 hr, the cells were treated with 10 µM rifampicin for 24 hr.

Coimmunoprecipitation

Nuclear extracts from ShP51 cells were prepared according to our previous report (Kurosawa et al., 2023). Briefly, ShP51 cells treated with rifampicin were lysed with buffer A [25 mM HEPES (pH 7.6), 5 mM MgCl₂, 25 mM KCl, 0.05 mM EDTA, 10% glycerol, 0.1% NP40, and 1 mM DTT] and incubated on ice for 10 min. The cells were centrifuged at 1,500 ×
g for 5 min at 4 °C, and the precipitate was collected as a nuclear fraction. The nuclear fraction was lysed with buffer C containing 0.3 M ammonium sulfate [10 mM HEPES (pH 7.6), 3 mM MgCl₂, 100 mM KCl, 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 0.3 M ammonium sulfate] and were incubated on ice for 20 min. The nuclear fraction was centrifuged at 100,000 × g for 10 min at 4 °C, and the supernatant was collected. Equal volumes of 0.6 g/mL ammonium sulfate were added to the supernatant and incubated on ice for 10 min. The supernatant was centrifuged at 100,000 × g for 10 min at 4 °C, and the precipitant was lysed with lysis buffer [20 mM HEPES (pH 8.0), 0.3 mM NaCl, 0.2 mM EDTA, 15% glycerol and 0.5% NP40] and collected as a nuclear extract for coimmunoprecipitation. Cell lysates from HEK293T cells were prepared as follows. The cells transfected with pTargeT/DAZAP1 plasmid and pcDNA3.1/FLAR-PXR plasmid followed by treatment with rifampicin as described above were lysed with lysis buffer and incubated on ice for 1 hr. The supernatant after centrifugation at 17,000 × g for 10 min at 4 °C was collected.

For coimmunoprecipitation, anti-DAZAP1 or anti-FLAG antibody was crosslinked with Dynabeads protein G using DMP. The nuclear extract or cell lysate was incubated with primary antibody-conjugated Dynabeads for 2 hr. After washing three times with wash buffer [10 mM HEPES (pH 8.0), 0.3 mM NaCl, 0.2 mM EDTA, 15% glycerol, and 0.1% NP40], the beads were boiled with SDS–PAGE sample buffer [0.2 M Tris-HCl (pH 6.8), 6% SDS, 0.3 mM bromophenol blue, 30% glycerol, and 15% 2-mercaptoethanol]. Immunoprecipitates were subjected to Western blotting using anti-PXR, anti-DAZAP1, anti-FLAG, or anti-His antibodies.

**RNA immunoprecipitation (RIP)**

ShP51 cells treated with rifampicin were collected and disrupted by freeze–thawing. RIP lysis buffer [100 mM KCl, 5 mM MgCl₂, 10 mM HEPES (pH 7.0), 0.5% NP40, and 200 units/mL RNase inhibitor] was added to the cell pellet and vigorously mixed to lyse the cells. The lysed cells were centrifuged at 20,000 × g for 10 min at 4 °C, and the supernatant was collected as a whole cell lysate. The whole cell lysate was precleared using Dynabeads protein
G. For immunoprecipitation, anti-PXR antibody was immobilized to the beads blocked with 10 mg/mL BSA and 1 mg/mL salmon sperm DNA. The PXR antibody-coupled beads were incubated with the whole cell lysate at 4 °C overnight. After washing the beads four times with NT-2 buffer [50 mM Tris, 150 mM NaCl, 1 mM MgCl₂, and 0.05% NP40] and twice with NT-2 buffer containing 2 M urea, RNAiso and chloroform were added to the beads and vigorously mixed. After centrifugation at 20,000 × g for 15 min at 4 °C, the supernatant was collected, and RNA was extracted using the Rneasy MinElute Cleanup Kit from Qiagen (Tokyo, Japan) according to the manufacturer’s protocol.

cDNA was synthesized from RNA using ReverTra Ace according to the manufacturer’s protocol. The enrichment of NEAT1_2 was evaluated by real-time RT–PCR. HNF4A-AS1 and GAPDH mRNA were evaluated as negative controls. The sequences of the primers and the PCR conditions for GAPDH were previously described (Tsuchiya et al., 2004). The primer sequences of the HNF4A-AS1 and NEAT1_2 were shown in Supplemental Table 1. The PCR conditions for HNF4A-AS1 and NEAT1_2 was as follows: after an initial denaturation at 95 °C for 60 sec, amplification was performed by denaturation at 95 °C for 15 sec, followed by annealing/extension at 58 °C for 20 sec for 40 cycles. Real-time RT–PCR was performed using QuantStudio1 (Thermo Fisher Scientific).

Fluorescence in situ hybridization (FISH) assay and immunofluorescence staining

FITC-labeled antisense RNA probe targeting NEAT1_2 was prepared as follows. DNA fragments were amplified using the NEAT1_2 FISH probe S primer and the NEAT1_2 FISH probe AS primer (Supplemental Table 1) using cDNA from ShP51 cells as a template. The DNA fragment was subcloned into the pT7Blue T vector for insertion in the antisense direction. To perform in vitro transcription, the plasmid DNA was linearized by EcoRI. By using FITC RNA Labeling Mix and ScriptMAX Thermo T7 Transcription Kit, FITC-labeled antisense RNA probe was transcribed according to the manufacturer’s instructions. The plasmid DNA was degraded by Dnase I, and the RNA probe was purified by phenol/chloroform extraction.

ShP51 cells were seeded on a cover glass in a 6-well plate. After transfection of siRNA
and/or treatment with rifampicin, the cells were fixed with 4% paraformaldehyde for 10 min. After fixation, the cells were permeated with 0.5% Triton X-100 for 10 min. Hybridization with 20 µg/mL FITC-labeled probes was carried out for 20 h at 55 °C in hybridization buffer [50% formamide, 2 × SSC, 1 × Denhardt’s solution, 5% dextran sulfate, 10 mM EDTA, and 0.01% Tween 20]. The samples were washed twice with wash buffer [50% formamide, 2 × SSC, and 0.01% Tween 20] at 55 °C for 30 min and then incubated at 37 °C for 1 h in RNase A buffer [5 µg/mL RNase A, 0.5 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, and 0.01% Tween 20]. The sample was further washed with 2 × SSC containing 0.01% Tween 20 at 55 °C for 30 min, followed by washing with 0.2 × SSC containing 0.01% Tween 20 at 55 °C for 30 min. After additional washing with 1 × PBS containing 0.01% Tween 20, the sample was incubated with blocking buffer [5% BSA, 0.1% Tween 20, and 1 × PBS] for 1 hr at r.t. The sample was incubated at room temperature with antibodies against FITC and PXR for 9 and 1 hr, respectively. After washing twice with 1 × PBS containing 0.01% Tween 20, the sample was incubated for 1 hr at room temperature with secondary antibody to visualize the FITC-labeled probe and PXR. The images were processed using a ZEISS LSM 900 (Carl Zeiss, Oberkochen, Germany).

**Preparation of cell homogenates and total RNA**

ShP51 and HepG2 cells were transfected with siRNA and treated with rifampicin as described above. Then, the cells were harvested to prepare cell homogenates or total RNA. In preparation of cell homogenates, the cells were suspended in TGE buffer [10 mM Tris-HCl, 20% glycerol, and 1 mM EDTA (pH 7.4)], disrupted by three rounds of freeze–thawing, and homogenized. The protein concentration was determined using Bradford protein assay reagent (Bio-Rad, Hercules, CA) with γ-globulin as a standard. Total RNA was prepared using RNAiso according to the manufacturer’s protocol.

**Real-time RT–PCR**

cDNA was synthesized from total RNA using ReverTra Ace. A 1-µL portion of the
The reverse-transcribed mixture was added to a PCR mixture containing 5 pmol of each primer and 10 μL of Luna Universal qPCR mix in a final volume of 20 μL. The primer and PCR condition of GAPDH and NEAT1_2 were described above. The primer sequences of the CYP3A4, CYP2C9 and WEE1 G2 checkpoint kinase (WEE1) were shown in Supplemental Table 1. The PCR conditions were as follows: The initial denaturation was at 95 °C for 60 sec, the denaturation step was at 95 °C for 15 sec, and the annealing/extension steps were at 57 °C (CYP3A4) for 30 sec, 50 °C (CYP2C9) for 30 sec, and 60 °C (WEE1) for 20 sec, respectively, for 40 cycles. Real-time RT–PCR was performed using QuantStudio1 (Thermo Fisher Scientific). The mRNA level was normalized to the GAPDH mRNA level.

**SDS–PAGE and Western blot analysis**

The cell homogenates, the whole cell lysate, the nuclear extract, and the immunoprecipitates were separated by 10% SDS–PAGE and transferred to Immobilon-P transfer membrane (DAZAP1, His-tag, FLAG-tag, TBP and GAPDH) (Millipore, Billerica, MA) or nitrocellulose (PXR) (Whatman GmbH, Dassel, Germany). The primary antibody and the fluorescent dye-labeled secondary antibody was probed with the membranes. The bands were detected by using an Odyssey Infrared Imaging system (LI-COR Biosciences).

**Formaldehyde-assisted isolation of regulatory element (FAIRE) assay**

ShP51 cells were transfected with siRNA and treated with rifampicin as described above. Then, the cells were cross-linked by 1% formaldehyde, lysed with ice-cold lysis buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 2% Triton X-100, 1% SDS, and 1 mM phenylmethylsulfonyl fluoride], and sonicated for 30 sec for 10 cycles using BioRaptor (Cosmo Bio, Tokyo, Japan) to shear chromatin DNA. Then, histone-free DNA extracted by phenol–chloroform extraction was subjected to real-time PCR analysis. Three regions in the CYP3A4 5′-flanking region, which are the proximal promoter, distal enhancer, and far enhancer, were evaluated using the respective primer pair (Supplemental Table 1). The initial denaturation was at 95 °C for 60 sec, the denaturation step was at 95 °C for 15 sec, and the annealing/extension...
steps in the CYP3A4 proximal promoter, distal enhancer, and far enhancer were at 58 °C for 30 sec, 52 °C for 30 sec, and 55 °C for 20 sec, respectively, for 40 cycles.

**Luciferase assay**

A reporter plasmid containing the CYP3A4 proximal promoter region and distal enhancer region (Fig. 4B, pGL3/CYP3A4-362-7.7k, Noracharttiyapot et al., 2006), which was kindly provided by Dr. Nagata and Dr. Yamazoe (Tohoku University), was used. ShP51 cells were seeded into 96-well plates with 5 nM siRNA using Lipofectamine RNAiMAX. After incubation for 24 hr, the cells were transfected with 50 ng pGL3/CYP3A4-362-7.7k plasmid and 10 pg NanoLuc plasmids using Lipofectamine 3000. After incubation for 24 hr, the cells were treated with 10 µM rifampicin for 24 hr. Luciferase activity was measured by a luminometer using the Nano-Glo Dual-Luciferase Reporter Assay System.

**Statistical analyses**

Comparison of two groups was made with an unpaired, two-tailed Student’s t-test. Comparisons of multiple groups were determined by analysis of variance followed by Tukey’s test. A value of $P < 0.05$ was considered statistically significant.
Results

PXR interacts with DAZAP1 and NEAT1_2, which are paraspeckle components

Our recent proteomic analysis revealed the possibility that PXR interacts with DAZAP1 (Kurosawa et al., 2023). To confirm the interaction, a coimmunoprecipitation assay was performed using the nuclear fraction of ShP51 cells and an anti-DAZAP1 antibody. As shown in Fig. 1A, PXR was detected in the immunoprecipitate, indicating that PXR interacts with DAZAP1. The interaction was not altered by rifampicin treatment (Fig. 1A), but this was an unexpected result because our previous proteomic analysis showed that the interaction between PXR and DAZAP1 was decreased by rifampicin. As a side note, the quality of nuclear-cytoplasmic fractionation was confirmed by Western blotting for TBP and GAPDH (supplemental Fig. S1). To investigate whether the interaction is truly not affected by rifampicin, coimmunoprecipitation using cell lysates of HEK293T cells, to which FLAG-PXR and DAZAP1-His were overexpressed, was performed. In the immunoprecipitate by anti-His antibody, FLAG-PXR was detected, and the band density was not affected by rifampicin (Fig. 1B). These results suggested that PXR interacts with paraspeckles via DAZAP1, and the interaction was not altered by rifampicin treatment.

Considering the possibility that PXR also interacts with NEAT1_2, a long noncoding RNA included in paraspeckles, we performed a RIP assay using an anti-PXR antibody and the lysate of ShP51 cells. The enrichment of NEAT1_2 was observed in the immunoprecipitate compared to IgG and was significantly decreased by rifampicin treatment (Fig. 1C). In contrast, the enrichment of neither a lncRNA HNF4A-AS1 nor GAPDH mRNA, which were evaluated as randomly selected controls, was observed (supplemental Fig. S2). These results suggest that PXR interacts with NEAT1_2 and DAZAP1, which are paraspeckle components, and that the activation of PXR by its ligand may cause PXR to dissociate from paraspeckles.

PXR is trapped in paraspeckles

To investigate whether PXR colocalizes with paraspeckles in situ, a FISH assay targeting
NEAT1_2 and immunofluorescent staining for PXR were performed using ShP51 cells. Among the 2 isoforms, NEAT1_1 (3,756 nt) and NEAT1_2 (22,743 nt), only NEAT1_2 is essential for paraspeckle formation. Therefore, in the FISH assay, an RNA probe that specifically recognizes NEAT1_2 was used (Fig. 2A). As shown in Fig. 2B, NEAT1_2 staining was observed in granules in the nucleus, which corresponds to paraspeckles. PXR was primarily observed in the nucleus rather than the cytoplasm, consistent with previous reports (Saradhi et al., 2005). Thus, it was demonstrated that some nuclear PXR colocalized with NEAT1_2 in paraspeckles (Fig. 2B).

**NEAT1_2 and DAZAP1 negatively regulate CYP3A4 induction by a PXR ligand**

It has been reported that paraspeckle formation is suppressed by knockdown of essential components of paraspeckles, such as NEAT1_2 and DAZAP1 (Naganuma et al., 2012). To examine whether the suppression of paraspeckle formation affects the induction of downstream genes of PXR, ShP51 cells were transfected with siNEAT1_2 or siDAZAP1 and treated with rifampicin. To evaluate the effects of siNEAT1_2 and siDAZAP1 on paraspeckle formation, FISH assays targeting NEAT1_2 and immunofluorescent staining for PXR were performed. Consistent with previous reports, the number of paraspeckle per cell was significantly decreased by transfection of siNEAT1_2 (Fig. 3A and B). To confirm knockdown efficiency, NEAT1_2 level and DAZAP1 protein level in siRNA-transfected ShP51 cells were evaluated. Unexpectedly, NEAT1_2 expression level was not altered by transfection of siNEAT1_2 in ShP51 cells (Fig. 3C). However, mRNA level of WEE1, which is positively regulated by NEAT1_2 acting as miR-101-3p sponge in Huh-7 cells (Chen and Zhang, 2019), was significantly decreased by transfection of siNEAT1_2 (Fig. 3D). Therefore, it was suggested that NEAT1_2 function, including regulation of WEE1 expression and paraspeckle assembly, was inhibited by siNEAT1_2 by hybridization to NEAT1_2 without decrease in NEAT1_2 expression level. By transfection of siDAZAP1, DAZAP1 protein expression was decreased, and NEAT1_2 level was slightly increased (Fig. 3E). Interestingly, PXR staining was decreased by transfection of siNEAT1_2 or siDAZAP1 (Fig. 3A). Western blotting using cell lysates also
revealed that PXR protein levels were decreased by transfection of siNEAT1_2 or siDAZAP1 (Fig. 3F and G). As shown in Fig. 3F and G, the induction of CYP3A4 mRNA by rifampicin was significantly increased by transfection of siNEAT1_2 or siDAZAP1, even though the PXR protein level was decreased by transfection of siNEAT1_2 or siDAZAP1. Moreover, the enhancement in CYP3A4 induction by siNEAT1_2 was further augmented by co-transfection of siDAZAP1 (Fig. 3H). As well as CYP3A4, the induction of CYP2C9, another PXR downstream gene, by rifampicin was significantly enhanced by transfection of siNEAT1_2 of siDAZAP1 (Fig. 3I and J). These results suggest that transcriptional activity by PXR was enhanced by the increased nuclear PXR, which is not trapped in paraspeckles, even though the overall PXR level was decreased. When the same experiments were performed using parental HepG2 cells, PXR protein levels were not altered by transfection of siNEAT1_2 or siDAZAP1 (Fig. 3K and L), but CYP3A4 induction by rifampicin was significantly increased by transfection of siNEAT1_2 and siDAZAP1 (Figs. 3K and 3L). These results suggest that the paraspeckle components NEAT1_2 and DAZAP1 negatively regulate PXR-mediated CYP3A4 induction.

**NEAT1_2 and DAZAP1 suppress the transcriptional activation of PXR**

In general, transcription is accelerated by relaxation of chromatin structure (Hübner et al., 2013). Our recent study revealed that the chromatin structure around the PXR response element in the 5′-flanking region of the CYP3A4 gene is relaxed by treatment with PXR ligands (Kurosawa et al., 2023). We surmised the possibility that the enhanced CYP3A4 induction by transfection of siNEAT1_2 or siDAZAP1 was mediated by alteration of chromatin status of the 5′-flanking region of the CYP3A4 gene. Here, the chromatin status of three regions in the 5′-flanking region of the CYP3A4 gene was evaluated. The chromatin structure in the far enhancer was significantly relaxed by rifampicin treatment, and those in the distal enhancer and the proximal promoter regions tended to be relaxed by rifampicin, consistent with our previous study (Kurosawa et al., 2023). Transfection of siNEAT1_2 or siDAZAP1 did not enhance the relaxation of chromatin structures in these regions (Fig. 4A), suggesting that NEAT1_2 and
DAZAP1 do not affect the chromatin state of the 5'-flanking region of the CYP3A4 gene. Accordingly, it was suggested that the enhanced CYP3A4 induction might be mediated via the activation of PXR itself by paraspeckle decomposition. We investigated, by luciferase assay using the pGL3/CYP3A4-362-7.7k plasmid, which contains the distal enhancer region and the proximal promoter region of CYP3A4, whether NEAT1_2 and DAZAP1 affect PXR-mediated transcriptional activation. The increase in luciferase activity by rifampicin was significantly enhanced by transfection of siNEAT1_2 or siDAZAP1 (Fig. 4C and D). These results suggest that the paraspeckle components NEAT1_2 and DAZAP1 suppress the activation of PXR itself and do not affect chromatin structure, leading to downregulation of the expression of downstream genes of PXR.
**Discussion**

Paraspeckles are nuclear membraneless organelles in which paraspeckle proteins interact with RNA binding proteins or transcription factors to regulate gene expression (Prasanth et al., 2005; Hirose et al., 2014). Recently, it has been reported that NONO, an essential paraspeckle protein, interacts with hypoxia inducible factor (HIF) 1 and HIF 2 to upregulate the expression of their downstream genes in hepatocellular carcinoma cell lines, leading to the progression of hepatocellular carcinoma (Shen et al., 2021). Although some studies have revealed the role of paraspeckle components in the regulation of protein function or gene expression related to hepatocellular cancer progression (Wang et al., 2018), the significance of paraspeckles in normal liver function, such as drug metabolism, remains to be elucidated. In this study, we examined the possibility that paraspeckle components may regulate PXR-mediated transactivation of genes encoding drug-metabolizing enzymes.

In our recent proteomic analysis to identify proteins interacting with PXR (Kurosawa et al., 2023), DAZAP1 was identified as a protein interacting with PXR, and the interaction was disassociated by rifampicin treatment. Supporting the proteomic analysis, the interaction between PXR and DAZAP1 was confirmed by the coimmunoprecipitation assay performed in the present study. However, the interaction was not diminished by rifampicin treatment in ShP51 cells and HEK293T cells (Fig. 1A and B). Meanwhile, the RIP assay revealed that PXR interacts with NEAT1_2, and the interaction was diminished by rifampicin treatment (Fig. 1C). It has been reported that DAZAP1 is distributed throughout the nucleus, whereas NEAT1_2 is localized only in the nuclear paraspeckle (Lin et al., 2012). Thus, it seems that PXR ligands interfere with the interaction of PXR with paraspeckle components such as NEAT1_2, which are localized in paraspeckles, but not the interaction of PXR with DAZAP1 distributed in the whole nucleus. The PXR ligand-mediated dissociation of PXR from paraspeckles was mainly due to the dissociation of PXR from NEAT1_2 (Fig. 1C), with the inconsistency in the dissociation of PXR and DAZAP1 by rifampicin between the two studies.

Supporting the results showing the interaction between PXR and NEAT1_2 (Fig. 1C), an *in situ* hybridization assay targeting NEAT1_2 revealed that some nuclear PXR was in
paraspeckles (Fig. 2B). We expected that the PXR signal overlapping with the paraspeckle would be more prominent than the other regions if PXR interacts with components of the paraspeckle, but such a phenomenon was not observed. Moreover, by immunofluorescence staining of PXR (Fig. 3A), neither constitutive nuclear accumulation nor disassociation from paraspeckles of PXR by the knockdown of paraspeckle components was observed in ShP51 cells. PXR in human primary hepatocytes is localized in the cytosol and translocates into the nucleus upon activation by ligands (Blumberg et al., 1998), whereas PXR in HepG2 cells is distributed in the nucleus and cytosol, and nuclear translocation by ligands is not observed (Saradhi et al., 2005). In contrast, it has been demonstrated that PXR in 3D-cultured HepG2 cells is localized in the cytoplasm and translocates into the nucleus upon activation by ligands (Yokobori et al., 2017). Therefore, it would be of interest to investigate whether PXR is colocalized with paraspeckles and disassociates from them by PXR ligands if ShP51 cells are 3D-cultured.

We found that transfection of siNEAT1_2 or siDAZAP1 resulted in decreased paraspeckle formation in ShP51 cells (Fig. 3A and B) and enhanced the induction of CYP3A4 (Fig. 3F and G) and CYP2C9 (Fig. 3I and J) by rifampicin. Unexpectedly, PXR protein levels were decreased by siNEAT1_2 or siDAZAP1 in ShP51 cells (Fig. 3F and G), although this phenomenon was not observed in parental HepG2 cells (Fig. 3K and L). The ShP51 cell line was established by overexpressing PXR in HepG2 cells using the pTargeT vector; therefore, exogenous PXR is transcribed via the CMV promoter (Kodama and Negishi, 2011). Thus, the knockdown of NEAT1_2 and DAZAP1 may interfere with CMV promoter activity, resulting in decreased ectopic PXR levels in ShP51 cells.

In the mechanisms of transactivation by ligand-activated PXR in primary hepatocytes, the translocation of PXR into the nucleus has widely been recognized as a key step (Lehmann et al., 1998; Kliwer et al., 2002). However, the transactivation of PXR-downstream genes by ligands is observed in cell lines such as HepG2 cells because PXR is constitutively distributed in nuclei. Accordingly, it is likely that mechanism(s) other than nuclear translocation also play an important role in the activation of PXR by ligands. Since we have recently revealed that
non-canonical brahma-related gene-associated factors, which are members of the chromatin remodeling switch/sucrose non-fermenting (SWI/SNF) complex, interact with PXR to open the chromatin structure of the 5’-flanking region of CYP3A4 (Kurosawa et al., 2023), we hypothesized that PXR dissociated from paraspeckles by rifampicin interacts with the SWI/SNF complex to relax the chromatin structure. However, the chromatin status of the 5’-flanking region of CYP3A4 was not altered by transfection of siNEAT1_2 or siDAZAP1 (Fig. 4A), suggesting that PXR disassociated from paraspeckle would not contribute to SWI/SNF-mediated chromatin remodeling. The luciferase assay revealed that the enhanced CYP3A4 induction by transfection of siNEAT1_2 or siDAZAP1 was due to the enhancement of transcriptional activation (Fig. 4C and D). Accordingly, the transcriptional activity of PXR would be enhanced by release from paraspeckles, resulting in enhancement of CYP3A4 induction by rifampicin. It has been reported that the SFPQ protein, which works as a transcription factor, is trapped by paraspeckles, resulting in the activation and suppression of transactivation of adenosine deaminase acting on RNA 2 and interleukin 8, respectively (Hirose et al., 2014; Imamura et al., 2014). Considering such studies reporting that transcription factors are trapped with paraspeckles to interfere with the regulation of their downstream genes, our proposal for the regulatory mechanism of the function of PXR, which is translocated to the nucleus, by trapping with paraspeckles in the nucleus would be reasonable. Since the behavior of PXR such as localization and nuclear translocation in ShP51 cells and HepG2 cells is different from that in normal hepatocytes, we performed the knockdown experiments for NEAT1_2 and DAZAP1 using primary human hepatocytes. However, the induction of CYP3A4 by rifampicin was not enhanced by transfection of siNEAT1_2 or siDAZAP1 (data not show), because of prominently lower (12-fold) NEAT1_2 level in the used primary hepatocytes. This is the weakness of this study. It would be worth to investigate the role of paraspeckle component on transcription by PXR using hepatocytes from an individual with high NEAT1_2 expression.

It has been reported that paraspeckle formation is promoted by oxidative stress, viral infection, and various diseases (Adriaens and Marine, 2017; McCluggage and Fox, 2021;
Szafranski et al., 2015). Paraspeckle formation is dependent on NEAT1_2 expression, and in other words, an increase in NEAT1_2 levels enhances paraspeckle formation (Mao et al., 2011). It has been reported that the number of speckle-like structures formed by NONO in the mouse liver is increased by feeding a normal diet or glucose injection after fasting (Benegiamo et al., 2018). In addition, it has been reported that NEAT1 expression was increased in HepG2 cells and mouse liver by free fatty acid treatment and high-fat diet feeding, respectively (Chen et al., 2019), and was significantly higher in the peripheral blood of diabetic patients than in healthy subjects (Asadi et al., 2021). Accordingly, it is possible that high-fat or high-glucose conditions promote paraspeckle formation in the liver to suppress the expression of drug metabolizing enzymes, which are controlled by PXR, such as CYP3A4. Since PXR positively regulates gluconeogenesis and lipogenesis by transactivating gluconeogenesis-related genes such as G6Pase and lipogenesis-related genes such as CD36, respectively (Bhalla et al., 2004; Kodama et al., 2004; Kodama et al., 2007), it is probable that the increased paraspeckle formation by high-fat or high-glucose conditions may result in the suppressed transactivation of PXR to reduce gluconeogenesis or lipogenesis as a negative feedback mechanism.

In conclusion, we found that the paraspeckle components NEAT1_2 and DAZAP1 trap PXR in the nucleus to negatively regulate its transactivation, resulting in suppression of the induction of downstream genes such as CYP3A4. This is the first study to demonstrate the function of paraspeckle components in the regulation of drug metabolism. Elucidating how to control the function of paraspeckles will allow us to understand or predict the variable drug response and toxicity.
Acknowledgments

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Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Authorship Contribution

Participated in research design: Mitamura, Nakano, Nakajima
Conducted experiments: Mitamura, Nakano, Isono, Kurosawa
Contributed new reagents or analytical tools: Mitamura, Kurosawa
Performed data analysis: Mitamura, Nakano, Isono Nakajima
Wrote or contributed to the writing of the manuscript: Mitamura, Nakano, Fukami, Nakajima
References


\textsuperscript{nrh}/NONO facilitates the hypoxia-enhanced progression of hepatocellular carcinoma. *Oncogene* 40: 4167-4183.


Footnotes
This study was supported by the World Premier International Research Center Initiative (WPI), MEXT, Japan.
No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

**Fig. 1.** Interaction between PXR and the paraspeckle components DAZAP1 and NEAT1_2. (A) Coimmunoprecipitation using an anti-DAZAP1 antibody. ShP51 cells were treated with 20 μM rifampicin for 24 hr, and the extracted nuclear fraction was subjected to coimmunoprecipitation with an anti-DAZAP1 antibody. DAZAP1 and PXR proteins in the immunoprecipitates were detected by Western blotting. (B) Coimmunoprecipitation using an anti-His-tag antibody. FLAG-PXR and DAZAP1-His co-overexpressed HEK293T cells were treated with 10 μM rifampicin for 24 hr, and prepared whole cell lysate was subjected to coimmunoprecipitation with anti-His-tag antibody. His-tag and FLAG-tag in the immunoprecipitates were detected by Western blotting. RNA immunoprecipitation using an anti-PXR antibody. ShP51 cells were treated with 20 μM rifampicin for 24 hr, and prepared whole cell lysates were subjected to RNA immunoprecipitation with an anti-PXR antibody. NEAT1_2 (C) level in the immunoprecipitates was evaluated by real-time RT–PCR. Each column represents the mean ± SD (n = 4). **P < 0.01.

**Fig. 2.** The interaction of PXR with paraspeckle in situ. (A) Schematic representation of NEAT1_2 RNA and the target region of the RNA probe. (B) FISH assay for NEAT1_2 and immunofluorescent staining of PXR in ShP51 cells.

**Fig. 3.** Effects of transfection of siNEAT1_2 or siDAZAP1 on the induction of CYP3A4 mRNA by rifampicin. ShP51 cells were transfected with 5 nM siNEAT1_2 or siDAZAP1. After incubation for 12 hr, the cells were treated with 10 μM rifampicin. (A) FISH assay targeting NEAT1_2 and immunofluorescent staining of PXR 12 hr after rifampicin treatment. (B) A box and whisker plot of the number of paraspeckles per 50 cells. The line in the box represents the median value, the box represents from the 25th to the 75th percentile, and the whiskers describe the maximum and the minimum values. The “+” sign represents the mean. ShP51 cells (C-L) or HepG2 (K and L) cells were transfected with 5 nM siNEAT1_2 or/and siDAZAP1. After 24 hr,
the cells were treated with 10 μM rifampicin. NEAT1_2 (C and E), WEE1 mRNA (D), CYP3A4 mRNA (F-H, K, and L), and CYP2C9 mRNA (I and J) levels were evaluated by real-time RT–PCR. PXR (F, G, K, and L) and DAZAP1 (C, E, and L) protein levels were determined by Western blotting. Each column represents the mean ± SD (n = 4). *P < 0.05, **P < 0.01, and ***P < 0.001.

**Fig. 4.** Effect of transfection of siNEAT1_2 or siDAZAP1 on transactivation by PXR. ShP51 cells were transfected with 5 nM siNEAT1_2 or siDAZAP1. After 24 hr, the cells were treated with 10 μM rifampicin for 24 hr. (A) FAIRE assay to evaluate the chromatin status of the far enhancer, distal enhancer, and proximal promoter regions of *CYP3A4*. Luciferase assay using a plasmid containing the distal enhancer and the proximal promoter regions of *CYP3A4*. (B) The structure of pGL3/CYP3A4-362-7.7k plasmid containing CYP3A4 proximal promoter region and distal enhancer region. siNEAT1_2 (C)- or siDAZAP1 (D)-transfected ShP51 cells were transiently transfected with 50 ng of pGL3/CYP3A4-362-7.7k plasmid. After 24 hr, the cells were treated with 10 μM rifampicin. Each column represents the mean ± SD (n = 4). *P < 0.05 and ***P < 0.001.
Figure 1

A

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<th>DAZAP1</th>
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<td>-</td>
<td>-</td>
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PXR

DAZAP1

(kDa)

Rifampicin

50

B

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FLAG-PXR

DAZAP1-His

(kDa)

Rifampicin

50

C

<table>
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<tr>
<td>DMSO IgG</td>
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<td>DMSO Anti-PXR</td>
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Rifampicin

**
A

Genomic DNA

<table>
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<td>NEAT1_2 (22,734 nt)</td>
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B

Hoechst 33342 | NEAT1_2 | PXR | Merge

![Imagery with Hoechst 33342, NEAT1_2, PXR, and Merge images with scale bar at 5 µm]
Figure 4

A

Far enhancer
Distal enhancer
Proximal promoter

-11,262
-7,779
-312
-11,038
-7,632
-98

ER6
ER6, DR3
ER6

CYP3A4

B

Distal enhancer
Proximal promoter

pGL3/CYP3A4-362-7.7k
ER6, DR3
ER6
Luciferase

C

D

Relative luciferase activity

Rifampicin
siControl
siNEAT1_2

***

Relative luciferase activity

Rifampicin
siControl
siDAZAP1

*
NEAT1_2 and DAZAP1, paraspeckle components, interact with PXR to negatively regulate CYP3A4 induction

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²WPI Nano Life Science Institute (WPI-NanoLSI), Kanazawa University, Kanazawa, Japan
Supplemental table 1. Sequence of primers.

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<td>CYP3A4 proximal promoter AS</td>
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<tr>
<td>CYP3A4 far enhancer AS</td>
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S: Sense primer, AS: Antisense primer
Fig. S1. Western blotting for PXR, GAPDH (cytosol marker), and TBP (nucleus marker) of cytosol and nuclear fraction of ShP51 cells. For detection of PXR and TBP, 45 µg cytosol and 15 µg nuclear fraction were applied. For detection of GAPDH, and 15 µg cytosol and 5 µg nuclear fraction were applied.
Fig. S2. Negative controls for RIP assay using anti-PXR antibody.

ShP51 cells were treated with 20 µM rifampicin for 24 hr, and prepared whole cell lysates were subjected to RNA immunoprecipitation with an anti-PXR antibody. A lncRNA HNF4A-AS1 (left panel) and GAPDH mRNA (right panel) levels in the immunoprecipitates were evaluated by real-time RT–PCR.