Protocadherin 19 (PCDH19) interacts with paraspeckle protein NONO to co-regulate gene expression with estrogen receptor alpha (ERα)

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Abstract

De novo and inherited mutations of X-chromosome cell adhesion molecule protocadherin 19 (PCDH19) cause frequent, highly variable epilepsy, autism, cognitive decline and behavioural problems syndrome. Intriguingly, hemizygous null males are not affected while heterozygous females are, contradicting established X-chromosome inheritance. The disease mechanism is not known. Cellular mosaicism is the likely driver. We have identified p54nrb/NONO, a multifunctional nuclear paraspeckle protein with known roles in nuclear hormone receptor gene regulation, as a PCDH19 protein interacting partner. Using breast cancer cells we show that PCDH19-NONO complex is a positive co-regulator of ERα-mediated gene expression. Expression of mutant PCDH19 affects at least a subset of known ERα-regulated genes. These data are consistent with our findings that genes regulated by nuclear hormone receptors and those involved in the metabolism of neurosteroids in particular are dysregulated in PCDH19-epilepsy girls and affected mosaic males. Overall we define and characterize a novel mechanism of gene regulation driven by PCDH19, which is mediated by paraspeckle constituent NONO and is ERα-dependent. This PCDH19-NONO-ERα axis is of relevance not only to PCDH19-epilepsy and its comorbidities but likely also to ERα and generally nuclear hormone receptor-associated cancers.

Introduction

PCDH19 Female Epilepsy (PCDH19-FE) (OMIM # 300088) is a frequent seizure disorder, which until recently has been under recognized (1,2). Unlike the classical X-linked male lethal disorder, PCDH19-FE affects heterozygous girls while hemizygous males are spared. The causative gene was originally mapped to X-chromosome in 1997 (3) and later identified to be PCDH19 in 2008 (4). PCDH19 belongs to the δ2 protocadherin...
subfamily of the cadherin adhesion molecule super family (5). This subclass of protocadherins also includes PCDH8, PCDH10, PCDH17 and PCDH18. The respective proteins are known (or speculated) to be involved in weak homotypic cell-cell adhesion and cell migration in a calcium dependent manner and are predominantly expressed in the brain with expression restricted to specific regions (6–8).

PCDH19 is the second most frequently mutated gene in epilepsy after SCN1A that causes Dravet Syndrome (9). PCDH19-FE is focal epilepsy characterized by early onset seizures (6–36 months), which cluster, are drug resistant and are often triggered by fever (10). PCDH19-FE is clinically variable, ranging from benign focal epilepsy with normal intelligence to severe generalized or multifocal epilepsy, resembling Dravet syndrome (1,9). The frequency of seizures often decreases at/after puberty and can stop altogether during adulthood. Several other comorbidities including intellectual disability, autism, schizophrenia and attention deficit hyperactivity disorder (ADHD) are common among PCDH19-FE affected individuals (9,10). So far in excess of 100 unique PCDH19 mutations have been reported that include missense changes, premature stop codons and complete gene deletions (Supplementary Material, Fig. S1). This also includes a single case of an affected male with an early somatic mutation leading to loss of PCDH19 in approximately half of his cells (11). The majority of PCDH19 mutations are within the extracellular domain with all missense mutations clustering exclusively within this region (Supplementary Material, Fig. S1). There is no obvious difference between the phenotype of patients with partial and complete loss of PCDH19 protein (11).

Determining the pathogenic mechanism of PCDH19 variants has been a major challenge. We have recently shown that neurosteroids may play a significant role in the pathology of PCDH19 has been a major challenge. We have recently shown that neurosteroids including progesterone and androgen receptors were among the most significantly dysregulated in patient primary skin cells (12).

Here, we show that the non-POU-domain-containing octamer binding protein (NONO)/P54nrb, a well-known multifunctional RNA- and DNA-binding nuclear protein, interacts with PCDH19. We also show that a fraction of the PCDH19 protein is localized in the cell nucleus where it interacts with NONO that genes regulated by estrogen, progesterone and androgen receptors were among the most significantly dysregulated in patient primary skin cells (12).

Results
NONO interacts with PCDH19

PCDH19 has six extracellular cadherin (EC) repeats in the N-terminus that resemble classical cadherins, a transmembrane domain (TM) and two conserved motifs known as CM1 and CM2 in the C-terminal region of the protein (Supplementary Material, Fig. S1) that are typical of α2 protocadherins (5). To identify PCDH19 interacting proteins, we performed a yeast two-hybrid (Y2H) screen using the C-terminal region (amino acids 706 to 1145) of mouse Pcdh19 (the least conserved region of Pcdh19 among its orthologs and paralogs) as a bait and E11 whole mouse embryo cDNA library as a prey (see Materials and Methods). Pcdh19 has been shown to be expressed in the mouse embryo during E9 to E14, a period important for early brain development (13). The Pcdh19 C-terminal region is highly similar (94% amino acid sequence identity) to human PCDH19, with the CM1 and CM2 motifs being 100% conserved (Supplementary Material, Fig. S2A).

One of the Pcdh19-interacting proteins identified by our screen was NONO, a 471 amino acid 54 kDa protein. NONO is a member of the Drosophila behavior human splicing (DBHS)-family of proteins with many roles in gene regulation including binding to a long noncoding RNA to form subnuclear bodies called paraspeckles (14,15). NONO was found to be the first nuclear protein to directly interact with a α2 protocadherin, a member of the cadherin superfamily which have been reported to involve in cell membrane functions (13,14,16–18). We used epitope-tagged human PCDH19 (96% amino acid sequence identical to mouse) protein for validating its interaction with NONO. Using co-immunoprecipitation experiments on HEK293T cell lysates expressing Myc-PCDH19 and HA-NONO we demonstrate that NONO and PCDH19 interact (Fig. 1A). The interaction between human NONO and PCDH19 was also validated by investigating Myc-PCDH19 interaction with the endogenous NONO. We detected the endogenous NONO in Myc-PCDH19 immunoprecipitates from HEK293T cells expressing this protein (Fig. 1B). This interaction was also confirmed by reciprocal detection of Myc-PCDH19 in NONO immunoprecipitated from the HeLa cells (Fig. 1C). Taken together these results showed that PCDH19 and NONO proteins interact in human cells.

PCDH19 localizes in close proximity to NONO in the nucleus

We next examined cellular localization of PCDH19 and NONO using immunofluorescence staining in cells expressing either full-length or a C-terminal PCDH19 region. In HeLa cells, the C-terminal region of PCDH19 (PCDH19CR) predominantly localized to the nucleus and in close proximity to NONO (Fig. 2A). This is consistent with the presence of predicted nuclear localization sequences (NLSs) within the PCDH19 protein (Supplementary Material, Fig. S2B). The full-length PCDH19 was predominantly perinuclear with some nucleoplasmatic localization (Fig. 3A and 3B). Next, we examined localization of the two proteins in mouse primary hippocampal neurons expressing the two proteins (Figs. 2B and 3B). We further examined localization of the two proteins in the common tight-junction cell model, Madin-Darby Canine Kidney (MDCK) cells (20,21). Unlike the classical cadherins that are known to be embedded within the cell membranes (22,23), PCDH19 was largely perinuclear and occasionally nucleoplasmic (Figs. 2C and 3C). We further examined PCDH19, NONO and ERα localization using a subcellular fractionation approach. Western blot analysis of control and Myc-PCDH19-expressing MCF-7 subcellular fractions showed that the majority of the endogenous and Myc-PCDH19 was present in the nuclear fraction along with the bulk of the cellular NONO and ERα (Supplementary Material, Fig. S4). These data suggest that PCDH19, NONO and ERα likely associate in the nucleus of the MCF-7 cells.

PCDH19 affects ERα transcriptional function

In our previous study, we gained indirect evidence of a likely role of nuclear hormone receptors in the pathology of PCDH19-
FE (12). To examine this further we took advantage of various breast cancer cell lines including MCF-7. MCF-7 cells express endogenous PCDH19 as well as the estrogen receptor alpha (ERα or ESR1), progesterone receptor (PGR) and androgen receptor (AR) (Supplementary Material, Fig. S5). ERα has been of particular interest because it is a well-known steroid receptor implicated in epilepsy, psychiatric and behavioral disorders (24–26).

Previously, we revealed that approximately one third of the significantly dysregulated genes in primary skin fibroblasts of PCDH19-FE girls have experimentally validated or predicted Estrogen Response Element (ERE) motifs in or near their promoters (12). To test whether PCDH19 influences ERα function, we used an ERE reporter assay. This assay employs an ERα-responsive luciferase (ERE-LUC) construct, in which the firefly luciferase coding sequence is cloned downstream of three tandem ERE repeats. We observed significantly increased luciferase reporter activity in MCF-7 cells ectopically expressing the wild type PCDH19 (Fig. 4A). This was further enhanced by the addition of the ERα ligand estradiol (E2) (Fig. 4A). Significant reduction in the ERE-LUC reporter activity in MCF-7 cells with PCDH19 knockdown indicated that regulation of ERα transcriptional activity was PCDH19-dependent (Supplementary Material, Fig. S6). Taken together the data showed that the PCDH19 protein enhances transcription from the ERE, and given that this effect is further enhanced in the presence of E2 supported a direct role of PCDH19 in ERα function.

Next, we aimed to determine whether selected PCDH19-FE disease-associated missense mutations [(Val441Glu, Asn557Lys, Val569Asp) were expressed in breast cancer cell lines. We found that PCDH19 expression was significantly increased in MCF-7 cells expressing the wild type PCDH19 protein compared to cells expressing the mutant PCDH19 protein. This suggests that PCDH19 may have a role in regulating ERα function in breast cancer cells.

To determine whether PCDH19 interacts with NONO, we performed co-immunoprecipitation assays using lysates from HEK293T cells expressing Myc-PCDH19, HA-NONO or both. Myc-PCDH19 was immunoprecipitated and endogenous NONO detected by western blotting with anti-HA antibody. (B) Lysates from HEK293T cells expressing Myc-PCDH19 were immunoprecipitated and endogenous NONO detected by western blotting with anti-PCDH19 antibody. (C) Endogenous NONO was immunoprecipitated from HeLa cells expressing Myc-PCDH19 and western blotted with anti-Myc antibody. Low level of Myc-PCDH19 immunoprecipitated with IgG was due to non-specific binding. Figure 1A–C was generated by cropping full-length western blots shown in Supplementary Material, Figure S10. Dotted lines separate the cropped areas of the western blots.

Figure 1. NONO interacts with PCDH19. (A) PCDH19 interaction with NONO was determined by co-immunoprecipitation assay using lysates from HEK293T cells expressing Myc-PCDH19, HA-NONO or both. Myc-PCDH19 was immunoprecipitated and endogenous NONO detected by western blotting with anti-HA antibody. (B) Lysates from HEK293T cells expressing Myc-PCDH19 were immunoprecipitated and endogenous NONO detected by western blotting with anti-HA antibody. (C) Endogenous NONO was immunoprecipitated from HeLa cells expressing Myc-PCDH19 and western blotted with anti-Myc antibody. Low level of Myc-PCDH19 immunoprecipitated with IgG was due to non-specific binding. Figure 1A–C was generated by cropping full-length western blots shown in Supplementary Material, Figure S10. Dotted lines separate the cropped areas of the western blots.

Figure 2. Cellular localization of the C-terminal PCDH19 and NONO. (A) HeLa cells expressing the C-terminal Myc-PCDH19 region (Myc-PCDH19CR) showing exclusive nuclear localization of PCDH19 (green) and partial co-localization with endogenous NONO (red). (B) Mouse primary hippocampal neurons expressing Myc-PCDH19CR showing PCDH19 (green) and endogenous NONO (cyan) nuclear localization. (C) MDCK cells expressing Myc-PCDH19CR showing predominantly nuclear and a small amount of cytoplasmic localization (green). Myc-PCDH19CR-endogenous NONO (cyan) nuclear co-localization was also observed. Cell nuclei and F-actin were visualized by staining with DAPI (blue) and phalloidin (red), respectively. Scale bars = 20 μm. Images are representative of >200 cells examined.
Ser276Pro) (4,27) and a small in-frame duplication (Ser139_Ala141dup) (28) have an effect. Whilst the wild-type PCDH19 enhanced transcription from the ERE-LUC reporter, none of the mutants did (Fig. 4). Addition of E2 increased the luciferase reporter expression for all PCDH19 variants tested, when compared to the empty vector transfected cells cultured without E2. To assess the physiological relevance of PCDH19 on ERα-mediated gene transcription, the effect of the wild-type or Asn557Lys PCDH19 was examined using four endogenous bona fide ERα target genes AKR1C3, APOD, ENC1 and OXTR.

Overexpression of the wild-type PCDH19 led to an increase in the expression of these genes, an effect which was lost in the cells expressing the Asn557Lys mutant (Supplementary Material, Fig. S7). Overall, mutations of the extracellular domain of PCDH19 lead to altered transcriptional regulation of ERE-LUC reporter and select endogenous cellular gene targets of ERα.

PCDH19 effect on ERE-LUC is ERα-dependent

To determine if the effect of PCDH19 on the luciferase reporter activity from the ERE-LUC plasmid is ERα-dependent, we compared the effect of PCDH19 between ERα deficient MDA-MB-231 and ERα positive MCF-7 cells (Supplementary Material, Fig. S8). We assayed ERE-LUC transcriptional activity in cells ectopically-expressing Ser139_Ala141dup (28) or wild-type PCDH19 and cultured in the presence and absence of the ERα ligand E2. While in the ERα positive MCF-7 cells the wild-type PCDH19 led to a significant increase of luciferase reporter expression in the presence or absence of E2 ligand, this effect was not detected in the ERα negative MDA-MB-231 cells irrespective of E2 addition (Supplementary Material, Fig. S8A). To further elucidate that the effect of PCDH19 on ERE-LUC reporter activity is ERα-dependent, we performed ERE-LUC reporter assay in MCF-7 cells with and without ERα knockdown and with or without exogenous Myc-PCDH19 expression. While PCDH19 positively regulated the ERE-LUC reporter activity in the control siRNA transfected cells, this effect was significantly reduced in cells treated with ERα siRNA (Supplementary Material, Fig. S9). Together, these data show that the PCDH19-mediated increase in ERE-LUC transcriptional activity is ERα-dependent.

PCDH19 regulates ERα through NONO

NONO is reported to be involved in the transcriptional regulation of PGR (29) and AR (30,31) but so far there is no direct evidence functionally linking NONO to ERα. We asked whether NONO was required for the PCDH19-mediated ERα-dependent regulation of the ERE-LUC reporter activity. LUC reporter activity was tested in the MCF-7 cells expressing wild type PCDH19, NONO individually or together in the presence or absence of E2. Overexpression of PCDH19 led to increased ERE-LUC reporter activity that was further enhanced in the presence of E2 (Fig. 5A). Interestingly, we observed that the presence of both PCDH19 and NONO enhanced the ERE-LUC reporter activity even further (Fig. 5A). This combined effect suggests the importance of both PCDH19 and NONO in the ERα-mediated gene expression regulation. Interestingly, the C-terminal PCDH19 fragment, the region which we showed to interact with NONO, did not show a noticeable effect on the ERE-LUC reporter activity. This suggested that while the C-terminal domain of PCDH19 is crucial...
pressed as relative luciferase activity and then for 6 h in the presence or absence of 10 nM estradiol (E2). Data are expressed as relative luciferase activity ± SD from four or more independent experiments. *P < 0.05 comparing E2 versus vector control, **P < 0.01 comparing E2 versus -E2 WT, ***P < 0.001 comparing E2 and -E2 WT versus vector or **P < 0.01 comparing -E2 WT versus -E2 MT, **P < 0.01 comparing -E2 WT versus -E2 MT using Bonferroni adjusted planned comparisons. (B) Levels of PCDH19 and endogenous-NONO protein were determined by western blotting with anti-Myc and anti-NONO antibodies. β-Actin was used as a loading control.

Discussion

Since the identification of PCDH19 as the gene responsible for PCDH19-FE (4), there has been little progress in the understanding of the molecular mechanisms driving the disease. PCDH19 is a member of the cadherin super family of cell adhesion proteins that play important roles in the brain morphogenesis and wiring including maintenance and plasticity of neuronal circuits that are important in learning and memory (32). In the present study, we identified a nuclear protein NONO as a novel interactor of PCDH19. This was surprising given our current understanding that Pcdh19/PCDH19 primarily functions as a cell-cell adhesion molecule (5). NONO is a member of the DBHS family of proteins which form essential structural component of ribonucleoprotein bodies found in the interchromatin space of mammalian cell nuclei called paraspeckles (14,15). Paraspeckle proteins including p54nrb/NONO, splicing factor proline-and glutamine-rich (SFPQ/PSF), paraspeckle protein 1 (PSPC1) and fused in sarcoma protein (FUS/TLS) have been shown to interact and form complexes with one another and are involved in both transcriptional and posttranscriptional regulation and DNA repair (30). Therefore interaction of two proteins previously shown to be localized in two different cellular compartments seemed unlikely. While we are yet to determine the specific region of interaction between PCDH19 and NONO, we demonstrate that the PCDH19 is present in the
perinuclear and nucleoplasmic region of the cell and in close proximity with NONO. NONO is a known co-regulator of steroid hormone receptors such as PGR and AR (30,31). Additionally, PGR knockdown has been shown to downregulate PCDH19 (33). These data are consistent with the role of PCDH19-NONO interaction in the regulation of steroid hormone receptors and also agree with our recently published findings showing nuclear hormone receptor regulated gene involvement in the pathogenesis of PCDH19-FE (12).

It is well known that the seizures in PCDH19-FE are associated with fever. As such it might be intriguing to speculate that this association could be, at least partly, modulated by NONO. It has been recently shown that silencing NONO expression in H295R human adrenocortical cells decreases the ability of the cells to increase intracellular CAMP production and subsequent cortisol biosynthesis in response to adrenocorticotropic hormone (ACTH) stimulation (34). It has also been shown that stressors like high ambient temperature result in the greatest rise in plasma ACTH as well as corticosterone synthesis and secretion in rats (35). As such it is plausible to speculate that this axis of PCDH19-NONO-ACTH is not working properly in PCDH19-FE, which may explain increased vulnerability of the PCDH19-FE girls to fever (or stress in general) and its associated effects, i.e. seizures.

A number of other PCDH19-interacting proteins have been identified recently, for example N-cadherin (36,37), Nck-associated protein 1 (NAP1) (38,39) and cytoplasmic FMRI interacting protein 2 (CYFIP2) (38,39). Interestingly, NAP1 and CYFIP2, like NONO, were shown to interact with the C-terminal region of PCDH19 (38,39). Notably, we used mouse and human PCDH19 while other studies used chicken and zebrafish Pcdh19. NAP1 and CYFIP2 proteins have been shown to form part of the WAVE regulatory complex (WRC) that controls actin cytoskeletal dynamics within the cell (39,40). In addition, WRC can also interact with the C-terminal region of a number of protocadherins including PCDH8, PCDH10, PCDH12, PCDH17, PCDH18, PCDH6 and PCDH19 (39). While this may facilitate a potential role of PCDH19 in actin cytoskeletal dynamics by associating with the WRC, the molecular mechanism mediating this initial association between PCDH19-WRC-induced rearrangements in the actin-cytoskeletal networks has not yet been established. These data along with our results suggested involvement of PCDH19 in more than one cellular pathway. We therefore postulated that while one role of PCDH19 in cell-cell adhesion and migration that is most likely mediated by PCDH19 interaction with WRC involving actin cytoskeletal networks (39), PCDH19 may also act as a signalling molecule linking cell adhesion and cell migration to gene expression regulation. The evidence presented here suggests that PCDH19 and NONO interact and regulate gene expression, at least partly through ERα nuclear steroid hormone receptor.

ER signalling has been associated with the activation of multiple neuroprotective pathways (41). Although ERs are predominantly intracellular, ~5% of cellular ERα and ERβ are associated with the membrane (42,43). Here they interact with neurotransmitters and growth factor receptors that are involved in non-genomic ER signalling and as such indirect transcriptional regulation (41,42). PCDH19, as a member of the cadherin superfamily of cell adhesion molecules, is assumed to be a cell membrane protein. However, this has not yet been demonstrated. Using subcellular fractionation studies of the MCF-7 cells, we show that PCDH19, NONO and ERs are present predominantly in the nuclear fraction (Supplementary Material, Fig. S4). It is plausible that these proteins associate with each other in at least MCF-7 cells in the nucleus, to regulate ERα transcriptional activity.

We have previously shown that the levels of the neurosteroid allopregnanolone are reduced in PCDH19-FE compared to normal control girls (12). The enzymes involved in reducing progesterone to allopregnanolone (through an intermediate metabolite 5α-DHP) are the aldo-keto reductases (AKR1C1-4). We have shown that at least AKR1C3 mRNA (and likely also AKR1C2) and protein were reduced in PCDH19-FE patients. The 17β-hydroxysteroid dehydrogenases (HSD) enzymatic properties of AKR1C3 are responsible for reducing androstenedione to testosterone that could then be converted to estradiol by cytochrome P450 aromatase enzyme in the brain (44). It appeared that the reduced expression of AKR1C3 not only affected the allopregnanolone level, but also testosterone, and in turn, estradiol (E2). In most tissues, endogenous ER expression is controlled by the abundance of estrogen; ER expression is low in the presence of lower estrogen levels and high when estrogen is high (44,45). For example, treatment of temporal lobe epilepsy...
patients with P450-inducing anti-epileptic drugs (phenytoin and carbamazepine) increased the level of E2 in patients causing a significant upregulation of ERα mRNA and proteins in their hippocampal pyramidal neurons (46,47). Here, we show that wild-type PCDH19 associates with NONO and, directly or indirectly, affects ERα-mediated gene expression, which is further enhanced in the presence of E2. In contrast, PCDH19-FE disease-associated variants including three missense mutations and one in-frame duplication abolished this function of PCDH19 or even led to lower than basal levels of the reporter expression. From clinical, patient phenotype perspective, there are no obvious yet identified differences between the group of patients with missense mutations (as tested here) and those with protein truncating mutations or entire gene deletions (10,11). This suggests that all of the currently reported PCDH19 mutations are loss of function mutations, which most likely lead, among other consequences, to altered ERα-regulated developmental and functional gene expression.

Estrogen signaling has been established to be crucial for normal brain development (48) and often with gender specific effects (49). ERα and its ligand estradiol (E2) have been shown to play important roles in various diseases including epilepsy, neurodegenerative disorders, psychiatric disorders, behavioral disorders and cancers (24,26,50,51). The role of estrogens in seizures susceptibility has been widely studied and is highly controversial. Findings among different studies are inconsistent due to biological complexity and variations in the experimental settings (26,52). In general, estrogens have proconvulsant and epileptogenic properties in both human and animal models (53–56). This is particularly well-demonstrated in women with catamenial epilepsy, where seizure activity has been linked to the menstrual cycle (56). In contrast, but consistent with our findings, other consequences, to altered ERα-regulated developmental and functional gene expression.

In summary, we have identified NONO as a novel PCDH19 interacting protein, which appears to be important in PCDH19-mediated ERα-dependent gene regulation (Fig. 7). This together with our recent finding of neurosteroid allopregnanolone deficiency in PCDH19- FE girls further underscores the importance of steroid nuclear hormone receptor, and ERα, specifically, signaling in epilepsy and likely also in autism, intellectual disability and other behaviours. Our study of a novel regulatory pathway of ERα regulation by a cell adhesion molecule has also potential relevance to other, apparently unrelated disorders like breast cancer and opens new possibilities of intervention.

**Materials and Methods**

**Cell culture and transfection**

Human embryonic kidney cells (HEK293T, ATCC CRL-1573) were cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% fetal bovine serum (Life Technologies), 2 mM glutamine, 0.2% (w/v) sodium bicarbonate, penicillin (1.2 mg/ml) and streptomycin (1.6 mg/ml). The cells were transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol and harvested 24 h post-transfection. Human cervical carcinoma (HeLa) cells were cultured, transfected with Lipofectamine 2000 (Life Technologies) and harvested in the same manner. MCF-7 (human mammary adenocarcinoma, ECACC# 86012803) and MDA-MB-231 (human mammary adenocarcinoma, ATCC# HTB-26) cells were

![Figure 7. Proposed model of PCDH19-mediated co-regulation of gene expression with NONO and ERα. PCDH19 is postulated to be a membrane bound protein of which a proportion (this work) is present in the nucleus. Our model suggests that the nuclear PCDH19 interacts with NONO and cooperates with ERα and its associated (in this case largely unknown) proteins to regulate target gene expression (e.g. OXTR, AKR1C3, APOD or ENC1, see Supplementary Material, Fig. S7). Whether PCDH19 interacts with ERα, directly or indirectly at the cell membrane or in the nucleus, remains to be determined. Estradiol – E2; ERE – Estrogen Response Element; ? – unknown proteins, protein-protein interactions or signalling pathways.](https://academic.oup.com/hmg/article-abstract/26/11/2042/3074031)
cultured in Dulbecco’s modified Eagle’s medium as described above.

**Yeast two-hybrid screen**

The Yeast two-hybrid screening was performed using the Matchmaker Gold Yeast Two-Hybrid system (Clontech) according to the manufacturer’s instructions. The C-terminal fragment containing the alternative spliced exon 2 and the conserved domains CM1 and CM2 of mouse Pcdh19 cDNA cytoplasmic region (amino acid 708 to 1146) was amplified with primers 5′- cacaccacacagcatccaATGGAGTCGCTCTCTATTTGAGC and 3′- cacaccacacacagcgcgT TAGAGACGATATCCTCAGGCACGTTCCACAGC-3′ and cloned into the GAL4 DNA binding sequence within the pGBKT7 vector at EcoRI and BamHI sites to generate pGBK7 + Pcdh19CR yeast bait expression construct. Sequencing verified the orientation and integrity of the mouse Pcdh19 cDNA sequences. This bait construct was then transformed into two strains of yeast (bait in Y2H Gold and prey in Y187) together with an E11 embryo Clontech’s Mate & Plate library as prey and Pcdh19CR as bait.

**Generation of expression constructs**

The full-length mouse Pcdh19 (NM_001105246.1) and human PCDH19 (NM_001184880) cDNA was amplified from mouse and human brain cDNA, respectively. The mouse Pcdh19 was amplified with primers 5′- cacaccacacagcagcattcaATGGAGTCGCTCTCTTCTACGC and 3′- cacaccacacacacagcgcT TAGAGACGATATCCTCAGGCACGTTCCACAC-3′ and cloned in frame with the GAL4 DNA binding sequence within the pGBK7 vector at EcoRI and BamHI sites to generate pGBK7 + Pcdh19CR yeast bait expression construct. Sequencing verified the orientation and integrity of the mouse Pcdh19 cDNA sequences. This bait construct was then transformed into two strains of yeast (bait in Y2H Gold and prey in Y187) together with an E11 embryo Clontech’s Mate & Plate library as prey and Pcdh19CR as bait.

**Immunoprecipitation and western blot analysis**

For western blotting, protein lysates were prepared by sonication in the presence of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM Na2VO4, 10 mM NaF, 0.8% Triton X-100 and protease inhibitors (Complete, Roche Applied Science). Protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Scientific). Lysates from HEK293T cells expressing the Myc-PCDH19, HA-NONO or both were prepared in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 50 mM NaF, 0.1 mM Na2VO4, 1% Triton-X-100 and 1× protease inhibitors. The lysates were incubated with Myc- conjugated agarose beads (Sigma), washed once with the lysis buffer, three times with wash buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% Sodium deoxycholate and 0.1% SDS), eluted with 1× SDS-loading buffer, subjected to SDS-PAGE and western blotting. The target proteins were detected with appropriate primary antibodies and horseradish peroxidase-conjugated anti-mouse (Pierce) or anti-rabbit (Pierce) secondary antibodies using an enhanced chemiluminescence kit (ECL, Amersham Biosciences). β-Tubulin was employed as a loading control using rabbit anti-β-tubulin antibody (Abcam, Cambridge, UK). Mouse anti-Myc (9E10; Santa Cruz Biotech), mouse anti-HA (clone HA-7; Sigma), mouse anti-HSP90 (AC88; Enzo Life Sciences), mouse anti-β-Actin (clone AC-74; Sigma), mouse anti-FLAG (clone M2; Sigma), mouse KCNT1 (clone S3-26; Sigma), rabbit anti-PCDH19 (Bethyl Laboratories), rabbit anti-NONO (Bethyl Laboratories), rabbit anti-β-Tubulin (Abcam), rabbit anti-ERα (Santa Cruz Biotech) and rabbit anti-Histone H3 (Abcam) primary antibodies were employed in the present study.

**Subcellular fractionation**

MCF-7 cells transfected with control or wild-type Myc-PCDH19 expression plasmids for 24 h were used for subcellular fractionation. Briefly, the cells were swollen in 0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl, 1.5 mM MgCl2 and 1× protease inhibitors on ice for 10 min and then resuspended in the same buffer with 0.2% Triton-X-100. Cell membranes were broken using a Dounce homogenizer and centrifuged at 1500 rpm for 10 min to pellet nuclei. Nuclear pellets were lysed in 1× lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1× protease inhibitors). Nuclear lysates were centrifuged at 70 000 rpm for 30 min to separate the cell membranes from the cytoplasmatic fraction. Supernatant was collected as a cytoplasmatic fraction and the cell membrane pellets were resuspended in lysis buffer with 0.8% Triton-X-100 or 1% DDM.
Quantitative RT-PCR (qPCR)

qPCR analyses were performed using the following profiles: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 30 s. Melt curve was included each run as quality control checkpoint. All primers were designed to generate a specific 70–250 bp amplicon (primers available on request). Data were acquired using StepOne Real-Time PCR System and software V 2.0 (Applied Biosystems) and expression of mRNA was normalized against either hypoxanthine-guanine phosphoribosyltransferase (HPRT) or actin, beta (ACTB).

Dual luciferase reporter assay

MCF-7 cells were transfected with luciferase reporter containing three copies of vitellogenin Estrogen Response Element (3× ERE TATA luc) and control, wild-type PCDH19, mutant PCDH19, C-terminal PCDH19 region, NONO, wild-type PCDH19 and NONO or C-terminal PCDH19 region and NONO expression vectors. MDA-MB-231 cells were transfected with luciferase reporter containing 3× ERE TATA luc and control, wild-type PCDH19 or mutant PCDH19 expression vectors. pRL-TK (Renilla) (Promega) was included in each transfection as transfection control. Cells were cultured in media containing 10% charcoal-stripped FCS for 16 h and then in the presence or absence of 10 nM estradiol (E2) for 6 h. Cells were lysed with the 1× lysis buffer and subjected to dual-luciferase reporter assay (Promega) according to the manufactures protocol. NONO and PCDH19 protein expression was determined by western blotting.

siRNA knockdown of NONO

MCF-7 cells were transfected with either NONO (annealed 5’-GGAAGCCACGCUUGGAAAAGCUUU-3’ and 5’-AGACCUUUCGCGAGCUGCUUCUU-3’) or scrambled (annealed 5’-UUCUGGACAGCUGCAACUGTT-3’ and 5’-ACGUGACAGCUUGGAGAAAT-3’) (GenePharma) using Lipofectamine RNAiMAX reagent according to the manufacturer’s specifications (Life Technologies). Following 24 h of siRNA transfection, MCF-7 cells were transfected with luciferase reporter containing 3× ERE TATA luc and control, wild-type PCDH19 or mutant PCDH19 expression vectors. Cells were cultured in charcoal-stripped FCS in the presence or absence of 10 nM E2, lysed and subjected to dual-luciferase reporter assay (Promega). NONO and PCDH19 protein expression was determined by western blotting.

Supplementary Material

Supplementary Material is available at HMG online.

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