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Separate necdin domains bind ARNT2 and HIF1α and repress transcription

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Abstract

PWS is caused by the loss of expression of a set of maternally imprinted genes including *NECDIN (NDN)*. *NDN* is expressed in post-mitotic neurons and plays an essential role in PWS as mouse models lacking only the *Ndn* gene mimic aspects of this disease. Patients haploid for *SIM1* develop a PW-like syndrome. Here, we report that NDN directly interacts with ARNT2, a bHLH-PAS protein and dimer partner for SIM1. We also found that NDN can interact with HIF1α. We showed that NDN can repress transcriptional activation mediated by ARNT2:SIM1 as well as ARNT2:HIF1α. The N-terminal 115 residues of NDN are sufficient for interaction with the bHLH domains of ARNT2 or HIF1α but not for transcriptional repression. Using GAL4-NDN fusion proteins, we determined that NDN possesses multiple repression domains. We thus propose that NDN regulates neuronal function and hypoxic response by regulating the activities of the ARNT2:SIM1 and ARNT2:HIF1α dimers, respectively.

Keywords: Necdin; Arnt2; Hif1α; Prader–Willi; Hypothalamus

Prader–Willi Syndrome (PWS) is caused by the loss of expression of a set of maternally imprinted genes located at 15q11–q13, including *NECDIN* (*NDN*) [1]. The PWS pathologies include infantile hypotonia, respiratory distress, hyperphagia, obesity, hypogonadotropic hypogonadism, and cognitive and behavioral abnormalities [2]. Mice lacking the *Ndn* gene have several features of this syndrome, including neonatal respiratory depression, behavioral defects, and reduced leutinizing hormone releasing hormone- and oxytocin-producing neurons in the anterior hypothalamus [3,4].

NDN is expressed in post-mitotic neurons, with highest levels in the hypothalamus [4]. NDN over-expression induces neuronal differentiation and neurite outgrowth in a neuroblastoma cell line [5]. By binding to TrkA and p75 receptor, NDN can also facilitate neurotrophin signaling [6,7]. Consistent with these proposed roles, *Ndn* mutant mice have defects of multiple axonal tracks in brain areas regulating rhythmic breathing [8] as well as a reduced number of sensory neurons in the dorsal root ganglion [7].

NDN belongs to a family of proteins called the MAGE proteins. The MAGE family consists of more than 25 members and each member possesses unique N- and C-terminal regions surrounding a central, conserved MAGE homology domain, MHD [9]. Whether and how the N-terminal and C-terminal unique domains of NDN contribute to its roles in neuronal differentiation remains undefined.

Several lines of evidence led us to investigate whether NDN regulates the transcriptional activity of the bHLH-PAS domain transcription factor SIM1. SIM1 is highly expressed in the hypothalamus where it, like NDN, plays an essential role in the development of oxytocin-expressing neurons [4,10]. Also, patients haploid for *SIM1* present with a PW-like syndrome similar to PWS patients [11,12], and *Sim1* heterozygous mice have defective paraventricular hypothalamic nuclei and develop obesity due to hyperphagia [13].

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Materials and methods

Plasmid construction. The mouse *Ndn* cDNA was cloned into the yeast vector pGBKT7, which contains the GAL4 DNA-binding domain (DBD). The mouse *Arnt*, *Arnt2*, *Bmal1*, *Hif1α*, *Sim1*, or *Sim2* cDNAs were ligated downstream of the GAL4 activating domain (AD) in pGADT7 (Clontech, Mountain View, CA). *Ndn* segments encoding amino acids (a.a.) 1–115, 1–170, 1–224, 1–281, 116–170, 116–224, 116–281, 190–281, and 190–325 were PCR amplified and ligated as EcoR1/XhoI fragments into pGBKT7. *Arnt2* segments encoding a.a. 1–129, 1–440, 130–440, 130–712, and 442–712 and *Hif1α* segments encoding a.a. 1–74, 1–359, 75–359, 75–823, and 362–823 were PCR amplified and ligated as EcoR1/BamHI fragments into pGADT7. The *Arnt2*, *Sim1*, and *Hif1α* cDNAs were inserted into pcDNA3 (Invitrogen, Carlsbad, CA, USA). Oligonucleotides encoding a Myc epitope, preceded by Kozak sequence 5′-AGCTTGCCACCATGGAGGAGCAGAAGCTG ATCTCAGAGGAGGACCTGG, were ligated N-terminal to HIF1α and NDN. The GAL4 DBD was transferred from pGBKT7 to pcDNA3 as a HindIII/EcoRI fragment. Various NDN segments were then ligated downstream of the GAL4 DBD as EcoRI/XbaI fragments. Cloned PCR segments were confirmed by sequencing. The pML/6C-WT reporter was described [14]. GAL4TKLUC was generously provided by Dr. A. Friedman.

Yeast two-hybrid assay. The yeast two-hybrid assay was carried out using the Yeastmaker Yeast Transformation System 2 (Clontech) in strain AH109 with 50–100 ng of yeast expression plasmids. Transformed yeast was plated on $leu^{-}trp^{-}$ minimal media plates. For quantitative re-spotting, one $leu^{+}trp^{+}$ colony was diluted to $\sim 2 \times 10^{4}$ yeast/μl and 1 μl was spotted on $leu^{-}trp^{-}ade^{-}his^{-}$ plates. After 3 days of incubation at 30 °C, the resulting growth was photographed using a ChemiDoc XRS System (Bio-Rad Laboratory, Hercules, CA).

Cell culture and transfection. 293T cells were cultured in DMEM (Invitrogen) with 10% fetal bovine serum (Hyclone, Logan, UT). Eighteen hours before transfection, cells were split 1:5 into 6-well dishes. Three microliters of Lipofectamine 2000 (Invitrogen) was incubated with 1 μg of total DNA in 200 μl OPTI-MEM (Invitrogen) for 45 min before application. CMV-βGal was included for control. Forty-eight hours after transfection, cell lysates harvested in Reporter Lysis Buffer (Promega, Madison, WI) were subjected to luciferase assay [15] and for β-galactosidase using 4 mg/ml *ortho*-nitrophenol-β-D-galactoside; the reaction was stopped by sodium bicarbonate and the OD_{420} was determined.

Co-immunoprecipitation and Western blotting. 293T cells were transfected with 3 μg of each expression vector as described in the text using 16 μl Lipofectamine 2000. Forty-eight hours later cells were collected, washed twice with 150 mM NaCl, 20 mM Tris, pH 7.5, and lysed for 20 min at 4 °C using 500 μl 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 7.5, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml chymostatin, and 1 μg/ml pepstatin A. After brief sonication, the supernatant was precleared for 1 h with protein A/G agarose beads (Pierce, Rockford, IL, USA). Fifty microliters was saved as input, and the remainder was split for IP by either specific antibodies or matched IgG controls. After incubation at 4 °C overnight, 20 μl of protein A/G beads (Pierce) were added, incubated for 1 hr, washed 3 times with lysis buffer, and collected in Laemmli sample buffer (LSB). For myc-NDN and GAL4-NDN proteins, 293T cells were transfected with 1 μg of each expression plasmid. The cells were harvested directly into LSB. Western blotting was carried out as described [16] using rabbit anti-ARNT2 (M165), rabbit anti-c-Myc (A14), mouse-anti-GAL4 (DBD), or goat-anti-NDN (N20) (Santa Cruz Biotechnology, San Diego, CA), or using anti-Myc antibody 9E10 (Upstate, Lake Placid, NY).

Results

NDN interacts with ARNT, ARNT2, BMAL1, and HIF1α in yeast two-hybrid assays

Full-length mouse *Ndn* cDNA was fused to the GAL4 DBD (as "bait"), and mouse *Sim1* was fused to the GAL4 AD (as "tester"). Yeast transformed with the "bait" and the "tester" were selected on the leu^{-};trp^{-} plates and then plates lacking leu, trp, his, and ade. Several other bHLH-PAS domain containing transcription factors were also fused to GAL4 AD and tested together with SIM1, including SIM2, ARNT, ARNT2, BMAL1/ARNT3, and HIF1α.

GAL4 DBD-NDN did not display detectable transcriptional activity with the empty AD vector (Fig. 1A). Similarly, none of the bHLH-PAS domain transcription factors trans-activated selection markers in conjunction with the empty DBD vector (Fig. 1A). As reported recently [17], an interaction between HIF1α and NDN was detected in our system. While we did not observe any indication of interaction (colony growth) between NDN and SIM1 or between NDN and SIM2, we did find that NDN interacted with ARNT, ARNT2, and BMAL1 (Fig. 1A).

NDN interacts with ARNT2 and HIF1α expressed in cultured cells

To confirm interactions between NDN and ARNT2 or HIF1α, we co-transfected a plasmid expressing ARNT2 and a plasmid expressing NDN with a Myc epitope tag at its N-terminus into 293T cells. Extracts were subjected to immunoprecipitation (IP) using the anti-Myc monoclonal antibody or mouse IgG, followed by Western analysis using rabbit anti-ARNT2. As shown in Fig. 1B (left panel), Myc antibody but not the control IgG brought down ARNT2. Conversely, IP using the rabbit anti-ARNT2 but not rabbit IgG brought down co-expressed Myc-NDN (Fig. 1B, right panel).

When an extract prepared from 293T cells expressing NDN and Myc-HIF1α was subjected to IP using a goat anti-NDN antiserum or goat IgG, Myc-HIF1α was brought down and detected by Myc antibody (Fig. 1C, left panel). Reciprocal co-IP using extracts containing untagged HIF1α and Myc-NDN showed that Myc antibody brought down HIF1α above the background level brought down by mouse IgG (Fig. 1C, right panel). Together with the yeast two-hybrid data, these results demonstrate physical association between NDN and ARNT2 and between NDN and HIF1α.

The N-terminal unique region of NDN interacts with ARNT2 and HIF1α

To determine the regions of NDN that mediate its interaction with ARNT2 or HIF1α, we fused NDN's unique N-terminal 115 residues (NDN 1–115), N-terminus plus region I (NDN 1–170), N-terminus plus regions I and II (NDN 1–224), N-terminus plus regions I–III (NDN 1–281), as well as region I alone (NDN 116–170), regions I–III (NDN 116–224), regions I–V (NDN 116–281), regions III–V (NDN 190–281), or region V plus the C-terminus (NDN 190–325) to the GAL4 DBD (Fig. 2A). These variants and wild-type (WT) GAL4 DBD-NDN (Fig. 2A,

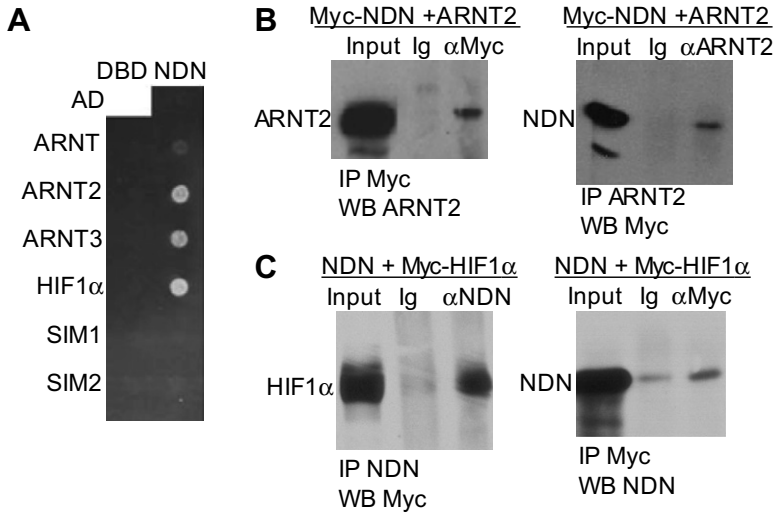

Fig. 1. NDN binds ARNT2 or HIF1α. (A) AH109 yeast transformed with plasmids expressing specific effectors are as indicated: GAL4-DBD, DBD; GAL4-DBD-NDN, NDN; GAL4-AD, AD; or GAL4-AD fused to ARNT, ARNT2, BMAL1/ARNT3, HIF1α, SIM1, or SIM2. $\sim 2 \times 10^4$ yeast cells were spotted on leu$^-$trp$^-$ade$^-$his$^-$ plates and cultured for three days. (B) Extracts from 293T cells expressing ARNT2 and Myc-NDN were subjected to IP with mouse anti-myc antibody or murine Ig followed by Western blotting (WB) with rabbit anti-ARNT2 antiserum (left panel). Similar extracts were subjected to IP with ARNT2 antiserum or rabbit Ig and then WB using anti-myc antibody (right panel). (C) Extracts from 293T cells expressing NDN and Myc-HIF1α were subjected to IP with goat anti-NDN antiserum or goat Ig followed by WB with mouse anti-myc antibody (left panel). Similar extracts were subjected to IP with anti-myc antibody or murine Ig and then blotted using anti-NDN antiserum (right panel).

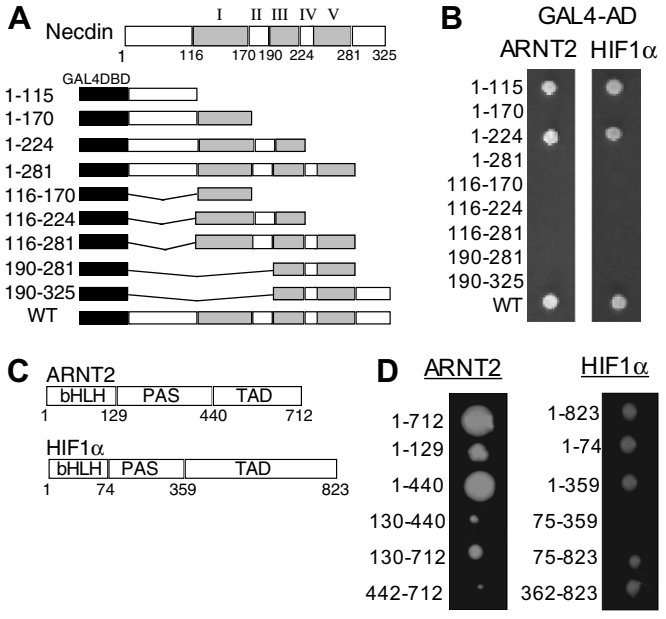

Fig. 2. Identification of the NDN and ARNT2 or HIF1α domains required for interaction. (A) Diagram of wild-type (WT) NDN. The MHD regions I–V are indicated. (B) WT NDN or indicated NDN segments linked to the GAL4 DBD were assayed for interaction with ARNT2 or HIF1α as in Fig. 1A. Results shown are representative of three experiments. (C) Diagrams of ARNT2 and HIF1α: their bHLH, PAS, and trans-activation domains (TAD) are indicated. (D) AH109 yeast was transformed with GAL4 DBD-NDN and GAL4 AD linked to various domains of ARNT2 or HIF1α as indicated. These were then spotted on leu$^-$trp$^-$ade$^-$his$^-$ plates and cultured for six days. Results shown are representative of two experiments.

bottom) were each individually co-transformed with GAL4 AD-ARNT2 or GAL4 AD-HIF1α into the yeast to assay for interaction (Fig. 2B) on the quadruple selection plates. In addition to the full-length NDN, only NDN 1–115 and NDN 1–224 strongly interacted with ARNT2 or HIF1α. The lack of interaction for NDN 1–170 and NDN 1–224 to ARNT2 and HIF1α may reflect abnormal folding of these variants. NDN segment 1–115 is apparently a minimal domain sufficient to interact with ARNT2 or HIF1α.

ARNT2 and HIF1α interact with NDN via their bHLH domains

We then conducted yeast two-hybrid assays to determine the domains of ARNT2 and HIF1α that interact with NDN. Both ARNT2 and HIF1α have a bHLH and a PAS domain, but their C-terminal regions are divergent. We made deletion constructs of ARNT2 containing the bHLH domain, the bHLH-PAS domain, the PAS domain, the PAS-C-terminal domain, or the C-terminal domain only. A similar set of deletion constructs was also made for HIF1α (Fig. 2C). These deletion variants were fused to the GAL4-AD to test for interaction with GAL4-DBD-NDN.

The bHLH domain of ARNT2 was sufficient for moderate interaction with NDN (Fig. 2D, left panel). The PAS domain or the C-terminus showed minimal interactions with NDN. PAS domain plus the C-terminus show somewhat stronger interaction with NDN than either alone. Strikingly, the bHLH and PAS domains together interacted with NDN as strongly as full-length ARNT2, suggesting that the PAS domain enhances the bHLH domain's interaction with NDN. Thus, NDN interacts with multiple regions of ARNT2, and the primary site of interaction is the bHLH-PAS region.

Different from ARNT2, the bHLH domain of HIF1α interacts with NDN as strongly as full-length HIF1α, and addition of its PAS domain did not enhance the interaction (Fig. 2D, right panel). However, the PAS domain of HIF1α also displays minimal interaction with NDN. NDN was reported to interact with the oxygen detection domain (ODD) of HIF1α located between a.a. 401 and 603 [17]. Consistently, we found that deletion constructs containing this region of HIFα also interact with NDN. Thus, NDN appears to interact with two distinct domains of HIF1α, the bHLH domain and the ODD.

NDN represses transcriptional activity of HIF1α and ARNT2

We next turned to assay the impact of NDN on the transcriptional activities of ARNT2:HIF1α and ARNT2:SIM1. Both ARNT2:HIF1α and ARNT2:SIM1 can activate transcription via binding to the CME site (Moffett and Pelletier 2000). A reporter plasmid, pML/6C-WT, containing six CME elements was activated more than 16-fold by ARNT2:HIF1α and 5-fold by ARNT2:SIM1 (Fig. 3A and B). When WT NDN was co-expressed in 293T cells, we observed 3-fold and 4-fold repression of ARNT2:HIF1α and ARNT2:SIM1 mediated transcription, respectively (Fig. 3A and B).

We then asked whether this repressive effect requires additional regions of NDN beyond the N-terminal 1–115 residues. Expression of full-length NDN, NDN 1–115, NDN 1–170, NDN 1–224, and NDN 1–281 was confirmed by Western blotting (Fig. 3C). While the full-length NDN repressed trans-activation of ARNT2:HIF1α 3-fold, NDN 1–115 mediated less than a 2-fold repression. Strikingly, NDN 1–170 and NDN 1–224 repressed trans-activation by more than 30-fold. NDN 1–281 repressed trans-activation of ARNT2:HIF1α by 6-fold (Fig. 3A). For ARNT2:SIM1 mediated transcription, NDN 1–115 acts as effectively as the full-length NDN, while NDN 1–170 and NDN 1–224 repressed their activity by ~20-fold. NDN 1–281 displayed 3-fold repression (Fig. 3B). The repressive effects on ARNT2:HIF1α or ARNT2:SIM1 imposed by NDN variants follow a similar trend. The greater effect of NDN 1–115 on ARNT2:SIM1 compared with ARNT2:HIF1α may reflect its interaction with only one subunit (ARNT2) in the former and with both subunits in the latter complex. The more potent repression affects seen with NDN 1–170 or NDN 1–224 compared to WT, NDN 1–115, and NDN 1–281 strongly implies the existence of at least one potent internal trans-repression domain between a.a. 115 and 170, the activity of which is masked by additional C-terminal sequences.

The MHD of NDN contains multiple repression domains

To further define the intrinsic repressive activity of the MHD internal domains independent of binding to ARNT2 and HIF1α, we fused a series of NDN segments to GAL4-DBD. A GAL4TKLUC reporter containing four GAL4 binding sites linked to the thymidine kinase promoter and luciferase was used. All of the proteins except for GAL4-DBD-NDN 116–224 were detected by Western blotting (Fig. 4A). The NDN 1–224 and 116–281 variants were expressed at lower levels. Unexpectedly, NDN WT linked to the GAL4 DBD did not repress transcription (Fig. 4B). On the other hand, GAL4 DBD-NDN 1–281 repressed promoter activity by 5-fold. This repression was

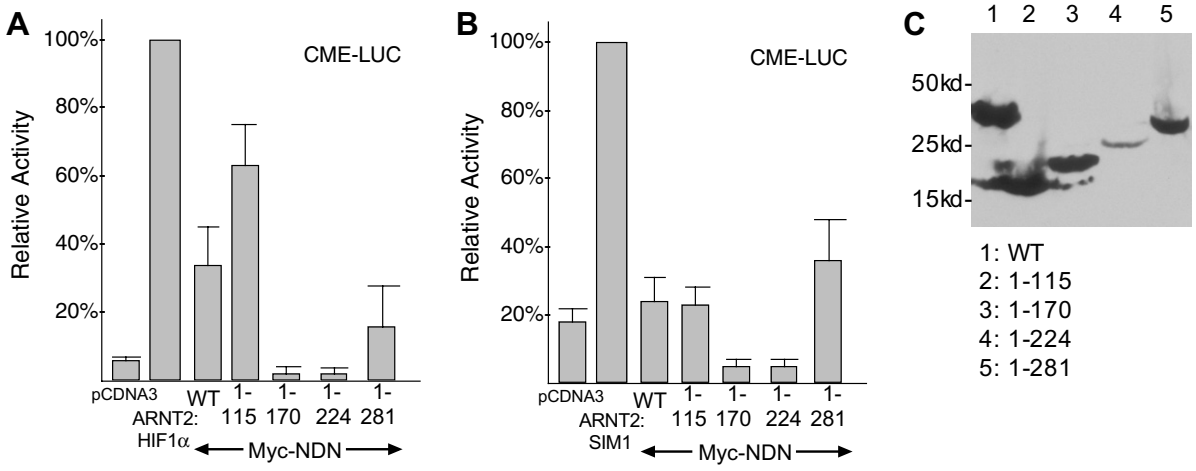

Fig. 3. Localization of NDN trans-repression domains for ARNT2:HIF1α or ARNT2:SIM1. (A) 293T cells were transfected with 700 ng pML/6C-WT and either 120 ng pcDNA3 (bar 1) or 10 ng pcDNA3-HIF1α, 10 ng pcDNA3-ARNT2, and 100 ng pcDNA3 (bar 2) or 100 ng pcDNA3-myc-NDN or the indicated pcDNA3-myc-NDN variants (bars 3–7). Each transfection also included 20 ng CMV-βGal. After 48 h cell extracts were subjected to luciferase and β-galactosidase assays. Luciferase activity, normalized for β-galactosidase, obtained when HIF1α and ARNT2 were expressed alone was set to 1.0 in each assay. Relative activities are shown (error bars = SE; $n = 3$). (B) 293T cells were transfected with 700 ng pML/6C-WT, 20 ng CMV-βGal, and either 120 ng pcDNA3 (bar 1) or 10 ng pcDNA3-ARNT2, 10 ng pcDNA3-SIM1, and 100 ng pcDNA3 (bar 2) or 100 ng pcDNA3-myc-NDN or the indicated pCDNA3-myc-NDN variants (bars 3–7). Relative activities are shown ($n = 4$). (C) 293T cells transfected with myc-tagged WT NDN or with NDN deletion variants were subjected to WB using rabbit anti-myc antiserum.

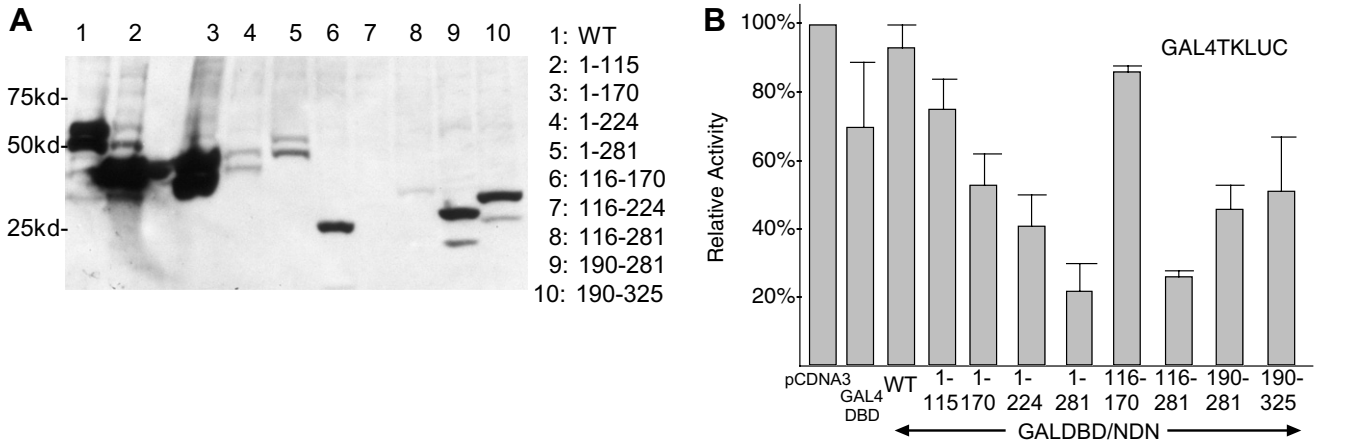

Fig. 4. Localization of NDN intrinsic trans-repression domains. (A) An equivalent number of 293T cells transfected with GAL4 DBD-NDN (WT) or the indicated GAL4 DBD-linked NDN variants were subjected to Western blotting using GAL4 antibody. (B) Seven hundred nanograms pGAL4TKLUC was transfected with 120 ng of pcDNA3, pcDNA3-GAL4 DBD, pcDNA3-NDN (WT), or pcDNA3-NDN variant, as indicated. The relative activity with pcDNA3 alone was set to 1.0 in each assay. Relative activities obtained with each construct are shown ($n = 3$).

gradually lost as C-terminal residues were progressively deleted, as seen by comparing the effects of GAL4-DBD fused NDN 1–281 to NDN 1–224 to NDN 1–170, and finally to NDN 1–115. Furthermore, a 5-fold repression was seen with GAL4-NDN 116–281, despite its low expression level. The lesser repressive activities of GAL4-DBD linked to either NDN 190–281 or NDN 116–170 compared with NDN 116–281 suggest that internal repression domains exist between residues 116 and 190 and between residues 170 and 281. The inactivity of WT compared with GAL4-DBD-NDN 1–281 again indicates that addition of a.a. C-terminal to 281 inhibits activity, as seen with the CME reporter. Finally, GAL4-DBD-NDN 1–115 only repressed activity by 20%, and removal of a.a. 1–115 from GAL4-NDN 1–281 to generate GAL4-NDN 116–281 did not alter repression, indicating that a.a. 1–115 do not display a strong intrinsic repression activity.

Discussion

Here, we report the repressive activity of NDN on the ARNT2:SIM1 as well as the ARNT2:HIF1α dimer complexes. We demonstrated that NDN utilizes its unique N-terminal region for physical binding to ARNT2 or HIF1α and portions of its MHD for repression. These findings have direct implications to NDN's function in the hypothalamus and in PWS.

NDN has previously been shown to also bind p53 and E2F and repress their transcriptional activity. Residues 102–110 within NDN's unique N-terminal region are required for binding to both E2F and p53 [18,19]. Here, we show that NDN also utilizes its N-terminal a.a 1–115 unique region for binding to HIF1α and ARNT2. Furthermore, we show that the propensity for NDN to interact with bHLH-PAS proteins is selective as NDN does not interact with SIM1 or SIM2.

NDN 1–115 had little effect when fused to GAL4-DBD, indicating that NDN 1–115 does not contain an intrinsic repression domain. On the other hand, NDN 1–115 did modestly repress ARNT2 or HIF1α activation of CME, perhaps by competing for the same docking sites as co-activators. The fact that NDN 1–170 and NDN 1–224 are repressive in all assays suggests that NDN's MHD contains *bona fide* repression domains. Our findings also indicate that NDN domains 115–170, 170–224, and 224–281 are each independent co-repression domains when fused to GAL4-DBD. Curiously, while full-length NDN was inhibitory when bound to ARNT2:SIM1 or ARNT2:HIF1α, it does not display repressive activity when fused to the GAL4-DBD. One possible explanation for these observations is that binding between NDN and ARNT2 or HIF1α via its 1–115 residues induces a conformational change of its MHD to expose internal repression domains. In the GAL4-DBD-NDN fusion assay, there is no binding partner for NDN and therefore full-length NDN is null for repression.

The association and modulation of ARNT2:SIM1 transcriptional complex by NDN has direct implications for the pathogenesis of the PWS. PWS patients have no NDN expression, while SIM1 haploid patients who have PW-like symptoms presumably have reduced SIM1 levels. Our findings and the link between NDN and SIM1 via a common partner ARNT2 suggests that the ARNT2:SIM1 dimer normally acts as a repressor via association with NDN, and reducing any of these components limits the repressive output and leads to related PW symptoms.

We also found that NDN binds to both the bHLH domain and the C-terminal region of HIF1α. Perhaps NDN can fine tune HIF1α activities in the CNS in response to oxygen concentrations. It is therefore tempting to speculate that lack of NDN function causes a compromise in HIF1α control, presumably at the midbrain respiratory rhythmic centers [8], and so may be partly responsible for the respiratory distress phenotype of PWS patients and *Ndn* (−/−) mice.

Acknowledgments

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