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The function of the *Aristaless-related homeobox* (Arx) gene product as a transcriptional repressor is diminished by mutations associated with X-linked mental retardation (XLMR)

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ABSTRACT

The *Aristaless-related homeobox* (Arx) is mutated in patients with X-linked mental retardation and a range of other neurological diseases. The molecular consequences of these mutations are unclear. Here, we show that two disease-associated mutations disrupt the function of Arx as a transcriptional repressor. We found that Arx contains two independent repression domains: an N-terminal octapeptide motif/engrailed homology domain and a novel domain located in the C-terminus. The octapeptide motif functions through interaction with members of the Groucho family of co-repressors. The C-terminal domain functions through interaction with C-terminal binding protein (CtBP).

The *Aristaless-related homeobox* gene, *Arx*, has come to the forefront of studies of the genetic regulation of brain development and has been recently described as one of the most important disease causing genes on the X chromosome [1]. Mutations in ARX have been implicated in a multitude of human neurological disorders including X-linked lissencephaly, X-linked infantile spasms or West syndrome, Partington syndrome, Proud syndrome, X-linked lissencephaly with abnormal genitalia (XLAG) and non-syndromic mental retardation [2,3]. Loss of function studies in mice have shown that Arx is involved in proper brain development and migration of GABA-ergic cortical interneurons [4–6].

Arx is a *paired*-type homeodomain transcription factor that is highly conserved among species especially within the octapeptide (OP), homeodomain (HD), and OAR (*Aristaless*) motifs [7,8]. The OP is involved in repression mediated by members of the groucho family of co-repressors [1]. Indeed, several studies have shown that Arx functions as a transcriptional repressor [9–12].

In this report, we focus on the function of Arx as a transcriptional regulator. Using disease-associated mutations as a guide, we identified two independently regulated repression domains within conserved regions of Arx. We also identify a novel interaction between Arx and the co-repressors Groucho and CtBP.

Materials and methods

Plasmids and mutagenesis. Gal4 DBD-Arx fusions were prepared using xArx amplified using the primers: F: 5′-GATCGAA TTCAGCAGCCACTACCAACAGGAG-3′; R: 5′-GATCGGATCCTCAGCA AACCTCTTTCCCTC-3′. The xArx PCR product was then TOPO-TA cloned (pCR 2.1-TOPO, Invitrogen), the sequence verified, and sub-cloned into pCS2 + Gal4 using EcoRI and SnaBI. The pCS2 + Gal4DBD vector was prepared by isolating the Gal4DBD from the pGBT9 plasmid (gift from David Moore) using Hind III (filled in) and EcoRI and then sub-cloned into pCS2 using BamHI (filled in) and EcoRI. The L33P mutation was made using the Quick Change XL Mutagenesis Kit (Stratagene) with primers: L33P F: 5′-TGTATAGACAGTATCCCGGGCAAGAAG-3′; L33P R: 5′-CTTCTTCCCG GGATACTGTCTATACA-3′. The Exon 5 truncation was amplified using primers designed to truncate a specific region, TA cloned and then sub-cloned into the Gal4 plasmid using EcoRI. Exon 5 F: 5′-GATCGAATTCAGCAGCCACTACCAAACAGGAG-3′; Exon 5 R: 5′-GATCCTCGAGTCTGCCGAAGGCCGGGCT-3′. The 398 truncation construct was made using a NotI site within the Gal4-Arx construct to remove the sequence following the site and re-ligate the remaining fragment. The xGrg4 and xGrg5 constructs were digested from a

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pGloMyc tagged vector (gift from Dan Kessler) and cloned into pCS2 plasmid. *Xenopus* CtBP1 coding region was sub-cloned into pCS2 from an EST purchased from Open BioSystems (#7012549) using EcoRI.

Cell culture and transfections. Cos7 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% Calf Bovine Serum, 100 U of penicillin/streptomycin, and 2 mM L-glutamine. Sub-confluent cells were trypsinized and plated the day before transfection at a density of 5×10^4 cells per well. Cells were transfected in triplicate with Fugene 6 transfection reagent at a 3:1 DNA to Fugene ratio per manufacturer's protocol (Roche). Empty pCS2-Gal4 plasmid was added to 400 ng total DNA per reaction. A *Renilla* luciferase reporter (pRL-TK, Promega) was co-transfected as an internal control at a 1:20 ratio to the Gal4-responsive reporter together with different effector DNAs. Cells were harvested 24 h later and lysates were assayed for firefly and *Renilla* luciferase reporter activities using the Dual Reporter Luciferase Assay Kit (Promega) according to manufacturer's protocol.

Immunoprecipitation. Immunoprecipitation experiments were performed using lysates prepared from transfected Cos7 cells. Cells were harvested 24 h later and lysed using M-Per reagent (Pierce) containing Complete Protease Inhibitor Cocktail (Roche) and phosphatase inhibitors (Roche).

Magenetic Protein G beads (Dynal) were used for the immunoprecipitation experiments. The beads were pre-bound with mouse anti-HA antibody (Sigma) as per protocol. Pre-bound beads (30 μl) were mixed with 150 μl of the cell lysate and 1× PBS to a final volume of 500 μl and incubated at 4 °C for 1 h on a rotator. Beads were recovered using a magnet and washed 3× with 1 ml cold 1× PBS + 0.1% Tween, eluted by boiling in SDS sample buffer (Bio-Rad), resolved on a SDS–PAGE gel, then analyzed by western blot. CtBP was detected with a rabbit polyclonal antibody (Bethyl Labs) and Groucho was detected with a goat polyclonal TLE antibody (Santa Cruz).

RNA microinjection. Xenopus laevis embryos were injected in one blastomere at the two-cell stage with RNA encoding wildtype or mutant forms of Gal4-Arx. Gal4-Arx was co-transfected with DNA encoding the Gal4-UAS responsive promoter and a *Renilla* luciferase plasmid that served as the internal control. Linearized DNA constructs transcribed using the SP6 mMessage Kit (Ambion). Embryos were harvested at stage 10, lysed, and assayed as described above. Graphs are representative of three independent experiments.

In situ hybridization. Xenopus Esg-1 [14] was isolated by PCR from an oocyte library (Stratagene) using primers spanning the coding region (accession# U18775). F: 5′-CTCGAGGATGTTCCCTC AAAACAGAC-3′; R: 5′-TCTAGAGGAGACTTCAGTAGATAACC-3′. The PCR product was cloned into pCS2, linearized with Hind III, and transcribed with T7 RNA polymerase to generate a digoxygenin-labelled antisense riboprobe that was used for in situ hybridization [15]. A xCtBP1 digoxygenin-labelled riboprobe was made by linearizing the pCS2/xCtBP1 plasmid with Kpn I and transcribed with T7. This riboprobe was used together with a fluorescein-labelled xArx riboprobe in double in situ hybridization analysis.

Results

Arx functions as a transcriptional repressor

Arx contains three conserved protein domains: an octapeptide (OP), a homeodomain (HD), and an OAR or *Aristaless* domain [4] (Fig. 1A). Previous studies have demonstrated that the OP

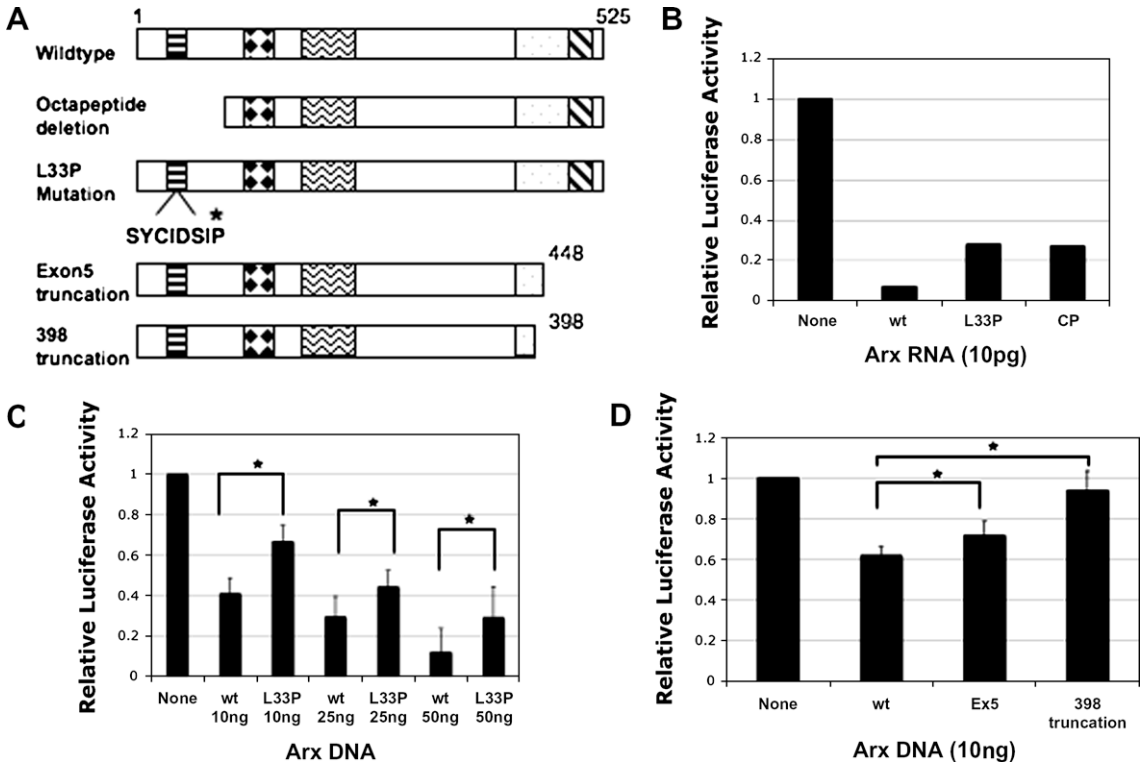

Fig. 1. *Xenopus* Arx functions as a transcriptional repressor. A. Schematic representation of Arx conserved domains. OP, octapeptide motif; HD, homeodomain; OAR, orthopedia-*Aristaless*-Rx domain; CR, conserved Region. (B–D) Luciferase assays performed using lysates from Cos 7 cells co-transfected (B–D) or *Xenopus* embryos injected (C) with wt or mutated Gal4 DBD-Arx expression plasmid, effectors as shown, and a Gal4-UAS-Luc reporter plasmid. (B) Luciferase assay performed using lysates from stage 10 *Xenopus* embryos micro-injected with 10 pg of RNA encoding Gal4-DBD-Arx-OP or L33P and a Gal4-UAS-Luc reporter plasmid. The graph represents a trend observed from three experiments. (C) Disruption of the OP by deletion or mutation (L33P) results in reduced repression activity. (D) Deletion of portions of the Arx C-terminus results in reduced repression activity. $^*p < 0.05$.

confers transcriptional repression [16,17]. We tested the hypothesis that Arx is a transcriptional repressor by assaying the activity of a Gal4-Arx fusion to regulate the activity of a Gal4-responsive luciferase. We found that the activity of the reporter was repressed in a dose-dependent manner, demonstrating that Arx can function as a transcriptional repressor (Supplementary Fig. 1A).

Arx repression is mediated in part by the octapeptide motif

The *Groucho* family of co-repressors mediates repression through direct interaction with the OP found in proteins such as Engrailed, Goosecoid, Pax, Nkx, and Six [18]. To determine whether the OP is necessary for the repression activity of Arx, we tested constructs containing a mutated OP in the luciferase reporter assay. Deletion of the OP decreased the repression activity of Arx by twofold (Fig. 1B). Interestingly, mutation of a highly conserved leucine residue within the OP (L33P) alleviated repression to a similar degree (Fig. 1B and C). The L33P mutation is of particular interest since it has been found in patients with X-linked mental retardation (XLMR) [1,19]. Interestingly, neither the OP deletion nor the L33P mutation completely alleviated repression, suggesting that there is an additional domain within Arx that is also required for repression.

Arx contains a second, groucho-independent, repression domain

To identify a second repression domain, we used the luciferase reporter activity assay to test a series of C-terminal truncation constructs. The first construct tested (Fig. 1A) mimics a truncation within exon 5 that has been described in patients with XLMR [7]. We found that the exon 5 truncation also partially reduced Arx repression activity, suggesting that this region contains a repression domain (Supplementary Fig. 1B).

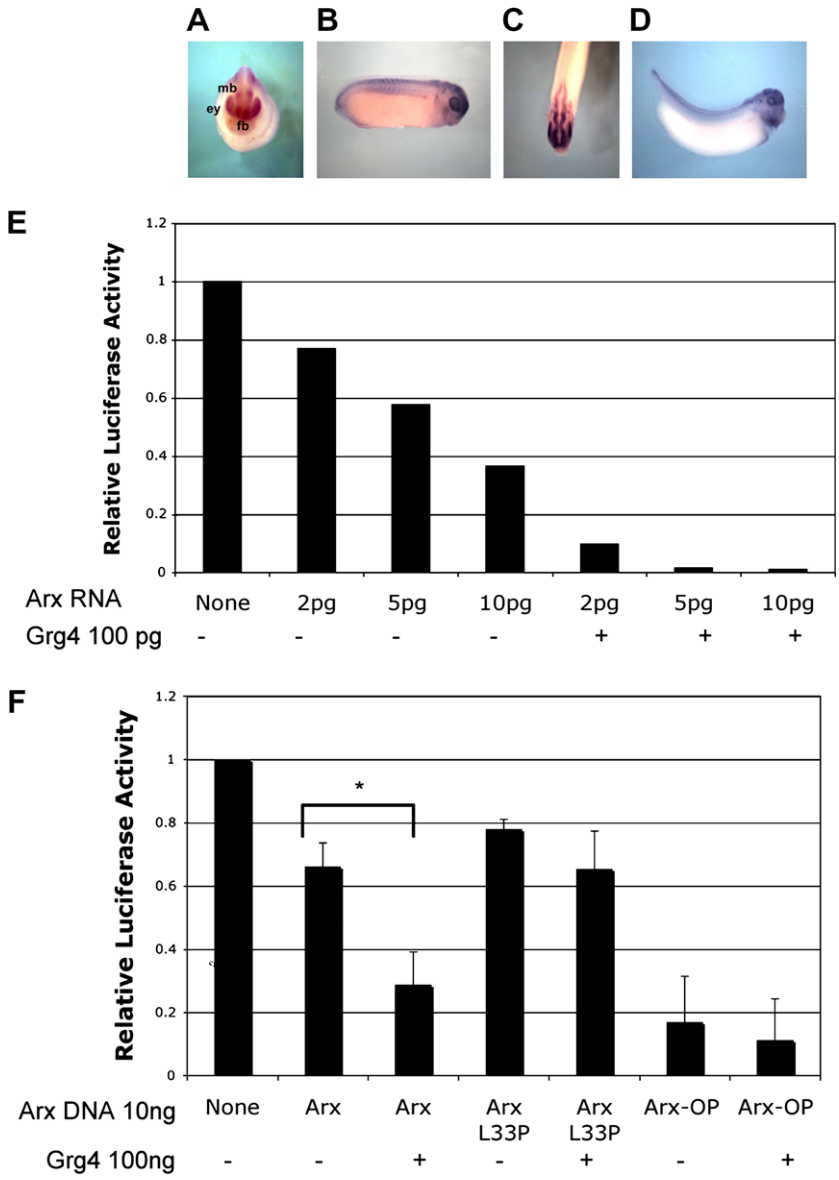

Fig. 2. The Octapeptide mediates repression via the Groucho family of co-repressors. (A–D) Whole mount in situ hybridization showing expression of *Xenopus* Groucho homolog Esg-1 in the brain of varying stages of *Xenopus* embryos at stages 21, 27, 32, and 35. Ey, eye; fb, forebrain; mb, midbrain. (E) The *Xenopus* Groucho homolog Grg4 enhances the repression of Arx. Luciferase assay performed using lysates from stage 10 *Xenopus* embryos that were co-injected with RNA encoding Gal4-DBD-Arx and xGrg4 and a Gal4-UAS-Luc reporter plasmid. The graph represents the trend observed from three experiments. (F) Luciferase assay performed using lysates from Cos7 cells co-transfected with Gal4 DBD-Arx, Gal4 DBD-Arx L33P, or Gal4 DBD-Arx-OP and xGrg4 expression plasmids along with a Gal4-UAS-Luc reporter plasmid. $^{*}p < 0.05$.

To further define the region within the C-terminus responsible for repression, we prepared and assayed the activity of a construct in which a larger portion of the C-terminus was deleted (Fig. 1A). This construct exhibited lower repression activity than the exon 5 truncation, indicating that the region between amino acid 398 and 448 is important for repression (Fig. 1D). Taken together, we conclude that Arx contains two repression domains, the OP and a second domain located in the C-terminus at amino acids (398–448) of the protein, termed ORD (for other repression domain).

The OP mediates repression via the Groucho family of co-repressors

The *Groucho* family of co-repressors is known to bind to transcription factors and mediate repression, specifically via the OP [20]. Two *Xenopus* Groucho homologs have been identified [14,21]. The first, xGrg4, is expressed in apparently overlapping regions of the developing forebrain as Arx [13,21]. The second *Xenopus* Groucho homolog, Esg-1 (Enhancer of Split Groucho-1), is most similar to human Tle-1 [14] and is also co-expressed with xArx in the developing brain (Fig. 2A–D). We were unable to identify additional Groucho genes by degenerate PCR (data not shown).

To test whether the *Groucho* family of co-repressors mediate repression of Arx through the OP, we co-injected wildtype and mutant Arx with xGrg4 in *Xenopus* embryos. When co-injected with Arx, Grg4 enhanced repression of the luciferase reporter in a dose-dependant manner (Fig. 2E). We obtained similar results using xEsg-1 (data not shown). We also found that Grg4 did not have an effect on the L33P Arx mutant in reporter assays (Fig. 2F) in agreement with published [12]. Further, Grg4 did not affect the repression activity of Arx lacking the OP (Fig. 2F). Taken together, our results indicate that Arx has two independently regulated repression domains, the OP and ORD.

C-terminal binding protein 1 (xCtBP1) mediates repression of Arx

C-terminal binding proteins (CtBPs) are co-repressors that bind the C-terminus of many transcription factors [22,23]. In addition, many proteins have been found to mediate repression by binding to both Groucho and CtBP [24–26]. We hypothesized that CtBP may mediate repression through the ORD in Arx. We found that xArx and xCtBP1 are co-expressed in the developing brain during tailbud stages (Fig. 3B and C). We found that CtBP1 enhances repression of the Gal4-dependent luciferase reporter ~3-fold compared to Arx alone (Fig. 3D). To determine whether xCtBP1 can physically interact with Arx, we performed co-immunoprecipitation experiments with Cos 7 cells transfected with HA-tagged Arx (Fig. 3E). HA-Arx precipitated endogenous CtBP, suggesting that CtBP1 mediates Arx repression via physical interaction.

The ORD mediates repression by CtBP1

To determine if the ORD was responsible for mediating repression via CtBP, we tested the exon 5 and 398 truncation constructs in a reporter assay with CtBP (Fig. 1A). We found that CtBP was able to enhance repression of these constructs (data not shown). We next tested constructs that contained both a C-terminal truncation and the L33P mutation. CtBP1 could still enhance repression of this doubly mutated Arx (data not shown). Previous studies report that the Arx L33P mutation can weakly bind Groucho [12]. We postulated that the observed repression could be due to residual Grg4 interaction with the mutated OP and that CtBP may be able to enhance repression through this complex. To address this possibility we tested constructs containing both the truncated C-terminus and the OP deletion (Supplementary Fig. 2A). CtBP1 could enhance repression of the -OP/exon5 truncation construct but not of the -OP/398 truncation construct, validating that CtBP1 can mediate

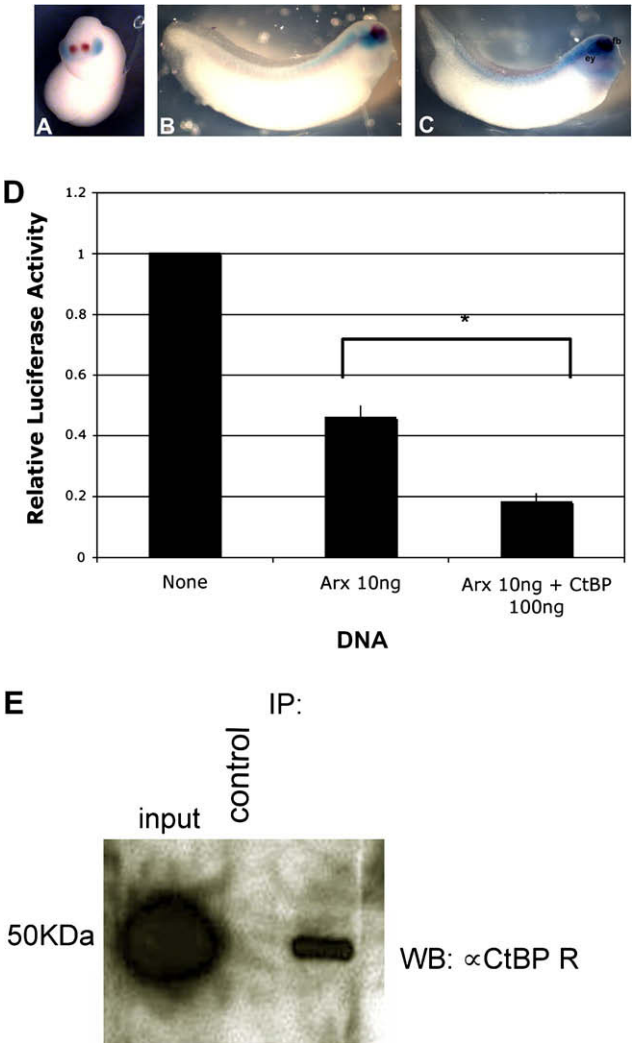

Fig. 3. C-terminal binding protein 1 (xCtBP1) mediates repression of Arx. (A–C) Double whole mount in situ hybridization showing overlapping expression of Arx and and xCtBP in the developing brain of *Xenopus* embryos at stages 21, 32, and 35. Ey, eye; fb, forebrain. Arx is stained magenta, xCtBP is stained turquoise, and areas of overlap are purple. (D) Luciferase assay performed using lysates from Cos7 cells co-transfected with Gal4 DBD-Arx and xCtBP expression plasmids and a Gal4-UAS-Luc reporter plasmid. $^{*}p < 0.05$. (E) Co-immunoprecipitation of CtBP performed using lysates prepared from Cos7 cells transfected with HA-Arx. Control IP was performed using mouse IgG.

repression through the ORD (Fig. 4A). This data also suggests that Arx, Groucho, and CtBP may collectively interact.

Arx contains a sequence within the ORD (PLGLS) that conforms loosely to the defined CtBP binding site (Supplementary Fig. 2B) [22]. Deletion of this motif did not affect the ability of CtBP to enhance repression by Arx (data not shown). Although we cannot rule out the involvement of the PLGLS motif, our data suggests that regions outside of this motif are necessary for CtBP to mediate repression through the ORD.

Arx interacts with both Groucho and CtBP

To investigate the possibility of an interaction between Arx, Grg4, and CtBP, we performed co-immunoprecipitation using Cos 7 cells transfected with HA-Arx (Fig. 4B). We found that endogenous CtBP could precipitate with endogenous Grg4 only in the presence of Arx. Further, CtBP could not precipitate Grg4 in untransfected control cells. This result indicates that Arx can interact with both co-repressors. Collectively, we found that Arx can

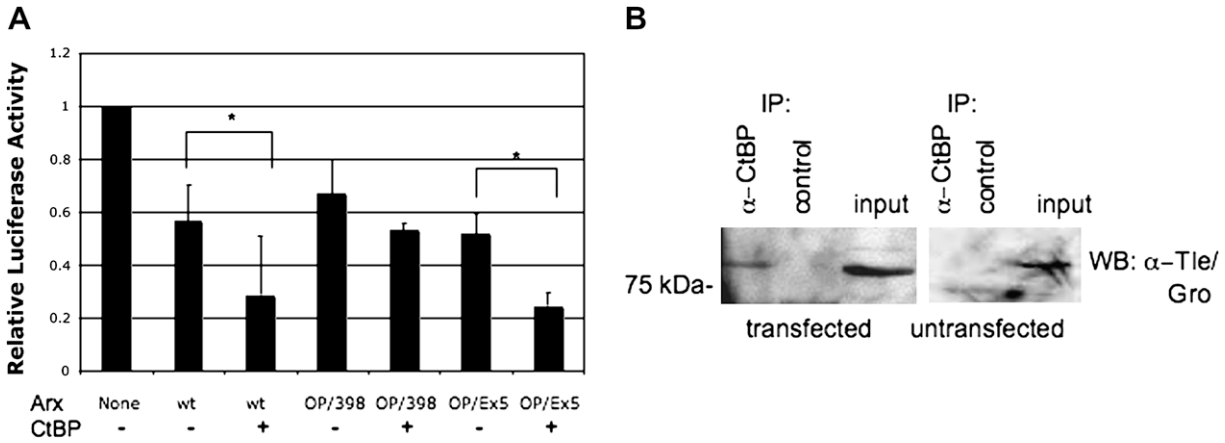

Fig. 4. The ORD mediates repression by CtBP1. (A) Luciferase assay performed using lysates prepared from Cos7 cells co-transfected with Gal4 DBD-Arx, Gal4 DBD-Arx-OP/Ex5 truncation, or Gal4 DBD-Arx-OP/398 truncation and xCtBP expression plasmids along with a Gal4-UAS-Luc reporter plasmid. $^{*}p < 0.05$. (B) Co-immunoprecipitation of Groucho performed using lysates prepared from Cos7 cells transfected with HA-Arx. Control IP was performed using mouse IgG.

physically interact with both Groucho and CtBP simultaneously since both co-factors could be precipitated in the presence of Arx. This result further supports our hypothesis that Arx is a transcriptional repressor regulated by both Groucho and CtBP.

Discussion

Arx plays a critical role in brain development and mutations that result in an altered protein cause neurological disorders. Despite the wealth of information known about the clinical manifestations of mutations in Arx, little is known about the molecular consequences of these mutations. In addition, it is not completely understood how Arx acts to repress its downstream targets. Our studies suggest that repression by Arx is complex and requires more than one co-factor.

In this report, we found that the octapeptide motif is necessary for Groucho-dependent repression activity. Also, the L33P mutation that is found in patients with XLMR [1,19] disrupts Groucho-mediated repression via the OP. Our results are consistent with previous findings that the L33P mutation in the Arx OP weakens the physical interaction with Groucho [12].

Through reporter assays with the exon 5 truncation construct, similar to a mutation found in patients with XLAG [7], we determined that Arx contains a second repression domain (ORD). We further narrowed the ORD to a region in the C-terminus corresponding to amino acids 398–448. Furthermore, our reporter assay data confirms that CtBP can mediate repression of Arx though the ORD.

The co-repressors Groucho and CtBP mediate repression by qualitatively different mechanisms. Groucho is involved in long-range repression by influencing enhancers that are located thousands of base pairs away and CtBP mediates short-range repression by interfering with activators at a nearby locus [20]. Recently, several transcriptional repressors have been reported to interact cooperatively with both Groucho and CtBP. Brinker, Hairy, and Single-minded can mediate repression of downstream genes by interacting with Groucho, CtBP, or both [24–26]. In addition, the *Drosophila* Hairless protein has been shown to confer repression of the Hairless-Su(H) complex via binding both CtBP and Groucho in combination [27]. In this paper, we demonstrate that Arx can physically interact with both Groucho and CtBP. Transcriptional repressors such as Brinker, Hairy, Hairless, and Single-minded, repress downstream targets in a context-dependent manner in which certain promoters require different combinations of CtBP and Groucho [24–27]. This type of regulation could also be a mechanism for repression by Arx, however, additional studies with downstream targets are necessary to test this possibility.

Previous studies of Arx deficient mice have shown that Arx is important for many developmental processes, including proper migration of GABA-ergic cortical interneurons and neuronal cell differentiation and proliferation [4,6,28]. Additionally, Arx is important for proper pancreatic endocrine cell specification through the inhibition of Pax4 [11]. Tcf3 is an example of a transcriptional repressor that partners with Groucho and CtBP to regulate developmental processes [29]. Additionally, temporal regulation of CtBP expression may result in tight regulation or fine tuning of repression at certain developmental stages [24]. In our report we show that the expression of Arx and CtBP in *Xenopus* embryos does not overlap until later stages of development (Fig. 3A–C). This finding indicates that there could be a temporal aspect to cooperative groucho and CtBP-mediated repression of Arx.

The data presented here supports the recent reports that repression is important for many developmental processes and in some instances several different co-repressors are necessary to regulate transcriptional repression. Further work is necessary to determine how XLMR-associated mutations affect the regulation of Arx target genes, leading to abnormalities in brain development and, ultimately, disease.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.116.

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