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AP-2α selectively regulates fragile X mental retardation-1 gene transcription during embryonic development

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Fragile X syndrome (FXS) is almost always caused by silencing of the *FMR1* gene. The defects observed in FXS indicate that the normal *FMR1* gene has a range of functions and plays a particularly prominent role during development. However, the mechanisms regulating FMR1 expression *in vivo* are not known. Here, we have tested the role of the transcription factor AP-2α in regulating Fmr1 expression. Chromatin immunoprecipitation showed that AP-2α associates with the Fmr1 promoter *in vivo*. Furthermore, Fmr1 transcript levels are reduced >4-fold in homozygous null AP-2α mutant mice at embryonic day 18.5 when compared with normal littermates. Notably, AP-2α exhibits a strong gene dosage effect, with heterozygous mice showing ∼2-fold reduction in Fmr1 levels. Examination of conditional AP-2α mutant mice indicates that this transcription factor plays a major role in regulating Fmr1 expression in embryos, but not in adults. We further investigated the role of AP-2α in the developmental regulation of Fmr1 expression using the *Xenopus* animal cap assay. Over-expression of a dominant-negative AP-2α in *Xenopus* embryos led to reduced Fmr1 levels. Moreover, exogenous wild-type AP-2α rescued Fmr1 expression in embryos where endogenous AP-2α had been suppressed. We conclude that AP-2α associates with the Fmr1 promoter *in vivo* and selectively regulates Fmr1 transcription during embryonic development.

INTRODUCTION

Fragile X syndrome (FXS) is the most common form of inherited mental retardation, affecting approximately one in 4000 males and one in 7000 females. The genetic defect underlying FXS is a massive CGG repeat expansion in the first exon of the fragile X mental retardation-1 (*Fmr1*) gene. The repeats themselves as well as nearby promoter regions are then hypermethylated, leading to chromatin silencing and suppression of Fmr1 transcription (1–3). The resultant loss of the protein product, FMRP, manifests in a wide range of clinical phenotypes including low-to-moderate intelligence quotient (IQ) (range: 20–80), craniofacial dysmorphism, seizures and neurobehavioral problems (4). Although the dysregulation in Fmr1 transcription has been firmly established as the key defect in FXS pathogenesis, the basic mechanisms underlying the transcriptional regulation of either the normal or the mutated *Fmr1* gene during *in vivo* development are still poorly understood.

A wide range of neural and non-neural tissues express Fmr1 during development. For example, during embryogenesis, the brain and testis express high levels of Fmr1 transcripts (5). Non-neural structures such as the eye, cartilage and gonads also exhibit strong to moderate levels of Fmr1 transcripts (6). Similarly, adult tissues show Fmr1 expression in the central nervous system (CNS) and testis as well as the ovaries, thymus, esophagus and spleen (5). Despite this broad expression pattern, Fmr1 levels in various tissues

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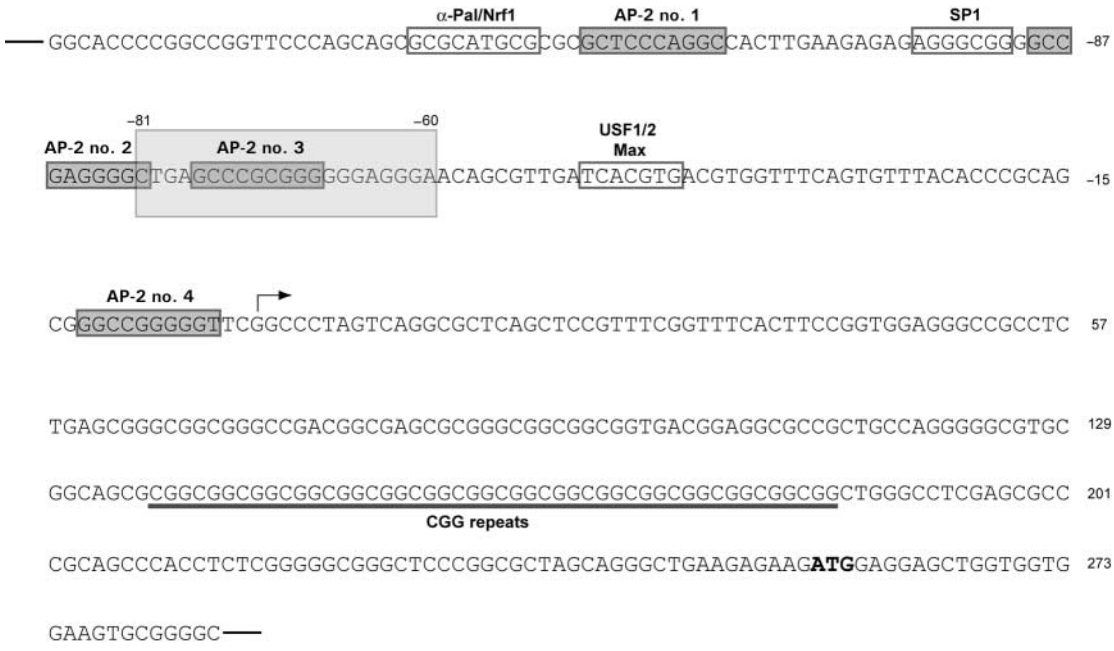

Figure 1. Human Fmr1 promoter showing transcription binding sites (boxed; α-Pal/Nrf1; AP-2; SP1; USF1/2; Max). Four potential AP-2 binding sites are shown as AP-2 nos 1–4 (dark gray boxes). The light gray box highlights the region (−60 to −81) protected in DNA footprint assay (11). The transcription start site is indicated by the arrow, and the start codon is bold-faced. CGG repeats are underlined.

display dynamic and specific changes during development (7). Such expression patterns suggest that a complex interplay among *cis* and *trans* regulatory elements underlie the unique nature of Fmr1 transcription during development.

The promoter region critical for the regulation of Fmr1 expression in cultured cells has been mapped and characterized in a number of studies (2,7–13). The binding of four transcription factors AP-2, SP1, α-Pal/Nrf1 and USF1/2 to the Fmr1 promoter has been previously noted *in vitro*, both in cell free extracts and in cell culture systems (2,9–12). Functional assays in cell culture system have shown that at least some of these factors can modulate Fmr1 transcription (2,11,12). However, no studies have yet shown that these or any other factors can regulate Fmr1 transcription in intact animals.

One of the potential regulators of Fmr1 expression, the AP-2 family of transcription factors, consists of AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε. These isoforms show overlapping yet distinct expression patterns in various tissues during development (14). In particular, AP-2α plays crucial roles in neural crest, craniofacial, ocular and CNS development (15–19). Interestingly, we noted that the clinical manifestations of FXS, such as craniofacial dysmorphism, and the CNS defects echo those attributed to the AP-2α function (17).

These correlations prompted us to test whether there is a functional link between AP-2α and *Fmr1* gene. Here, we show that Fmr1 is a target of AP-2α *in vivo*. We use chromatin immunoprecipitation (ChIP) to show that AP-2α binds to the human Fmr1 promoter in living cells. In addition, we use both conventional and conditional AP-2α mutant mice as well as developing *Xenopus* embryos to show that AP-2α selectively regulates Fmr1 expression during embryonic development.

RESULTS

AP-2α binds to the Fmr1 promoter *in vivo*

AP-2 family members bind to several *cis*-acting DNA sequences: 5′-TCCCCANGCG-3′, 5′-(G/C)CCCA(G/C)(G/C)(G/C)-3′ and 5′-GCCN$_{3/4}$GG(G/C)-3′ (20–22). Although a number of potential AP-2 binding sites are present between −120 and +1 of the Fmr1 promoter (Fig. 1; AP-2 nos 1–4), DNase I footprinting analysis has shown that AP-2 associates with the region between −60 and −81 (11). This area contains an AP-2 binding site with the sequence: GCCCG CGGG (Fig. 1; AP-2 no. 3). We used ChIP analysis in HeLa cells to test whether AP-2α binds to this region *in vivo*. AP-2α-immunoprecipitated chromatin showed specific enrichment of Fmr1 DNA fragments containing AP-2 site no. 3 (Fig. 2). Thus, AP-2α binds directly to the Fmr1 promoter in living cells.

AP-2α null mice have reduced Fmr1 expression

We next used AP-2α mutant mice to test the role of this transcription factor in regulating Fmr1 expression in intact animals. We analyzed Fmr1 transcript levels in the head tissue of E18.5 AP-2α wild-type, heterozygous and homozygous null animals (Fig. 3). The relative Fmr1 expression levels found in these genotypes were 1 ± 0.0 (AP-2$\alpha^{+/+}$); 0.50 ± 0.20 (AP-2$\alpha^{+/-}$) and 0.21 ± 0.20 (AP-2$\alpha^{-/-}$; all values are mean ± SEM), respectively. Thus,

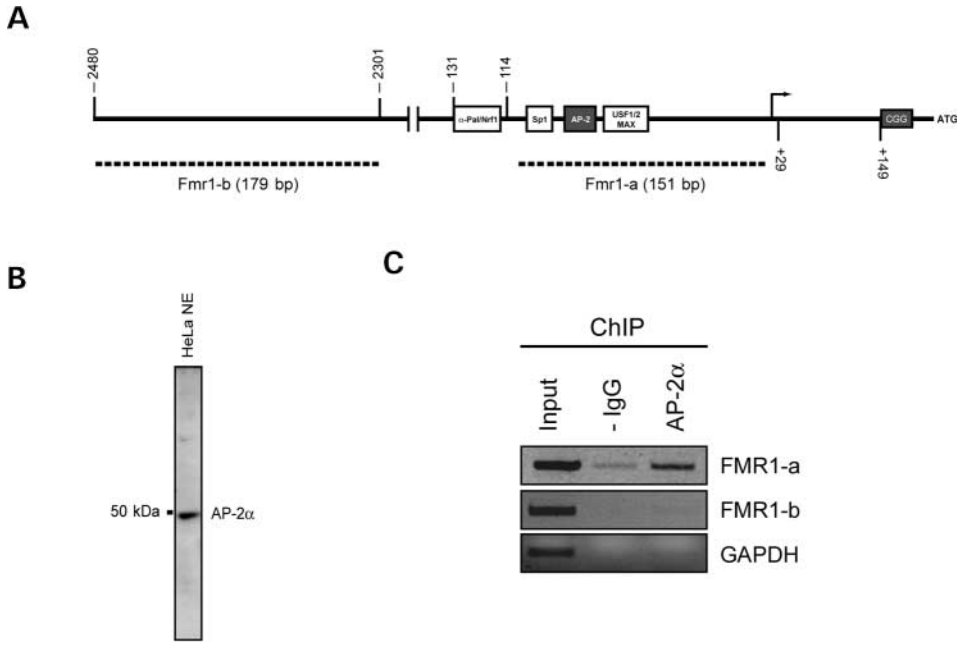

Figure 2. ChIP analysis demonstrating the binding of endogenous AP-2α to Fmr1 promoter in HeLa cells. (**A**) Schematic of the Fmr1 promoter depicting the AP-2α binding site and the location of PCR primers used in the ChIP assay. (**B**) Western blot analysis showing the presence of AP-2α protein in HeLa nuclear cell extracts (NE). (**C**) ChIP assay shows enrichment of the Fmr1 DNA fragment (Fmr1-a; 151 bp) in AP-2α-immunoprecipitated chromatin when compared with non-immune IgG-immunoprecipitated chromatin. The input preparation is also shown. An 179 bp region (Fmr1-b) that lies ~2.4 kb upstream of the Fmr1-a region and does not contain an AP-2 site was used to demonstrate the appropriate shearing of the chromatin. Furthermore, an 174 bp genomic region flanking the GAPDH and the chromosome condensation-related SMC-associated protein (*CNAP1*) gene, which also does not have an AP-2 site, was used as an additional negative control.

AP-2α shows a strong, dose-dependent regulation of *Fmr1* gene expression.

AP-2α selectively regulates Fmr1 transcription during development

AP-2α-null mice die at birth with multiple congenital defects including cranioabdominoschisis and severe craniofacial dysmorphogenesis (15,23). Therefore, the examination of Fmr1 transcript levels beyond late embryonic stages was not possible in these conventional AP-2α null animals. We generated conditional null mutants to study the role of AP-2α in adults as well as in developing animals. Mice carrying a conditional (floxed) version of the *AP-2α* gene were crossed with animals expressing Cre recombinase under the control of *Six3 cis*-regulatory sequences (referred as 'floxdel/KI'). In these mice, the AP-2α allele is selectively mutated in the eyes of embryonic (Fig. 4A) and adult animals (data not shown). We observed significant reduction in the relative levels of Fmr1 transcripts in the eyes of E18.5 floxdel/KI mice (0.15 ± 0.27) when compared with the wild-type controls (1.0 ± 0.0; Fig. 4B). Moreover, in the legs of E18.5 floxdel/KI mice, where *Six3* is not expressed and thus AP-2α allele is not deleted, the levels of Fmr1 were unchanged (Fig. 4B; wild-type: mean ± SEM; 1.0 ± 0.0; floxdel/KI: mean ± SEM; 1.1 ± 0.37). Remarkably, and in contrast to the embryonic eyes, Fmr1 transcript levels were not significantly changed in the adult eyes of floxdel/KI mice (Fig. 4B; mean ± SEM; 1.51 ± 0.63). Therefore, these results demonstrate that AP-2α regulates Fmr1 transcription during embryonic eye development, but is not required for its expression in the adult.

Co-localization of AP-2α and Fmr1 in *Xenopus* embryos

To further investigate the regulation of Fmr1 transcription by AP-2α during embryonic development, we used a *Xenopus* embryo system, which has been a powerful model for studying the transcriptional regulation of genes during embryogenesis (24–26). Moreover, the expression and function of AP-2α has been well characterized during early development in these animals (18,27–29). Whole-mount *in situ* hybridization of mid-neurula stage (st. 15) *Xenopus* embryos showed that Fmr1 expression pattern is broader than AP-2α. However, both transcripts are localized in the cranial neural crest region (compare Fig. 5A and A′). At late tailbud stage (st. 25), Fmr1 co-localized with AP-2α in the pharyngeal arches and craniofacial regions (compare Fig. 5B and B′). Fmr1 levels were also high in the CNS and eye at this stage.

A dominant-negative form of AP-2α downregulates Fmr1 expression in *Xenopus* embryos

Our finding that the expression patterns of AP-2α and Fmr1 overlap in the cranial neural crest regions in *Xenopus* embryos suggested that AP-2α might also regulate Fmr1

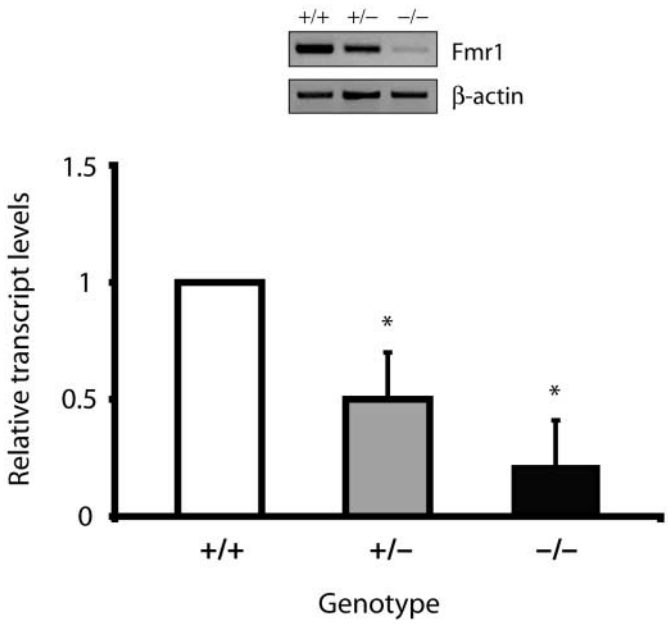

Figure 3. Reduction of Fmr1 transcripts in heads of AP-2α null mutant mice. When compared with wild-type (+/+) littermate controls, AP-2α homozygous (−/−) and heterozygous (+/−) mice show ∼4-fold and 2-fold reduction in Fmr1 transcript levels, respectively. Results from both semi-quantitative and quantitative RT–PCR are shown (Student's *t*-test: $^*P < 0.05$). Fmr1 levels are normalized to GAPDH levels.

transcription in *Xenopus* embryos. To test this idea, we used the animal cap assay. Microinjection of 500 and 1000 pg of a dominant-negative *Xenopus* AP-2α (ΔXAP2), which lacks the transactivation domain (18), significantly repressed Fmr1 transcript levels (Fig. 6A). However, this manipulation had no effect on the levels of Fxr1, an autosomal homolog of Fmr1. The expression pattern of Fxr1 is similar to Fmr1 in *Xenopus* embryos, but with more prominent staining in the somitic mesoderm (30). Thus, AP-2α action is selective for Fmr1 expression (Fig. 6A).

AP-2α rescues Fmr1 expression in AP-2α-suppressed *Xenopus* embryos

As a second test of the role of AP-2α in controlling Fmr1 expression, we performed a rescue experiment. We took advantage of the observation that the bone morphogenetic protein (BMP) signaling pathway is required for AP-2α expression (18,31). Accordingly, BMP antagonists such as chordin (32) can inhibit AP-2α expression in *Xenopus* embryos (18). We therefore injected embryos with chordin mRNA and performed animal cap assays. Chordin injection alone inhibited Fmr1 expression. However, the addition of either 100 or 250 pg of RNA encoding wild-type *Xenopus* AP-2α (XAP2) restored Fmr1 expression to levels comparable to those in the uninjected caps (Fig. 6B). Taken together with the co-localization and dominant-negative studies, these rescue experiments provide strong evidence that AP-2α regulates Fmr1 expression in developing *Xenopus* embryos.

DISCUSSION

In the current study, we used a range of experimental approaches and animal systems to demonstrate that AP-2α regulates Fmr1 transcription *in vivo*. Our study is the first study to demonstrate a role for a specific transcription factor in mediating Fmr1 expression in intact animals. Furthermore, our findings verify and extend earlier DNA footprinting, element-mapping and *in vitro* DNA binding analyses (9–11). Of particular note is our finding that AP-2α can selectively regulate Fmr1 expression during embryonic development. Furthermore, our data underscore the importance of context-dependent (i.e. developmental) regulation of Fmr1 expression.

The human Fmr1 promoter harbors four potential AP-2 binding sites between regions −120 and +1 (Fig. 1). The consensus DNA binding sequences in these sites are well conserved in other vertebrates (12). Our present results as well as earlier work (11) strongly suggest that AP-2 site no. 3 (region −76 to −68) is the critical AP-2α binding site for regulating Fmr1 expression. Nonetheless, we note a previous study that failed to detect AP-2α binding to the Fmr1 promoter using adult mouse brain nuclear extracts in gel shift assays (12). However, the levels of AP-2α in those preparations were not determined. Such information is important as brain regions are known to differentially express AP-2α during development (14,33). For example, AP-2α levels are high in embryonic olfactory bulb and hippocampus, but decline sharply during postnatal life (14). In contrast, AP-2α levels are relatively stable throughout development in the eye (16) and epidermis (34). Therefore, the age and the type of the tissue used in the assay may explain the discrepancy between the previous study (12) and others (11; and our data here). These differential findings further emphasize the importance of examining the regulation of Fmr1 transcription in the context of organism's developmental milieu.

Our results indicate that distinct transcriptional mechanisms regulate Fmr1 expression in developing when compared with adult animals. AP-2α is a critical mediator of Fmr1 expression during embryonic and perinatal development. However, it is dispensable in the adult. Therefore, other transcription factors (e.g. α-Pal/Nrf1, Sp1, USF1/2 and Max) are likely to play prominent roles in mature animals. Another possibility is that other AP-2 subtypes (i.e. β, δ, γ and ε) which exhibit highly conserved DNA contact domain and overlapping expression patterns (14) can influence Fmr1 transcription. It seems likely that a variety of *trans*-elements, both positive and negative, will collaborate in distinct combinations to produce the nuanced expression of Fmr1 necessary for its function during development.

Our results also shed light on potential roles for FMRP during embryonic development. As described earlier, AP-2α regulates a wide range of genes that are particularly important in craniofacial, neural crest, eye and neural development. FMRP could play a prominent role in these processes. For example, the finding that Fmr1 co-localized with AP-2α in neural crest cells (Fig. 5) is particularly interesting as many tissue structures affected in FXS are neural crest derivatives. Many diseases classified as 'neurocristopathies' arise from defects in crest development (35). The most common features of neurocristopathies are mental retardation and craniofacial

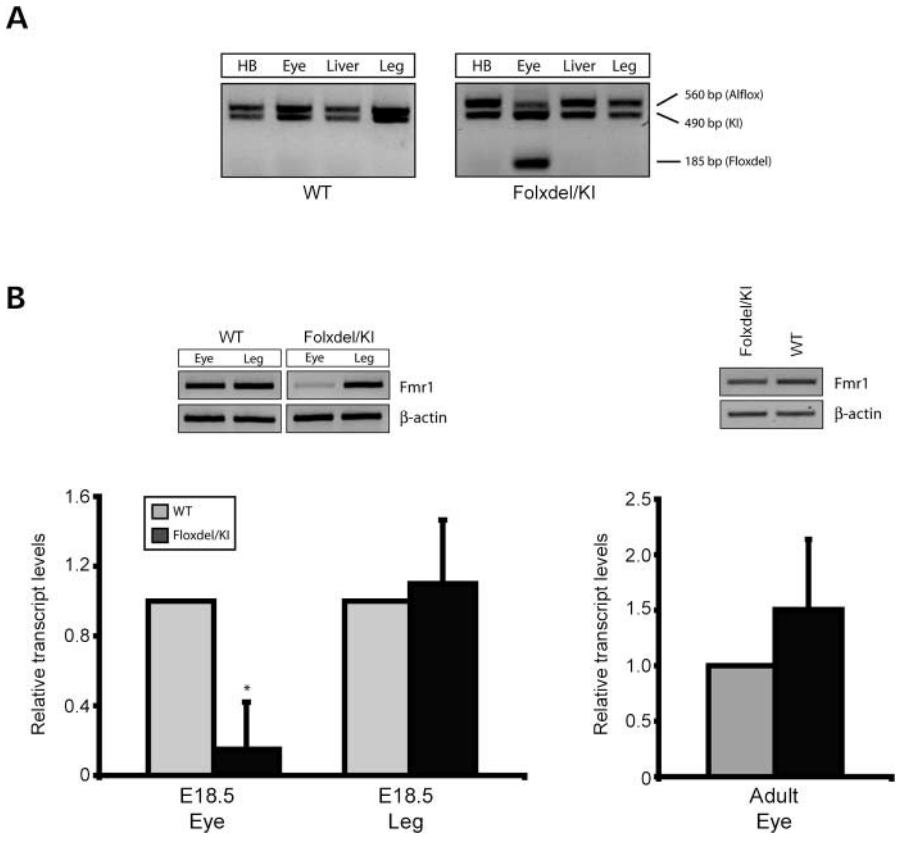

Figure 4. Six3–Cre-mediated deletion of AP-2α in the eye reduces Fmr1 transcript levels in E18.5 mouse embryos. (**A**) PCR analysis of Six3–Cre-mediated deletion of the Alflox allele using primers Alflp, Alflox4 and Alfscsq (19). All mice contain the AP2α:LacZ KI allele, which generates a 490 bp product with primers Alflox4 and Alfscsq. Alflox/KI and Floxdel/KI mice both have one conditional AP-2α allele, but only the Floxdel/KI mice also contain the Six3–Cre transgene. The undeleted Alflox allele generates a band of 560 bp with primers Alflox4 and Alfscsq. The Floxdel allele produces a 185 bp band from primers Alflp and Afscsq. The deleted conditional allele is only detected in the eye in the presence of Six3–Cre, HB: hindbrain. (**B**) Semi-quantitative and quantitative PCR analyses of Fmr1 transcript levels in E18.5 and adult wild-type and Floxdel/KI mice. Fmr1 transcript levels are significantly reduced in the eye of E18.5 floxdel mice when compared with the wild-type control mice (Student's *t*-test; $^{*}P < 0.05$). In contrast, Fmr1 levels are not changed in the leg where the conditional allele is undeleted. Moreover, adult Floxdel/KI mice do not show significant changes in Fmr1 levels in the eye.

dysmorphism—the most recognizable features of FXS. Thus, it should be fruitful to further investigate the role of Fmr1 expression in neural crest development.

Finally, our results have important implications to the fragile X-associated tremor/ataxia syndrome or FXTAS, which is a late-onset neurodegenerative disorder (36,37). Individuals who develop FXTAS usually carry premutation Fmr1 allele (55–200 CGG repeats). Although these individuals show normal FMRP expression, they display elevated Fmr1 transcript levels (36). It is possible that altered chromatin structure (i.e. open promoter conformation) brought on by the expanded CGG repeats, in concert with increased transcription factor(s) binding affinity, underlies the enhanced Fmr1 transcriptional activity observed in these patients (38). On the basis of our study, it is likely that the developmental environment will dictate which *trans*-elements will be involved in such process.

In the present study, we have demonstrated the importance of developmental environment in regulating Fmr1 expression. The finding that AP-2α selectively regulates Fmr1 expression during development carries important implications for potential therapies aimed at reactivating *Fmr1* gene expression in FXS or at repressing it in FXTAS. Indeed, the clinical outcomes of potential therapies based on modulating the levels of transcription factors will likely depend upon the age of the individual. Therefore, the identification of specific *trans*-factors such as AP-2α that act at a particular developmental window to regulate Fmr1 expression will be critical to the success of such therapeutic approaches.

MATERIALS AND METHODS

Western blot analysis

HeLa cell nuclear extracts were prepared as described previously (39). Nuclear extracts were resolved on 7.5% polyacrylamide gel and transferred to nitrocellulose membrane, probed with anti-AP-2α antibody (3B5, Santa Cruz Biotechnology, Inc.), followed by an HRP-conjugated secondary antibody. Immunoreactive bands were visualized with ECL plus (Amersham Biosciences).

ChIP assay

ChIP was performed using the ChIP-IT^{TM} kit (Active-Motif) with the following modifications. HeLa cells were grown to

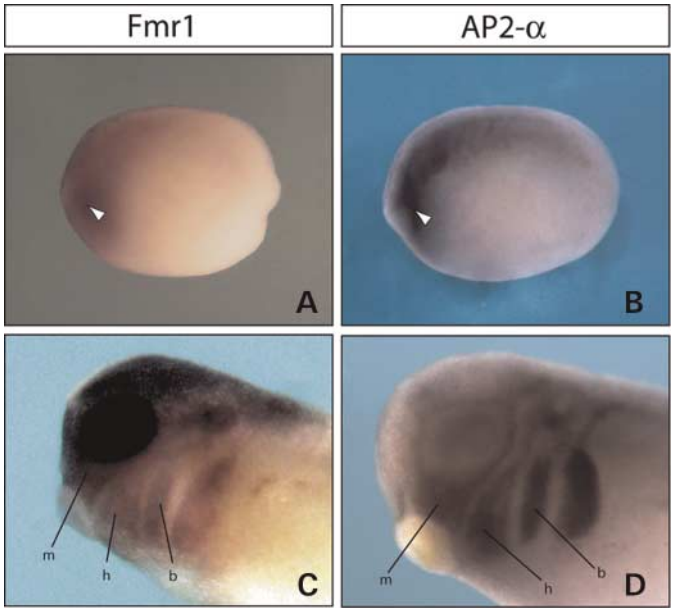

Figure 5. Co-localization of Fmr1 and AP-2α transcripts in developing *Xenopus laevis* embryos. (**A** and **B**) Whole-mount *in situ* hybridization of Fmr1 (A) and AP-2α (B) at late neurula stage (lateral view). Neural crest and epidermal regions show co-localization of Fmr1 and AP-2α transcripts. (**C** and **D**) Whole-mount *in situ* hybridization of Fmr1 and AP-2α at early-mid tailbud stage (lateral view). Both Fmr1 (C) and AP-2α (D) show robust expression in the cranial crests (m, mandibular; h, hyoid and b, branchial). The eye and the CNS display higher transcript levels of Fmr1 than AP-2α at this stage. Cement gland is negative for both Fmr1 and AP-2α transcripts.

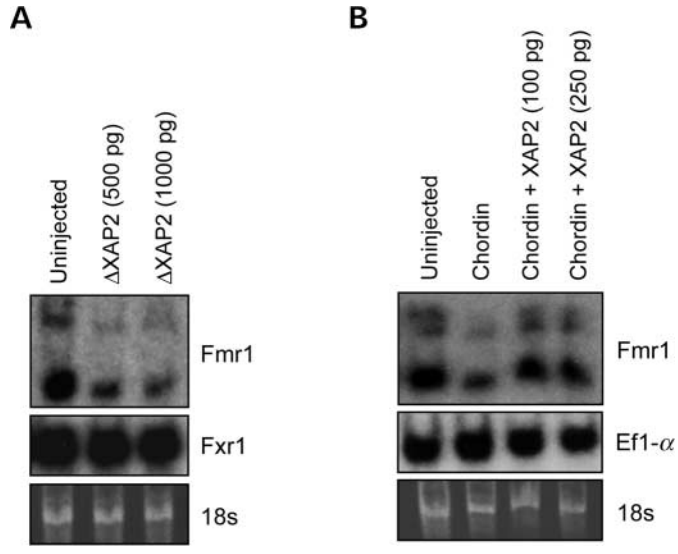

Figure 6. Regulation of Fmr1 expression by transcription factor AP-2α in *Xenopus laevis*. (**A**) Dominant-negative AP-2α (ΔXAP2) represses Fmr1 transcription. Either 500 or 1000 pg of ΔXAP2 was microinjected into one-cell stage embryos. Animal cap ectoderm was excised at blastula stage and cultured to stage 14 or 15 (mid-late neurula). RNA was then isolated and northern blots probed for the indicated transcripts. Animal cap RNA from uninjected embryos is shown for comparison. (**B**) AP-2α rescues Fmr1 expression. Fertilized embryos were microinjected with RNA encoding chordin (250 pg) to suppress endogenous AP-2α expression (18). Micro-injection of 100 pg of AP-2α restores Fmr1 transcript levels comparable with uninjected embryo cap.

70–80% confluency, fixed with 1% formaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4 at 37°C for 10 min. The samples were then sonicated using Virsonic 100 Ultrasonic Cell Disruptor to shear chromatin to an average length of about 700–800 bp. The isolated chromatin was run on a 1.2% agarose gel to check for shearing efficiency. Subsequently, AP-2α bound chromatin complexes were immunoprecipitated using anti-AP-2α antibody (C-18, sc-184; Santa Cruz Biotechnology, Inc.). Negative control IgG was used to control for specificity. PCR was performed with the eluted genomic DNA using the following primers: Fmr1-a forward: 5′-CAG GCC ACT TGA AGA GAG AG-3′; Fmr1-a reverse: 5′-AGT GAA ACC GAA ACG GAG C-3′; Fmr1-b forward: 5′-GCT AGA GAT CAG AGT AAG GCT G-3′; Fmr1-b reverse: 5′-AGA CAG GGC AAA TGT CTT TTC-3′; GAPDH forward: 5′-ATG GTT GCC ACT GGG GAT CT-3′; GAPDH reverse: 5′-TGC CAA AGC CTA GGG GAA GA-3′. PCR conditions were: 95°C, 30 s; 55–60°C, 30 s; 72°C, 20 s for 24–27 cycles.

Generation of eye-specific AP-2α conditional knockout mice

The generation of AP-2α floxed mice (Alflox; 18) and Six3–Cre mice (40) is described previously. Briefly, mice heterozygous for the AP-2α : LacZ KI allele, a null allele (41), were bred with Six3–Cre transgenic mice (40). Then, the offspring that were heterozygous for both the *Cre* gene and the AP-2α : LacZ KI allele were bred with homozygous Alflox mice. DNA from embryo or adult tissue samples were prepared using DNeasy tissue kit (Qiagen) and genotyped as described previously (19).

RNA and cDNA preparation

Tissues were dissected in 0.1 M PBS, pH 7.4. They were then transferred to a microcentrifuge tube and quick-frozen in liquid nitrogen. RNA extraction was performed using the Trizol method (Invitrogen). Genomic DNAs were subsequently removed by incubating the RNA samples with DNase I (Invitrogen) for 30 min at 37°C. A second round of RNA extraction with Trizol was performed to remove the DNase I. The purified RNAs were then converted to cDNA using the Superscript III First-Strand Synthesis System Kit (Invitrogen).

Quantitative PCR analysis

PCR reactions were performed with LUX™ fluorogenic primers (Invitrogen) using ABI PRISM 7700 real-time thermocycler. Primer sequences are as follows: Fmr1 forward: 5′-GAC GAT CAT TCC CGA ACA GAT AA-3′; Fmr1 reverse: 5′-GCG GAA GAG GAC AAG GAG GA-3′; GAPDH forward, 5′-CAA CAG CAA CTC CCA TTC TTC C-3′; GAPDH reverse: 5′-AAG GGC ATC CTG GGC TAC AC-3′; Fmr1 and GAPDH primers were labeled with 6-carboxy-fluorescein

(FAM) and 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxy-fluorescein (JOE), respectively. PCR conditions were 95°C, 15 s; 55°C, 30 s and 72°C, 30 s for 45 cycles. The data from PCR reaction were quantified using the $2^{-\Delta\Delta Ct}$ method (42).

Fmr1 antisense probe generation and *in situ* hybridization

A 732 bp Fmr1 fragment was synthesized from pGEM72-xFmr1 plasmid (46) containing the full-length *Xenopus* Fmr1 using primers: forward 5′-cgg cga tct aga CTC CAA TGG AGC TTT CTA C-3′; reverse 5′-gca taa gga gct cTC AAT TGC AGT CAC CCC AG-3′ (*Xba*1 and *Sac*1 sites are underlined in the primer sequence, respectively). The PCR product was digested with *Xba*1 and *Sac*1, gel purified and subcloned into *Xba*1 and *Sac*1 sites in pBluescript SK-vector (Stratagene). It was then linearized with *Xba*1 and *in vitro* transcribed in the presence of digoxygenin and T3 polymerase (Roche Molecular).

Whole-mount *in situ* hybridization was performed as described previously (44) using digoxygenin antisense probes for *Xenopus* Fmr1 and AP-2α (29). Embryos were then visualized using a Zeiss Axioskop and images were captured using a Hamamatsu color-chilled 3CCD camera system and exported to Adobe Photoshop.

Xenopus embryo manipulation

Embryos were obtained from adult *Xenopus laevis* by hormone-induced egg laying and artificial fertilization using standard methods and staged according to Nieuwkoop and Faber (1967). For ectodermal explants, full-length capped transcripts encoding ΔXAP2 (18), AP-2α (18) and chordin (32) were injected into two sites in the animal hemisphere of the one-cell embryos. After injection, the embryos were cultured in 4% Ficoll/1× modified Ringer's solution (MR) for 90 min and then transferred to 0.3× MR. Ectodermal explants were removed at blastula stage and cultured until stage 14 and then processed for northern blot analysis.

Northern blot hybridization

RNAs were isolated and analyzed by using denaturing methylmercury hydroxide RNA gels as described (46).

Probes for Fmr1, Fxr1 and EF1α were labeled with [^{32}P]dCTP by primer extension (Life Technologies, Inc.).

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Conflict of Interest statement. None declared.

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