Expanded View Figures

Figure EV1. Evolution of the *Fx-mir* cluster.
The location of *Fx-mir* miRNAs relative to each other and nearby protein-coding genes. Drawn to scale unless indicated. miRNAs deposited in miRBase are indicated for mouse, human, and all but *miR-509* for dog. For dog *miR-509* and all elephant *Fx-mir* family members, we assigned names to the genes indicated in the Ensembl database based on phylogenetic similarity to the mouse and human entries. Note that the paucity of *Fx-mir* family members for both dog and elephant is likely artifactual due to poorer miRNA characterization in these two taxa relative to mouse and human.

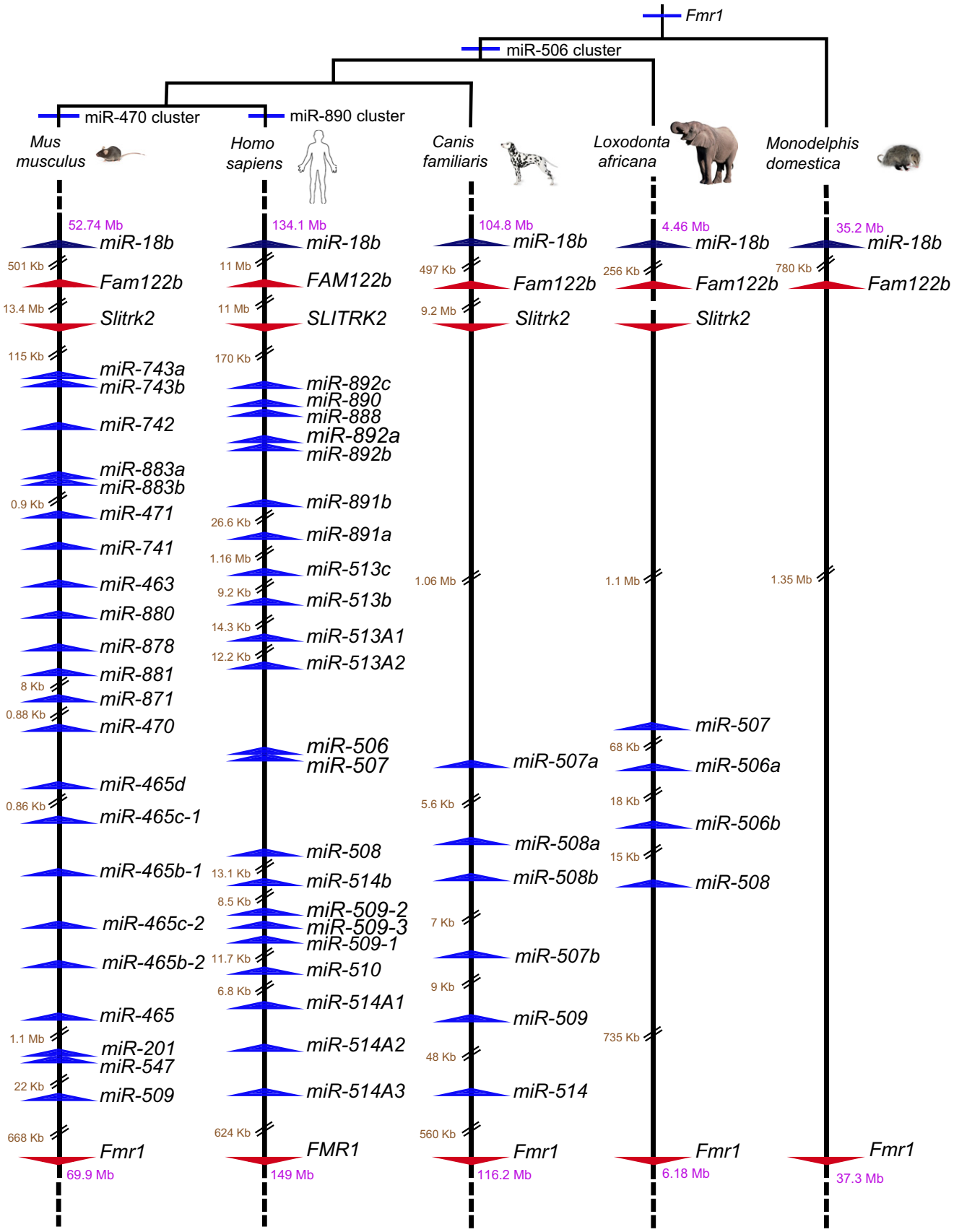
Fmr1
miR-506 cluster
miR-470 cluster
miR-890 cluster
Mus musculus
Homo sapiens
Canis familiaris
Loxodonta africana
Monodelphis domestica
52.74 Mb
miR-18b
501 Kb
Fam122b
13.4 Mb
Slitrk2
115 Kb
miR-743a
miR-743b
miR-742
miR-883a
miR-883b
0.9 Kb
miR-471
miR-741
miR-463
miR-880
miR-878
miR-881
8 Kb
miR-871
0.88 Kb
miR-470
miR-465d
0.86 Kb
miR-465c-1
miR-465b-1
miR-465c-2
miR-465b-2
miR-465
1.1 Mb
miR-201
miR-547
22 Kb
miR-509
668 Kb
Fmr1
69.9 Mb
134.1 Mb
miR-18b
11 Mb
FAM122b
11 Mb
SLITRK2
170 Kb
miR-892c
miR-890
miR-888
miR-892a
miR-892b
miR-891b
26.6 Kb
miR-891a
1.16 Mb
miR-513c
9.2 Kb
miR-513b
14.3 Kb
miR-513A1
12.2 Kb
miR-513A2
miR-506
miR-507
miR-508
13.1 Kb
miR-514b
8.5 Kb
miR-509-2
miR-509-3
miR-509-1
11.7 Kb
miR-510
6.8 Kb
miR-514A1
miR-514A2
miR-514A3
624 Kb
FMR1
149 Mb
104.8 Mb
miR-18b
497 Kb
Fam122b
9.2 Mb
Slitrk2
1.06 Mb
miR-507a
5.6 Kb
miR-508a
miR-508b
7 Kb
miR-507b
9 Kb
miR-509
48 Kb
miR-514
560 Kb
Fmr1
116.2 Mb
4.46 Mb
miR-18b
256 Kb
Fam122b
Slitrk2
1.1 Mb
miR-507
68 Kb
miR-506a
18 Kb
miR-506b
15 Kb
miR-508
735 Kb
Fmr1
6.18 Mb
35.2 Mb
miR-18b
780 Kb
Fam122b
1.35 Mb
Fmr1
37.3 Mb

Figure EV1.

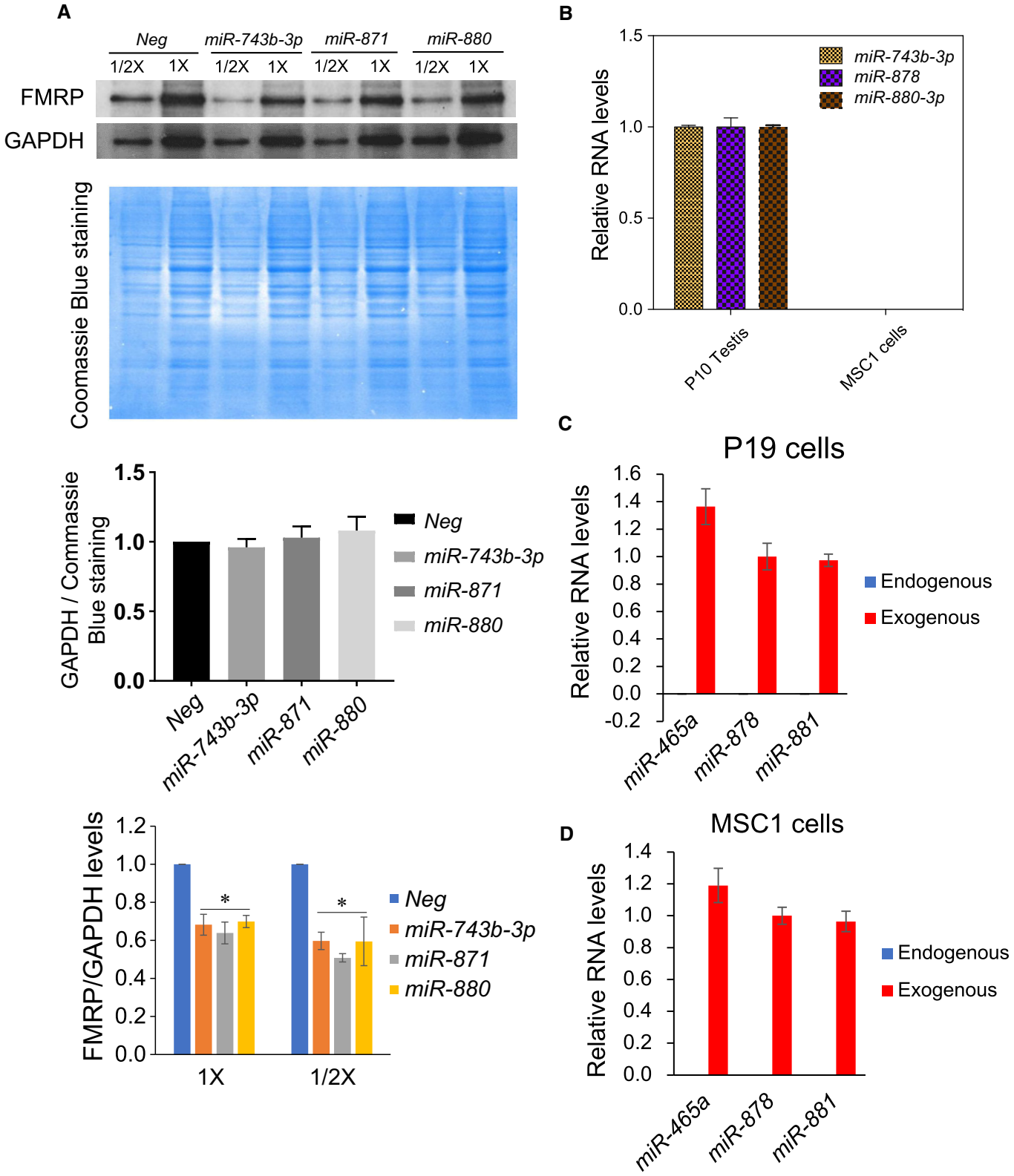

Figure EV2.

◀ **Figure EV2. *Fx-mir* family members directly regulate *Fmr1*.**

A *Fx-mir* miRNAs repress FMRP protein expression. Top: Western blot analysis of endogenous FMRP protein levels in P19 cells transfected with the indicated *Fx-mir* miRNA precursors or a negative-control miRNA precursor (Neg). The loading sample amounts are 15 μg (1 X) and 7.5 μg (1/2 X) total protein, based on the Bradford assay. The Coomassie Blue (CB)-stained blots are also shown to indicate loading. Middle: quantification of GAPDH/CB-stained protein ratio, showing that GAPDH protein levels are not statistically altered by expression of the miRNAs. Bottom: quantification of FMRP protein level normalized against GAPDH level.

B MSC1 cells lack detectable expression of *Fx-mir* miRNAs. The expression levels of *miR-743b-3p*, *miR-878*, and *miR-880-3p* in MSC1 cells and postnatal day (P) 10 mouse testes assayed by TaqMan-qPCR analysis. U6 snRNA levels were used for normalization.

C, D TaqMan-qPCR analysis of *miR-465a*, miR-*878*, and miR-*881* expression levels in P19 and MSC1 cells transfected with indicated *Fx-mir* miRNA precursors. U6 snRNA levels were used for normalization.

Data information: In (A–D), the bars in the histogram represent three independent biological replicates. Data are presented as mean ± SEM. *$P < 0.05$ (Student's *t*-test).

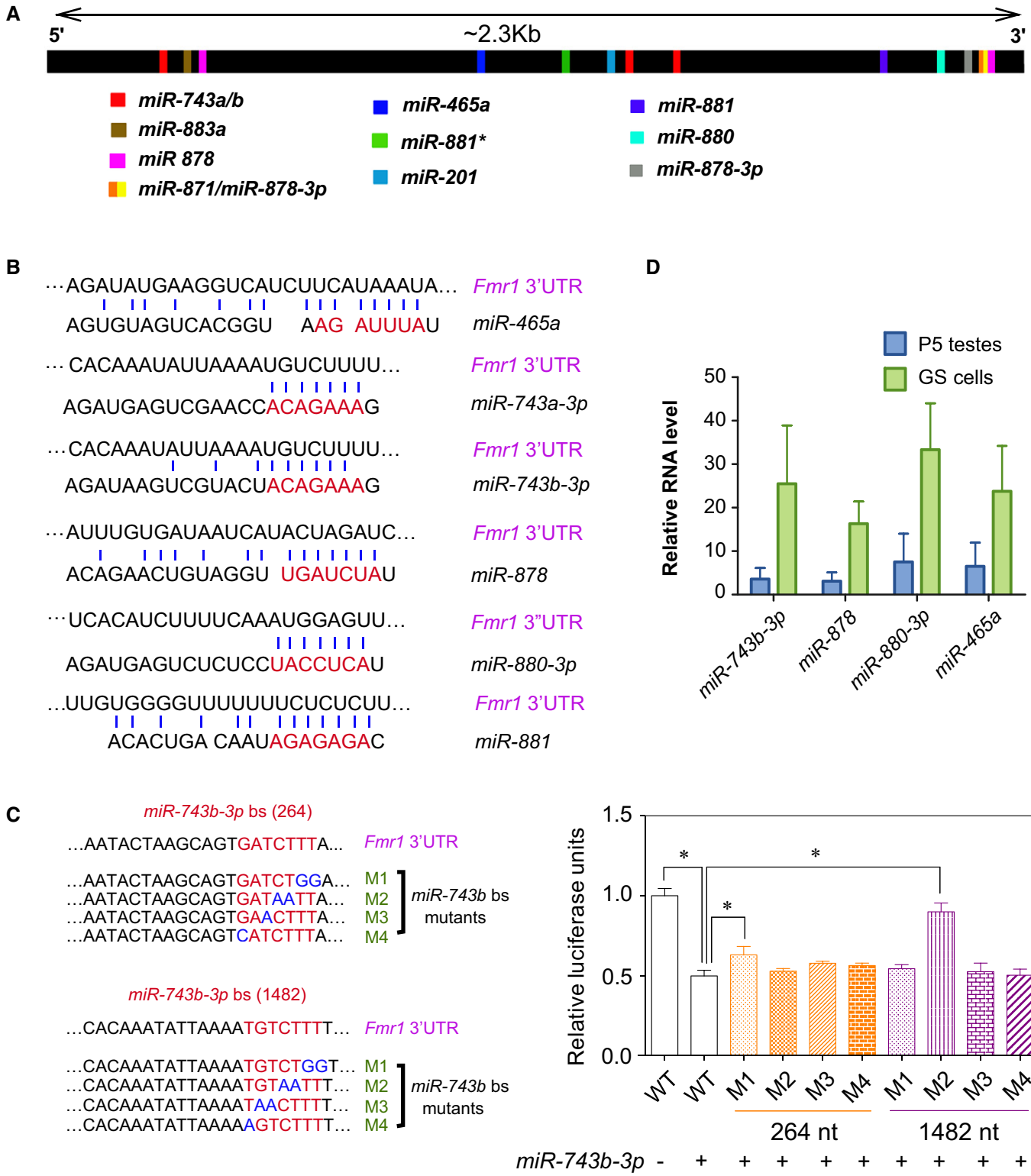

Figure EV3.

Figure EV3. *Fx-mir*-binding sites in the *Fmr1* 3′UTR.

A Location of *Fx-mir* miRNA-binding sites, as predicted by microRNA.org. Most *Fx-mir* miRNAs have only one predicted binding site. *miR-743b-3p* has three predicted binding sites (starting at positions 264, 1,368, and 1,482 nt relative to the beginning of the 3′UTR), while *miR-878* has two predicted binding sites (positions 357 and 2,225).

B Predicted base pairing between the indicated mature *Fx-mir* miRNAs with their predicted binding sites in the mouse *Fmr1* 3′UTR. The seed region of the miRNA is indicated in red. The *Fmr1* 3′UTR is presented in the 5′ to 3′ orientation while the miRNAs are in the 3′ to 5′ orientation.

C Left: Two predicted binding sites of *miR-743b-3p* in the *Fmr1* 3′UTR act redundantly. *miR-743b-3p*-binding site mutants in the 3′UTR of *Fmr1* are shown. Right: luciferase analysis of MSC1 cells co-transfected with (i) a miRNA precursor or a negative-control scrambled miRNA precursor and (ii) the pMIR luciferase reporter with the wild-type version of the mouse *Fmr1* 3′UTR or mutant versions with the indicated predicted miRNA-binding site mutations. The binding site mutants were generated for *miR-743b-3p* sites at 264 nt and 1,482 nt. A Renilla luciferase vector was co-transfected to normalize for transfection efficiency.

D Several *Fx-mir* miRNAs are expressed in GS cells. TaqMan-qPCR analysis of the expression of Fx-mir family members in GS cells. P5 testes are as a control. U6 snRNA levels were used for normalization.

Data information: In (C and D), the bars in the histogram represent three independent biological replicates. Data are presented as mean ± SEM. *$P < 0.05$ (Student's *t*-test).
Source data are available online for this figure.

Figure EV4. Expression pattern of selected *Fx-mir* family members.

A *Fx-mir* miRNA levels in different adult rat tissues assessed by TaqMan-qPCR analysis. U6 snRNA levels were used for normalization.

B *Fx-mir* levels in the testes of germ cell-deficient Klinefelter syndrome (XXY) mice. TaqMan-qPCR analysis of three XXY mice and three control littermate mice are presented. U6 snRNA levels were used for normalization.

C qPCR analysis of germ cell, Leydig, and SC marker genes in cell fractions isolated from mouse testis. The mRNA values were normalized to L19, and the expression values from total testis are considered as 1.

D *Fx-mir miRNA* levels in total mouse testis and different testicular cell fractions assessed by TaqMan-qPCR analysis. U6 snRNA levels were used for normalization. SgPr, primitive type A spermatogonia; SgA, type A spermatogonia; SgB, type B spermatogonia; SpPr, preleptotene spermatocytes; SpLZ, leptotene and zygotene spermatocytes; SpPaJ, pachytene spermatocytes from juvenile mice at postnatal day 18; SpPaA, pachytene spermatocytes from adult mice; SdR, round spermatids; SdE, elongated spermatids and residual bodies.

Data information: In (A–D), the bars in the histogram represent three independent biological replicates. Data are presented as mean ± SEM.

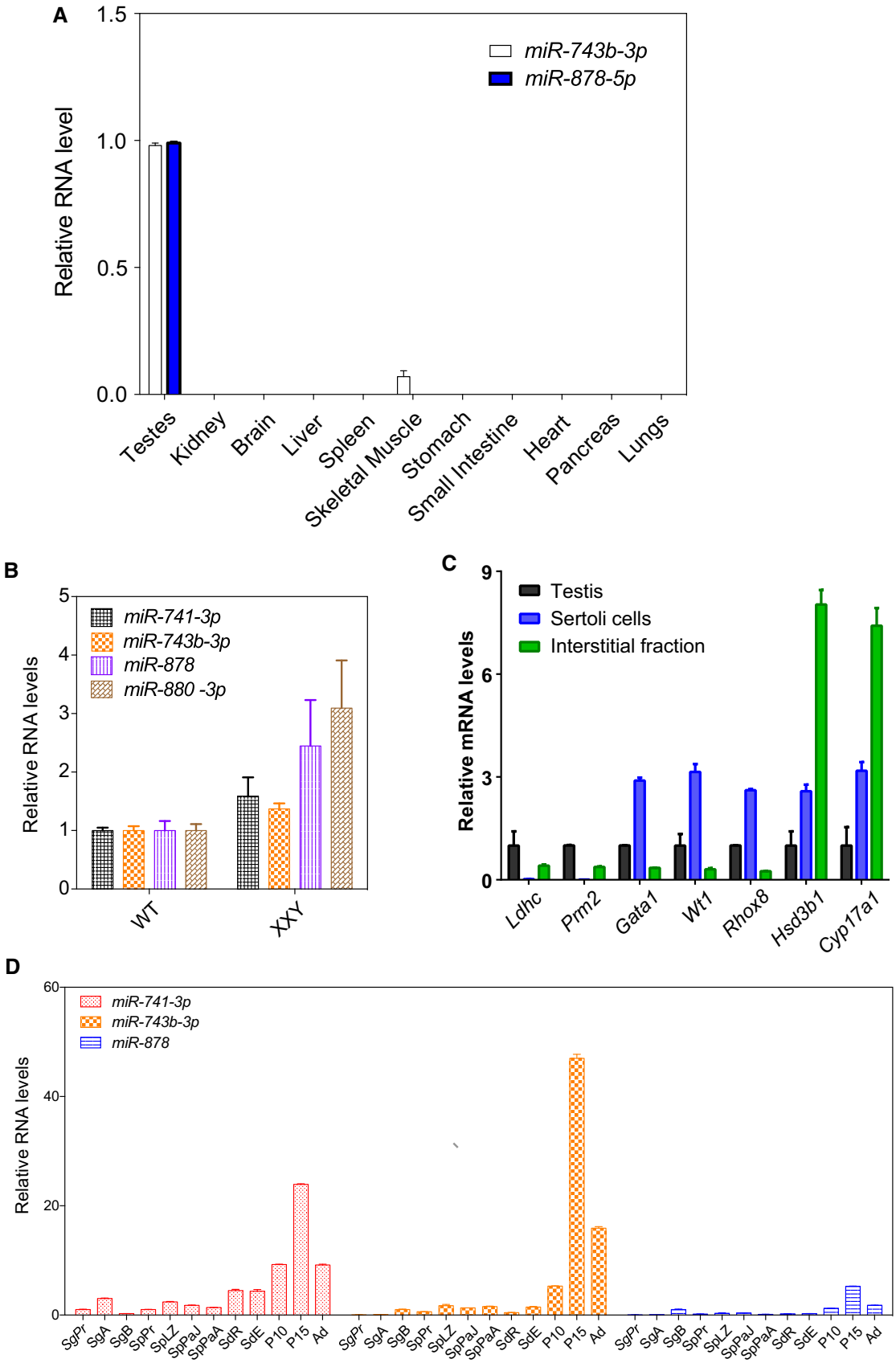

Figure EV4.

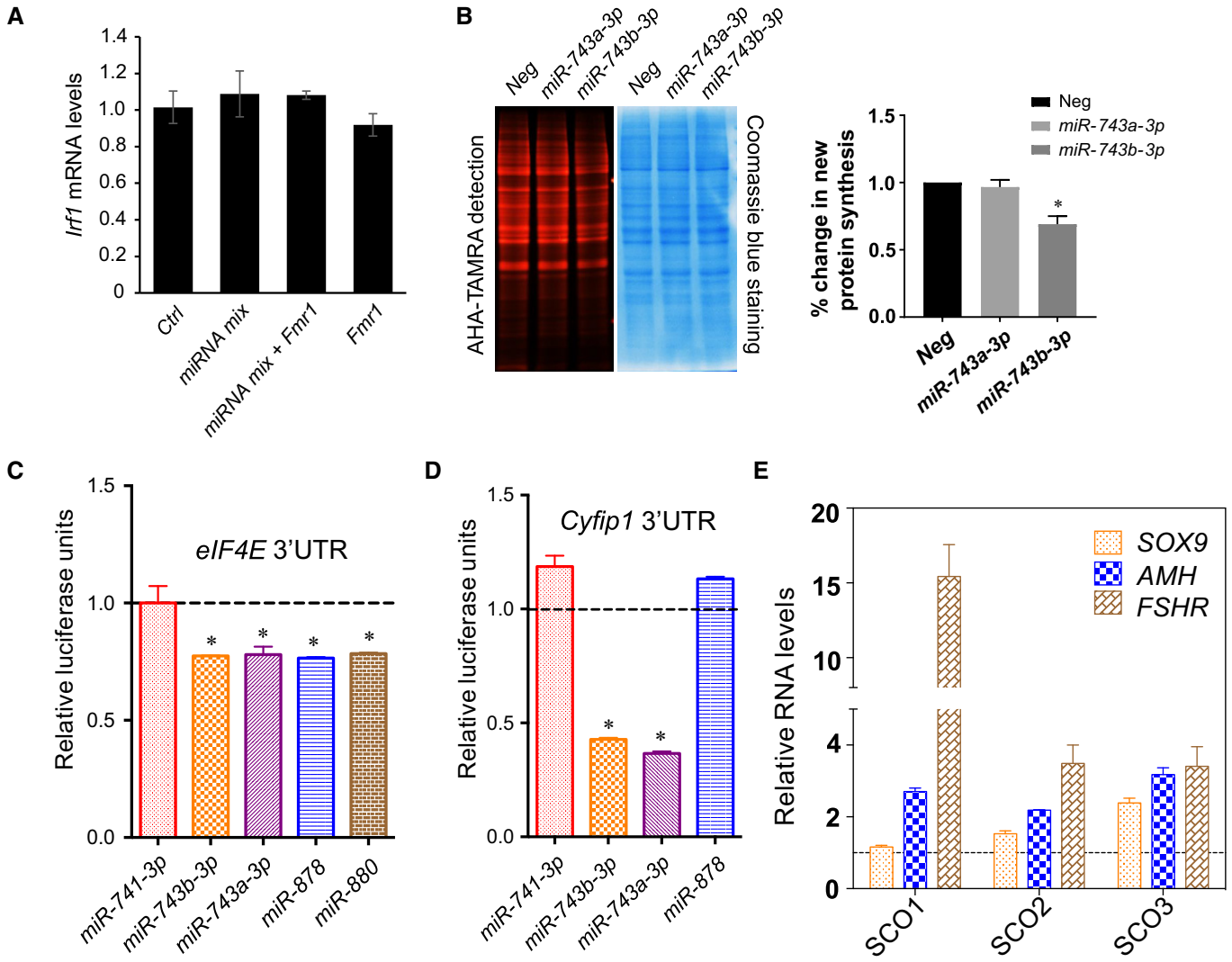

Figure EV5. *Fx-mir* miRNAs regulate factors that form a translation regulatory complex.

A qPCR analyses of *Irf1* in P19 cells transfected with Fx-mir miRNA mix and/or *Fmr1* overexpression (OE) vector. The mRNA values were normalized to L19. All values are relative to P19 cells co-transfected with a scrambled miRNA precursor and OE empty vector, which is set to 1.

B *miR-743b-3p* reduces proteins synthesis. Left: P19 cells were transfected with negative-control miRNAs or the indicated *Fx-mir* precursor miRNAs 48 h prior to the addition of Click-iT AHA in the culture medium. Newly synthesized proteins labeled with Click-iT AHA were conjugated with the TAMRA and detected using 532 nm excitation. The gel was then stained with Coomassie Blue to assay protein loading. Right: % change in new protein synthesis.

C *Fx-mir* miRNAs target *Eif4e*. Luciferase analysis of P19 cells co-transfected with pMIR luciferase reporter harboring mouse *Eif4e* 3′UTR and the indicated *Fx-mir* miRNAs or a scrambled miRNA precursor that served as control. *miR-741-3p* was used as a control to demonstrate specificity, as it does not have a predicted binding site in *Eif4e 3′UTR*. A Renilla luciferase vector was co-transfected to normalize for transfection efficiency.

D *Fx-mir* miRNAs target *Cyfip1*. Luciferase analysis of P19 cells co-transfected with pMIR luciferase reporter harboring mouse *Cyfip1* 3′UTR and the indicated *Fx-mir* miRNAs or a scrambled miRNA precursor that served as control. *miR-741-3p* and *miR-878* were to demonstrate specificity, as they do not have binding sites in *Cyfip1* 3′UTR. A Renilla luciferase vector was co-transfected to normalize for transfection efficiency.

E SCO patient testes express elevated levels of Sertoli cell marker genes. qPCR analysis of *SOX9*, *AMH*, and *FSHR* (SC marker genes) in SCO patient samples was performed. The mRNA values were normalized to GAPDH, and the expression values from controls are considered as 1.

Data information: In (A–E), the bars in the histogram represent three independent biological replicates. Data are presented as mean ± SEM. *$P < 0.05$ (Student's *t*-test).