Nuclear localization of folate receptor alpha: a new role as a transcription factor

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Folic acid (FA) has traditionally been associated with prevention of neural tube defects; more recent work suggests that it may also be involved in the prevention of adult onset diseases. As the role of FA in human health and disease expands, it also becomes more critical to understand the mechanisms behind FA action. In this work we examined the hypothesis that folate receptor alpha (FR α) acts as a transcription factor. FR α is a GPI-anchored protein and a component of the caveolae fraction. The work described here shows that FR α translocates to the nucleus, where it binds to *cis*-regulatory elements at promoter regions of *Fgfr4* and *Hes1*, and regulates their expression. The FR α recognition domain mapped to AT rich regions on the promoters. Until this time FR α has only been considered as a folate transporter, these studies describe a novel role for FR α as a transcription factor.

Traditionally folic acid (FA)¹ has been associated with prevention of neural tube defects; however more recently FA has been associated with the prevention of adult onset disease, such as Alzheimer's disease, dementia, neuropsychiatric disorders, cardiovascular diseases, and cerebral ischemia (reviewed in ref. 2). Cellular uptake of folate is mediated by specific carriers or receptors, including FR α (folate receptor alpha; also known as Folr1 and Folbp1)³, proton-coupled folic acid transporter (PCFT), and reduced folic acid carrier (RFC) (see ref. 4 for review). FR α , a GPI-anchored protein⁵ is critical for embryonic development⁶. Disruption of both FR α alleles in mice results in pups with a range of malformations and is lethal to the embryos at the time of neural tube closure⁶. FR α is one of the components of the caveolae fraction⁷, which includes EGFR⁸, caveolin-1 (Cav-1)⁹, the β subunit of heterotrimeric GTP binding protein (G β) and protein kinase C α subunit (PKC α)². Cav-1⁹ and EGFR¹⁰ act as transcription factors by binding to *cis*-regulatory elements of downstream target genes. EGFR binds to the promoters of *cyclin D1*, *iNOS*, *B-Myb*, *Aurora-A*, *thymidylate synthase*, *COX-2*, *c-Myc*, and *BCRP* which are involved in tumorigenesis, chromosome instability, and chemo-resistance¹¹. Cav-1 binds to the promoters of *cyclin D1* and FR α ⁹, *IGF-1* receptor¹², *BRCA1*¹³. This study examines a possible role of another caveolar protein, FR α as a transcription factor for key developmental genes.

Previous data from our lab^{2,14,15} demonstrated that FA remodels chromatin structures¹⁵. A second mechanism of FA action may be through FR α translocating to the nucleus and acting as a transcription factor. Bozard and colleagues¹⁶ reported the presence of FR α on the plasma membrane, the nuclear membrane and within endosomal structures; however the relevance of nuclear FR α was not determined. In this work we tested the hypothesis that in response to FA, FR α translocates to the nucleus and acts as a transcription factor.

To test the role of FR α as a transcription factor we examined nuclear localization in cell lines and interaction of FR α with two candidate genes *FGFR4* and *Hes1*. These candidate genes were chosen because in our previous studies, working with neural stem cells from Pax3 mutant (also known as or *Splotch* (*Sp*^{-/-})) mouse embryos, we found that FA up-regulates *Fgfr4* and *Fgfr4* receptor protein² and increases levels of *Hes1*¹⁴.

Results

Nuclear localization of FR α . To test the hypothesis that FR α translocates to the nucleus, a time course (0 min, 15 min and 30 min) for FR α nuclear localization was performed in DAOY cells treated with FA. The results of FR α immunoblots using mouse monoclonal antibody on nuclear extracts (Fig. 1a, b) showed that FR α

Figure 1 | Nuclear localization of FRα. (a) Nuclear extracts from DAOY cells treated with FA (200 μg/ml) for zero, 15 and 30 min at 37°C were subjected to immunoblotting using monoclonal anti-FRα (recognizing a 38 kDa band) and polyclonal anti-pRB (recognizing 110 kDa band) (see Supplementary Fig S1). (b) Ratio of FRα/pRB average band intensity (densitometry data is an average + SEM of three experiments). (c) Subcellular fractions from DAOY cells not-treated or treated with FA (200 μg/ml) for 30 min were immunoblotted with NCAM, N-cadherin, ICAM-1, vimentin, hsp90, pRB, H3 and FRα (rabbit polyclonal) antibodies. The FRα polyclonal antibody is made against an epitope corresponding to amino acids 1-257 representing full length FR α of human origin. This antibody is reported to recognize multiple types of FR, α, β and perhaps γ. Rabbit IgG was used as a negative control. M, membrane enriched; C, cytosolic enriched; P, insoluble cytoskeletal enriched; N, nuclear enriched; CB, chromatin bound fraction. The data above is a representative example of 5 different western blots. (d) The data is the average of 5 different western blot experiments +/− standard error mean. The ratio of average band intensity of FRα (42 kDa and 38 kDa) to subcellular fraction markers: FRα/ICAM-1; FRα/hsp90; FRα/vimentin; FRα/pRB; FRα/H3 was determined using densitometry. Statistical significance was calculated using Student's t test. (e) The data in “d” is presented as total non-nuclear fraction comprising of membrane, cytosol and insoluble cytoskeletal pellet, and total nuclear fraction comprised of nuclear and chromatin bound fractions. Statistical significance was calculated using Student's t test. (f) DAOY cells were grown in 8 well chamber slides in DMEM with 10% FBS for 24 h, then switched to serum free media in the absence or presence of FA (200 μg/ml) for 30 min at 37°C and immunostained using FRα monoclonal antibody and polyclonal pRB antibody and subjected to confocal microscopy. Secondary antibodies were donkey anti-rabbit Cy3 (red) and donkey anti-mouse Alexa488 (green). Yellow signals indicate co-localization of FRα (red) and pRB (green) in the nucleus (also stained blue with DAPI). The data is a representative of five separate experiments.

translocates to the nucleus within 15 min of FA incubation. It is to be noted that a very faint band of immunoreactivity for FRα (38 kDa band) was present in the nucleus even in the absence of FA.

To study FRα distribution in the absence and presence of FA (30 min), we isolated different subcellular fractions of DAOY cells—membrane, cytosol, cytoskeletal, nuclear and chromatin enriched fractions and performed western immunoblots (Fig. 1c) using FRα antibody along with antibodies against subcellular markers NCAM, N-cadherin and ICAM-1 (for membrane enriched fraction), hsp90 (for cytosolic enriched fraction), vimentin (for cytoskeletal enriched fraction), pRB (for nuclear enriched fraction) and histone H3 (for chromatin bound fraction). The ratio of the average band intensities of the two immunoreactive bands of FRα (42 kDa and 38 kDa doublet) with the marker of individual subcellular fraction (FRα/ICAM-1, FRα/hsp90, FRα/vimentin, FRα/pRB, and FRα/H3 bands) were determined using densitometry (Fig. 1d). It is to be noted that all the membrane markers used here also showed strong immunoreactivity in the nuclear enriched fraction. The hsp90 immunoreactivity was highest in the cytosolic enriched fractions (C) and the vimentin antibody cross-reacted with the insoluble cytoskeletal pellet (P). The pRB immunoreactivity was highest in the nuclear enriched fractions (N) and histone H3 antibody immunoreacted with the chromatin bound fraction (CB). In the absence of FA, FRα was predominantly

present in the cytosolic fraction whereas in the presence of FA, the FRα (42 kDa band) appeared to translocate to the non-nuclear fraction (membrane, cytosol and insoluble cytoskeletal pellet) and the 38 kDa band to the nucleus. In the nucleus the FRα (38 kDa band) appeared to be present in the chromatin bound fraction in the presence of FA. When the data in Fig. 1e is presented as total non-nuclear fraction (membrane + cytosol + insoluble cytoskeletal pellet) and nuclear fraction (nuclear + chromatin bound) we observe that even in the absence of FA, FRα is present in the nucleus and in the presence of FA, there is a significant increase in the translocation of FRα to the nuclear fraction.

Presence of FRα in the nucleus was further confirmed by confocal microscopy in DAOY cells (Fig. 1f). pRB was chosen as a nuclear marker. Increased co-localization of FRα and pRB was observed in the presence of FA. These results suggest the following: (i) In the absence of FA, there is a more FRα in the cytosolic fraction; (ii) Upon FA treatment, FRα is distributed significantly to the non-nuclear membrane fraction as well to the nuclear enriched and chromatin bound fractions; (iii) Of the two immunostained FRα-42 kDa and 38 kDa bands, the 42 kDa band seems to translocate to the membrane enriched fraction in the presence of FA. Although both 42 kDa and 38 kDa bands of FRα appear to translocate to the nucleus, only the 38 kDa band translocates

Figure 2 | Activation of Pax3 downstream target genes by FR α . FR α -pcDNA3 or control pcDNA3 (0.2 ng/well) constructs were co-transfected with *Hes1* promoter-luciferase construct (a), human *FGFR4* promoter-luciferase construct P-535/+99 (b) or a control *PGL3* (promoter-less luciferase gene) into DAOY cells. FA (200 μ g/ml) was added 24 h post transfection. Luciferase activity was assayed 48 h post-transfection. *pRLnull* (5 ng/well) was used as a transfection control in all wells. For both *Hes1* promoter luciferase and *FGFR4* promoter-luciferase construct P-535/+99 FR α significantly increased promoter activity, with the highest increases observed in the presence of FA. Experiments were performed in quadruplicate with each data point in duplicate. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ (Student T-test).

significantly to the chromatin bound fraction in the FA treated cells.

FR α binds to *cis*-regulatory elements of gene promoters. The above studies suggested that FR α translocates to the nucleus and in the presence of FA, it is enriched in the chromatin bound fraction. To determine whether FR α activates *FGFR4*, *FGFR4* promoter-luciferase constructs P-535/+99 from human *FGFR4* promoter¹⁷ were transiently transfected into DAOY cells, treated or not treated with FA. *FGFR4* promoter-luciferase reporter activity increased ($p < 0.05$) in the presence of FA (Fig. 2a). FR α significantly increased *FGFR4* promoter-luciferase in the absence of FA ($p < 0.001$), with a further increase in the presence of FA ($p < 0.0001$). This demonstrates that FR α activates *FGFR4* promoter by binding to *cis*-regulatory elements.

To establish if FR α activates other FA modulated genes by binding to *cis*-regulatory regions, mouse *Hes1* promoter-luciferase construct¹⁸ was co-transfected with FR α expression vector into DAOY cells treated or not treated with FA. *Hes1* is a Pax3 downstream target gene¹⁸, FA increases *Hes1* mRNA and protein levels¹⁴. *Hes1*-promoter-luciferase reporter activity increased ($p < 0.05$) in response to FA. FR α significantly increased ($p < 0.001$) *Hes1*-promoter-luciferase without FA (Fig. 2b), with a further increase with FA treatment. These data indicate that FR α transcriptional activation is not limited to *FGFR4*.

To confirm FR α binding to *cis*-regulatory elements of *Hes1* and *Fgfr4* promoters in intact embryos, chromatin immunoprecipitation (ChIP) experiments were performed using the lower lumbar region of the neural tube from *wild-type* (WT) mouse embryos (E10.0, 30 somite stage), an area where both of these genes are expressed. FR α bound to *cis*-regulatory regions of *Hes1* and *Fgfr4* promoters *in vivo* (Fig. 3a).

FR α binds to AANTT consensus sequence on *Hes1* or *FGFR4* promoter. To identify putative FR α binding sequences in *Hes1* and *Fgfr4* promoters, ³²P-labeled oligonucleotides were made from appropriate promoter regions and EMSA was performed using affinity-purified GST-FR α fusion protein (Fig. 3b, c). GST-FR α fusion protein bound the *Hes1* oligonucleotide 5'-AAATTAT-TTTTTTTTGCCTGAAG-3' which had AANTT or NAAAAN and/or NTTTTN sequences. When this sequence was mutated as in 5'-AACCCCTATTCCCTTTGCCTGAAG-3' there was no shift. Similarly, on the *Fgfr4* promoter the GST-FR α binding site mapped to AANTT or NAAAAN and/or NTTTTN in the

oligonucleotide 5'-CAAACAAACAAAAAGAAACAACAAAAA-ACTTTTTTA-3' and NTTTTN in the oligonucleotide 5'-ATA-AAAGCACAACTTTTTACAAAGTTTAAAGTTTTTT-3'. When the oligonucleotide sequence did not have the AANTT or TTNAA and NAAAAN consensus GST-FR α did not show a shift as in the case of 5'-CGTTCGCGTGCAGTCCGAGATAT-3'.

To further confirm the identity of FR α binding sites on *Hes1* and *FGFR4* promoters, AANTT sites were mutated on *Hes1* and *FGFR4* promoter-luciferase reporter constructs P-535/+99. Mutated constructs were transfected into DAOY cells as above. Luciferase activity did not increase with these constructs for either *Hes1* or *FGFR4* promoters (Fig. 4a). The results confirm that FR α binds *Hes1* and *FGFR4* promoters at AANTT or TTNAA and NTTTTN or NAAAAN sites.

Discussion

Previous work from our lab demonstrated that in the absence of functional Pax3, FA increased KDM6B, through up-regulation of *KDM6B* targeting micro-RNAs. This in turn altered H3K27 methylation marks on the promoters of Pax3 downstream targets, *Hes1* and *Neurog2*, and affected gene transcription¹⁴. Thus one mechanism of FA action is through remodeling of chromatin structures. In this study we have elucidated a second mechanism for FA action, through activation of FR α and its subsequent action as a transcription factor. A hypothetical model showing FR α internalization is presented in Fig. 4b. FR α is internalized in a caveolar structure as early endosome¹⁹. The endosome becomes increasingly acidic²⁰ and fuses with a lysosome²¹. In the lysosome FA is released²² and lysosomal GPI-specific phospholipase D²³ cleaves off the GPI anchor on FR α , which is then set free. Free FR α translocates into the nucleus where it binds to *cis*-regulatory elements of target genes and directly activates transcription. This model does not take into account FR α recycling and it is still unclear exactly how FR α translocates into the nucleus.

Binding to *Hes1* and *Fgfr4* promoters suggests FR α involvement in stem cell maintenance and skeletal muscle differentiation, respectively. The list of putative FR α targets shown in Table 1, suggests that FR α may be involved in regulating a plethora of developmental genes involved in myogenesis, skeletogenesis, cell mobility, neural crest cell migration, cranial and cardiac neural crest formation, hair morphogenesis, oligodendrogenesis, spermatogenesis²⁴, melanogenesis²⁵, and epithelial to mesenchymal transformation²⁶. A survey of the promoter regions of *c-Met*, *PDGF α* , *TGF β 2*, *MITF*, *N-CAM*, *c-RET*, *MyoD* and *Tyrrp-1* indicates that the FR α binding motif AANTT

Figure 3 | FR α binds to murine *Hes1* and *Fgfr4* promoter *cis*-regulatory elements. (a) ChIP assays were performed using E10.0 (30 somite) lumbar neural tube. Anti-FR α polyclonal antibody was used to immunoprecipitate (IP) the protein–DNA complex. This antibody is made against epitope corresponding to amino acids 1–257 representing full length FR α of human origin. This antibody is reported to recognize multiple types of FR, α , β and perhaps γ . Primers used to amplify *cis*-regulatory elements in *Hes1* and *Fgfr4* promoters are shown in Supplementary Information Table 1. Rabbit IgG was used as an IP negative control. ChIP experiments were performed in triplicate using one lumbar neural tube region per assay with a total of $n=4$. (b) EMSA of binding reactions performed using GST-FR α fusion protein and 32 P-labeled double-stranded oligonucleotides. Mouse *Hes1* oligo #1 (with AANTT): 5'-AAAAAATTATTTTGTGCGTGAAG-3'; Mouse *Hes1* oligo #2 (mutant AAA>CCC): 5'-AAACCCTATTTCCCTTTGCGTGAAG-3'; (c) Mouse *Fgfr4* oligo #3 (with AANTT): 5'-CAAACAAACAAAAAGAAACAACAAAAAACTTTT-3'; Mouse *Fgfr4* oligo #4 (with AANTT): 5'-ATAAAAGCACAACTTTTACAAAGTTTAAAGTTT-3'; Mouse *Fgfr4* oligo #5 (deletion mutant without AANTT) 5'-CGTTCGCGTGCAGTCCGAGATAT-3'. The arrow shows GST-FR α binding to oligonucleotides which have the AANTT sequence.

Figure 4 | FA does not activate *Hes1* or *FGFR4* promoter luciferase activity when the FR α consensus sequence (AANTT) is mutated. (a) AA>CC substitution mutations were made at the putative FR α binding sites on *Hes1* or *FGFR4* promoters (mutated sites are shown in the supplemental information). *Hes1* promoter-luciferase containing plasmid or plasmids containing mutated sequences (20 ng) were transiently co-transfected with FR α -*pcDNA3* or *pcDNA3* vector control into DAOY cells treated or not treated with FA and luciferase assays were performed. Renilla luciferase plasmid, pRLnull (5 ng/well) was simultaneously transfected as an insertional control for transfection efficiency. FR α -*pcDNA3* values were expressed as the activity of Firefly-luciferase, minus values obtained for control *pcDNA3* transfection. Experiments were performed in triplicate with each data point in duplicate; * $p<0.05$; ** $p<0.001$; *** $p<0.0001$ (Student T-test). (b) A hypothetical working model depicting FR α as a transcription factor. FR α , a GPI-anchored protein, gets internalized in a caveolar structured early endosomes, which undergo acidification and subsequent fusion with lysosomes. GPI-specific phospholipase D cleaves FR α from its GPI-anchor. FR α is released and translocates to the nucleus via an unknown mechanism(s) where it binds *cis*-regulatory elements of different gene promoters.

Table 1 | Promoters of genes contain Pax3 and FR α binding sites in close proximity to each other

Gene Promoter	Accession number	Promoters of Pax3 downstream target genes containing the Pax3 and FR α binding sites in close proximity to each other
Human <i>MITF</i>	AF034755.1	1. GT[GAAAAATAAAAAAGT]ATTACAGTAAAGAG 2. GAAC[GTITTTTTTT]ACATGCATAACTAATTAGCTTAGGTTATTATAA 3. AACATTATTGAACCTTCAAAATTTTAA[GCTTAA 4. TGAATTACATTTATTTTTAAACAGAACTCTTTT[CTTTTTAA]GTGAATAGT 5. CTACTAATGTAATAATCGTGTTT[TCAAAAAT]CTCAATTTTAAATTCIAATATGC 1. GATATTTTAGTGAAACGTT[TTTTTG]TCATAGATTGAATAAAACTT 1. GGATTACAGGTTGAGCCCTTAGCCTGGCCCTTGAACTTTTTTTTTAA[CATGGATTTTTAG 2. CCAGAC[CTTTTTT]CAAGGAGTAAATG 1. TCTG[CTTTTTT]GATTACGTA 2. AATAGATTAGGAAGCGT[CAAAAAA]TACA 3. CTCGAAAGGCTACAGGCGTCT[GTITTTT]TTTCTTCCATTACTAG 4. CGTCT[GTITTTT]TTCTTCCATTACTAGTTGCAAGGCATGCTTCAAAAAAGCAG
Human N-CAM Human c-RET	BE019307: AA364465 NM_020630.4	1. CAGGCTAAT[GA AAAA]CAAC 1. CTGCAGTGCTCTGCTGAAGGA[TTAAAA]AAAAAAAT 2. TGCAC[CTTTTTT]TACCCCTTGCCCT 3. TTAACCCCTTGATAATCCCTCAAG[GA AAAA]CAAAA[CAAAAA]CCC 4. CAAAAA[CA AAAA]CCCATCATGTCATATTGGACT 5. TG[ATTTT]GGGGGATCGT[TTGT]CCC 1. TCTTGATTAT[CTTTTTT]TTGGGCT 2. ACAT[AA TTTT]TGAATTAAT
Murine c-ret	AY255629.1	3. AGA[GTITTTT]CAATGTGTGATAAT[GA AAAA]CTGTAT[TCAA]GGGA 4. CCAGTGAGCAGATC[ATTTTTT]TACGGGCTGTTATCC 1. AAATAATTACAGCTAGTATTACAAAGCAAGCGCTGCATGCCAGGTTACACATACTGCTAACATGCTCTACTTTTTCACAC 1. GAGTCCAGGACAGCCAGGCTATACAGAGAAACCCCTGCTTT[GA AAAA]CAAAAAC[CA AAAA]C 1. CTTTACC[TTGTT]CCCTC[CTTTTTT]CAA 2. AAAT[ATTTT]CAATGAACCTTATTATACA 3. ACATAATAAACCT[TTCAA]CTGCTCAGTA[GTITTTT]CTT 4. ATA[TTTTT]CAATGAACCTTATTATACA 1. GCTTAAIGTGAATAATCAATCCATAT[TA AAAA]TG 2. GCTAATCAATAGCTGCTCTCCCTGGGTGGATC[GTITTT]CAG 1. CTTAATTATAGATATATCCCTAGTTGT[CTTTTTGGTTGTTAAAT]TAC 2. TTTGGTTATTTTATAT[AA TTTT]GTAT 3. ATCAGTGCTGACCTTTTCTTAAGAC[TTTAA]CCATCACAAGGAA 1. GAA[AACTT]CTCCACCTAGAAAGT[TTACCTT]TGC
Murine <i>TGFβ2</i> Human <i>PDGFRα</i>	NW_001030678.1 AJ278993.1	
Human <i>TGFβ2</i>	NM_009367.3	
Human <i>FGFR4</i> Murine <i>Fgfr4</i> Human <i>HES1</i>	Y13901.1 NT_039589.8 NM_005524.3	
Chicken <i>MyoD</i>	L34006.1	
Murine <i>Myrpl</i>	AF087673.1	
Human <i>C-MET</i>	Z26936.1

or NTTTTN and/or NAAAAAN map close to Pax3, a transcription factor and multifunctional regulatory protein, expressed early in embryogenesis, binding sites, which map to ATTA, GTTCC, TAAT, CCTTG, CAAGG, GTTAT, TATTG, GTGTGA, and CAGTGT²⁷. These observations suggest that FR α and Pax3 might appear as a complex regulating target genes synergistically. However, up-regulation of *Hes1* or *Neurog2* in FA-rescued *Sp*^{-/-} embryos¹⁴ suggests that FR α may also have a role independent of Pax3. Future work shall test this hypothesis.

The observation that FR α acts like a transcription factor is relevant to our understanding of the mechanisms of FA action during development and has significant implications for disorders associated with FA deficiency and FR α misregulation and for management of human cancers which express FR α as a tumor antigen. FA has been associated with the prevention of adult onset diseases (reviewed in ref. 2). In some of these cases FA deficiency may not be the problem. The issue may be an inability to respond to FA due to misregulation of FR α . Cazzaniga and colleagues²⁸ compared levels of serum folate and assessed differences in folate binding ability with primary fibroblast cultures, from Alzheimer's disease (AD) patients and age-matched healthy subject. Circulating folate was significantly lower in AD patients, whereas folate binding to fibroblasts was significantly higher, possibly due to enhanced expression of FR α in AD fibroblasts. Cerebral folate transport deficiency is an inherited brain-specific folate transport defect caused by mutations in the folate receptor 1 (*FOLR1*) gene which codes for FR α ²⁹. This disorder generally has a late infantile onset and symptoms include progressive movement disturbance, psychomotor decline, epilepsy and disturbed brain myelination, as well as a depletion of white matter choline and inositol³⁰. Grapp and colleagues²⁹ reported that whereas WT FR α was localized in the plasma membrane, in cerebral folate deficiency FR α mutants were mistargeted to intracellular compartments. The data presented in this paper provides relevant insight to these clinical situations. If FA interaction with FR α is misregulated, key transcriptional events may be affected. This in turn can lead to a series of developmental consequences or to adult onset disease associated with FA levels. Further work needs to be done to examine direct transcriptional activation of FR α responsive genes by FR α and its' role in these multifactorial diseases.

Another clinical role for FR α is in cancer, where it is recognized as a tumor antigen/biomarker³¹. Because of this, diagnostic and therapeutic methods which exploit FR α are being developed for cancer treatment, including the use of folate-drug conjugates³¹. The knowledge that FR α acts as a transcription factor can be exploited to target FA-siRNA or FA-drug conjugates to silence downstream targets in appropriate cancers. For instance, two Pax3 downstream targets *c-MET* and *MITF* are associated with melanoma³². MET promotes the melanoma phenotype by stimulating migration, invasion, resistance to apoptosis, and tumor cell growth. PAX3 mediates MET induction through direct activation of the gene, and indirect regulation through MITF. FA-drug conjugates exploiting the proximity of Pax3 and FR α binding sites could potentially silence *c-met* and/or *MITF* expression.

In summary, our study shows that FR α is localized in the nucleus, where it binds to *cis*-regulatory elements (AANTT or TTNAA and NTTTTN or NAAAAAN) on FA modulated genes and activates their transcription. This novel role of FR α as a transcription factor provides insight into developmental mechanisms associated with FA responsiveness. It also provides an exciting new avenue to explore for treatment of diseases associated with FA deficiency, FR α misregulation and cancers which express FR α as a biomarker.

Methods

Antibodies and reagents. pRb antibody-rabbit polyclonal; 1:1000, Cell Signaling Technologies; Ser807/811, FR α antibody-rabbit polyclonal; 1:500. Santa Cruz: sc-28997, NCAM (Santa Cruz; sc-1507), N-Cadherin (Santa Cruz; sc-1502), I-CAM-1 (Santa Cruz; sc-1510), vimentin (BD Pharmingen; 550513); pRB (Cell Signaling Technologies, Ser807/811 rabbit polyclonal; 1:1000); Histone H3 (Cell Signaling

Technologies, 9701, rabbit polyclonal 1:1000); Mouse monoclonal anti-FR α antibody from Lifespan biosciences (cat # LS-C23683). Donkey anti-rabbit IgG-HRP (sc-2305), and Donkey anti-mouse IgG-HRP (sc-2306) were from Santa Cruz. DAPI was purchased from Sigma. Secondary antibodies for immunostaining procedures were donkey anti-rabbit Cy3 (red) (1:200) and donkey anti-mouse Alexa488 (green) (1:200). Primers and oligonucleotides for EMSA were from Operon. *Wild type* (WT) C57BL/6J male and female mice were from Jackson Labs. For timed embryos, females and males were mated, the morning a vaginal plug was observed was noted as E0.5. Pregnant dams were euthanized by cervical dislocation with CO₂ inhalation, and moniliform uterine beads were removed at E10.5. Neural tubes were dissected out as described earlier³³. All animal experiments were approved by IACUC -Children's Hospital of Chicago Research Center, Chicago (Approval # Mayanil, IACUC ID: 13-001.0 09) and all experiments were performed in accordance with relevant institutional guidelines and regulations.

Statistical analysis. Values given are means $\pm$ SEM. Probabilities (p) were calculated with Student's unpaired t test using GraphPad Prism version 4.0. p values < 0.05 were considered statistically significant. One-way ANOVA with Bonferroni's multiple comparison tests were used for multiple comparisons between data.

Nuclear localization of FR α . DAOY cells were treated with FA (200 μ g/ml) for zero, 15 and 30 minutes at 37°C. For the western blots studies, the DAOY cells not-treated or treated with FA for 30 min were used. Subcellular fractions, membrane enriched, cytosolic, insoluble pellet cytoskeletal fraction, nuclear and chromatin bound fractions (30 μ g) were immunoblotted with antibodies against FR α (rabbit polyclonal, 1:500), NCAM (1:500); N-Cadherin (1:1000); I-CAM-1 (1:1000); vimentin (1:10,000); pRB (1:500) and Histone H3 (1:500). This rabbit polyclonal anti-FR α antibody is made against epitope corresponding to amino acids 1-257 representing full length FR α of human origin. This antibody is reported to recognize multiple types of FR, α , β and perhaps γ . The average band intensity of FR α /ICAM-1; FR α /hsp90; FR α /vimentin; FR α /pRB; FR α /H3 was determined using densitometry. For immunostaining, DAOY cells were plated and grown in DMEM with 10% FBS for 24 hours and then changed to serum-free media. FA (200 μ g/ml) was added to appropriate wells. The cells were allowed to grow for an additional 30 min. Cells were immunostained with anti-FR α (mouse monoclonal antibody; 1:100) and pRB (rabbit polyclonal; 1:100). This mouse monoclonal FR α antibody recognizes only the 38 kd band of FR α . Rabbit IgG was used as a negative control. Secondary antibodies were donkey anti-rabbit Cy3 (red) (1:200) and donkey anti-mouse Alexa488 (green) (1:200). Confocal microscopy was done with a Zeiss 510 META Confocal Laser Scanning Microscope.

Real time quantitative RT-PCR. Real time quantitative RT-PCR was done as described earlier²⁷. Primers and probes used in this study were designed using Primer Express software (PerkinElmer Life Sciences). Primers were synthesized by Operon Inc. probes were synthesized by MegaBases Inc (Refer Supplementary Information-Table 1 for primers).

Chromatin immunoprecipitation (ChIP) assays. ChIP assays using lumbar neural tube from WT embryos (E10.0) were performed as described earlier³⁴. PCR was performed with primers for murine *Hes1* and *Fgfr4* promoter regions (Supplementary Information-Table S1 for primers). All ChIP samples were tested for false-positive PCR amplification by sequencing the 200-bp amplified product to ascertain the specificity of FR α binding to *cis*-regulatory elements.

Analysis of *Hes1* and *Fgfr4* promoter activity. A 2.5 kb *Hes1* promoter-luciferase construct was provided by Dr. R. Kageyama, Institute for Virus Research, Kyoto University Kyoto, Japan. Human *FGFR4* promoter constructs were provided by Dr. Shereen Ezzat, Departments of Medicine, Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada. The FR α expression construct in *pcDNA3* was kindly provided by Dr. Asok Antony, Indiana University Medical School Indianapolis, IN, USA. *Renilla* luciferase plasmid, *pRL-null* (0.5 μ g) (dual luciferase system, Promega), was simultaneously transfected as an insertional control for transfection efficiency in all of the studies. DAOY cells were seeded at 5 $\times$ 10⁴ cells/60 mm diameter dish in DMEM supplemented with 10% fetal calf serum for 24 h prior to transfection. For co-transfections: human FR α cDNA and mouse *Hes1-Luc* promoter or human FR α cDNA and human *FGFR4-Luc* promoter were transfected into the cells using MegaTran 1.0 from OriGene; Cat#: TT200003. After 24 h, FA was added to the treatment wells. The cells were washed 3 times with phosphate-buffered saline (PBS) and lysed with Passive Lysis Buffer (PLB) 48 h post transfection. Luciferase activity was measured using a Vector 2 Luminometer (PerkinElmer Life Sciences). The AA>CC substitution mutations of the putative FR α binding sites on murine *Hes1* or human *FGFR4* promoters were made with the QuickChangeXL site-directed mutagenesis kit (Stratagene). *Hes1* promoter-luciferase containing plasmid or plasmids containing the mutated sequences (20 ng) were transiently co-transfected with FR α -*pcDNA3* or *pcDNA3* vector control into DAOY cells and luciferase assays were done as described earlier³⁴ using the Dual Luciferase kit from Promega. *Renilla* luciferase plasmid, *pRL-null* (5 ng/well) (Dual Luciferase System Promega), was simultaneously transfected as an insertional control for transfection efficiency.

Purification of GST-FR α fusion protein. GST-FR α fusion plasmid was kindly provided by Dr. Asok Antony. *Escherichia coli* was transformed with GST-FR α fusion

plasmid, and the cells were grown overnight in LB medium. GST-FR α fusion protein production was induced with 0.1 mM isopropyl-D-thiogalactopyranoside (Sigma) for 2 hr. Cells were pelleted and sonicated to release the protein in 50 mM Tris-HCl pH7.8 buffer. Supernatant containing GST-FR α fusion protein among other proteins was loaded onto glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). Unbound proteins were washed with 50 mM Tris-HCl pH7.8 buffer and eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 9.5. Eluted protein was concentrated using Centricon 10 (Amicon) as per the manufacturer's instruction.

Electro-mobility shift assays (EMSA). Probes were prepared for EMSA by annealing complementary oligonucleotides representing selected regions of murine *Hes1* and *Fgfr4* promoters, followed by 5'-end labeling with [γ - 32 P] ATP by T4 polynucleotide kinase. EMSA was done as described earlier¹⁸. Double-stranded oligonucleotide probes produced, covered the following regions: WT *Hes1* promoter oligo: 5'-AAAAAATTATTTTTTTTTCGCTGAAG-3' and mutant *Hes1* promoter oligo: 5'-AAACCCCTTATCCCTTTTTCGCTGAAG-3'; WT *Fgfr4* promoter oligo #1: 5'-CAACAAACAAAAAGAAACAACAAAAAAGTTTAA-3' WT promoter oligo #2: 5'-ATAAAAGCACAACTTTTACAAAGTTTAAAGTTT-3' and an oligo without AANTT sequence #3: 5'-CGTTCGCTGCAGTCCGAGATAT-3'. For shift assays 4 μ g GST-FR α fusion protein was pre-incubated at room temperature for 30 min with 4 M urea and EMSA reaction buffer (100 mM Tris-HCl pH7.5, 500 mM KCl, 6.5% glycerol, 50 mM pyrophosphate, 25 mM DTT in 2.5% Tween 20, 1 μ g/ μ l salmon sperm DNA) prior to addition of labeled oligonucleotides. For hot reactions GST-FR α fusion protein and radio-labeled oligonucleotides were added and pre-incubated for 30 min at room temperature. For cold reactions GST-FR α fusion protein and unlabeled oligonucleotides were added, and labeled oligonucleotides were added after 30 min. After pre-incubation, free DNA and DNA protein complexes were resolved in 6% polyacrylamide gels (these gels were pre-run at 1000 V and 5 mA for 16 h in the continuous cooling system) using 0.25 $\times$ TBE as the running buffer. Electrophoresis was performed at 1000 V and 25 mA for 2 h in a continuous cooling system. To visualize shifted bands, gels were dried at RT and transferred to Phosphor Imager Screens (Amersham Biosciences). Gels were exposed overnight at 4°C.

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Author contributions

V.B., K.S., C.S.M. designed research; V.B., E.S. and S.I. performed confocal microscopy, V.B., K.S. and T.T. performed EMSA and luciferase assays. V.B., X.I. and B.M.F. made promoter luciferase mutants. K.S. and T.T. purified GST-FR α fusion protein. V.B., G.X. and S.I. did statistical analysis. T.T., D.G.M. and C.S.M. coordinated the work. B.M.F. and C.S.M. interpreted the data and C.S.M. wrote the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

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