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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 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 (FRα) acts as a transcription factor. FRα is a GPI-anchored protein and a component of the caveolae fraction. The work described here shows that FRα translocates to the nucleus, where it binds to *cis*-regulatory elements at promoter regions of *Fgfr4* and *Hes1*, and regulates their expression. The FRα recognition domain mapped to AT rich regions on the promoters. Until this time FRα has only been considered as a folate transporter, these studies describe a novel role for FRα 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 FRα (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). FRα, a GPI-anchored protein[5] is critical for embryonic development[6]. Disruption of both FRα alleles in mice results in pups with a range of malformations and is lethal to the embryos at the time of neural tube closure[6]. FRα is one of the components of the caveolae fraction[7], which includes EGFR[8], caveolin-1 (Cav-1)[9], the β subunit of heterotrimeric GTP binding protein (Gβ) and protein kinase C α subunit (PKCα)[2]. Cav-1[9] and EGFR[10] 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 tumorogenesis, chromosome instability, and chemo-resistance[11]. Cav-1 binds to the promoters of *cyclin D1* and *FRα*[9], *IGF-1* receptor[12], *BRCA1*[13]. This study examines a possible role of another caveolar protein, FRα as a transcription factor for key developmental genes.

Previous data from our lab[2,14,15] demonstrated that FA remodels chromatin structures[15]. A second mechanism of FA action may be through FRα translocating to the nucleus and acting as a transcription factor. Bozard and colleagues[16] reported the presence of FRα on the plasma membrane, the nuclear membrane and within endosomal structures; however the relevance of nuclear FRα was not determined. In this work we tested the hypothesis that in response to FA, FRα translocates to the nucleus and acts as a transcription factor.

To test the role of FRα as a transcription factor we examined nuclear localization in cell lines and interaction of FRα 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 protein[2] and increases levels of *Hes1*[14].

Results

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

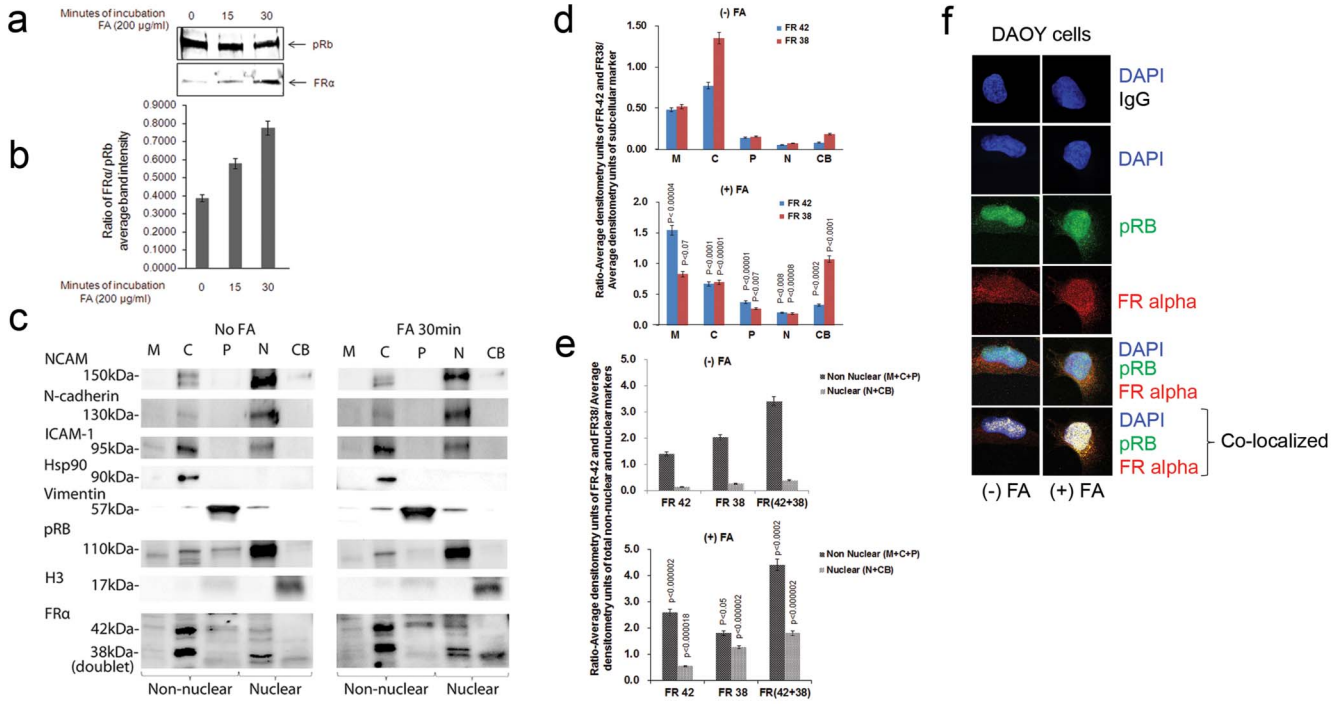

Figure 1 | Nuclear localization of FRα. (a) Nuclear extracts from DAOY cells treated with FA (200 µg/ml) for zero, 15 and 30 min at 37°C were subjected to immunoblotting using monoclonal anti-FRα (recognizing a 38 kd band) and polyclonal anti-pRB (recognizing 110 kd band) (*see Supplementary Fig S1*). (b) Ratio of FRα/pRB average band intensity (densitometry data is an average + SEM of three experiments). (c) Subcellular factions 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 kd and 38 kd) 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 kd 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 I-CAM1 (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 kd and 38 kd 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 kd band) appeared to translocate to the non-nuclear fraction (membrane, and cytoskeletal pellet fraction) and the 38 kd band to the nucleus. In the nucleus the FRα (38 kd 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 kd and 38 kd bands, the 42 kd band seems to translocate to the membrane enriched fraction in the presence of FA. Although both 42 kd and 38 kd bands of FRα appear to translocate to the nucleus, only the 38 kd band translocates

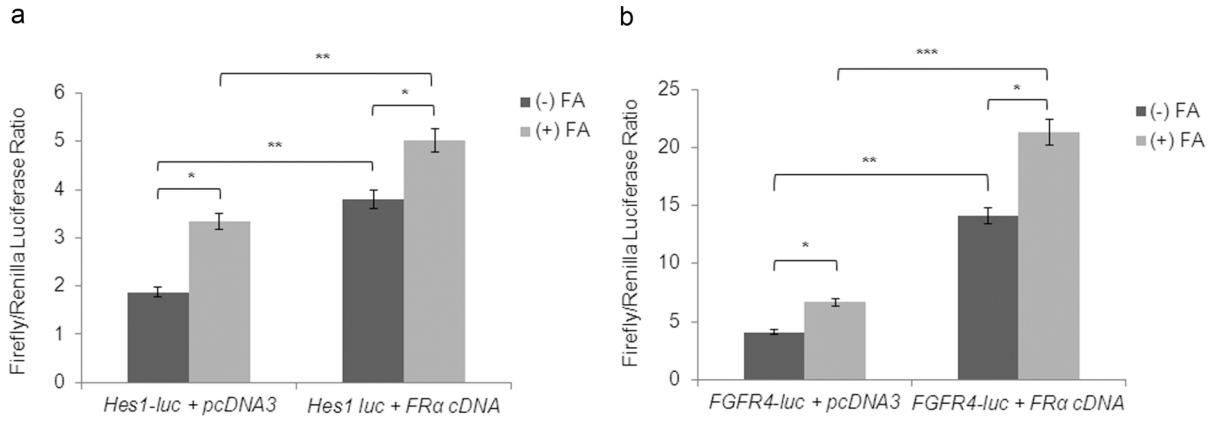

Figure 2 | Activation of Pax3 downstream target genes by FRα. *FRα-pcDNA3* or control pcDNA3 (0.2 ng/well) constructs were co-transfected with *Hes1* promoter-luciferase construct (a), human *FGFR4* promoter-luciferase construct P-535/+99[19] (b) or a control *PGL3* (promoter-less luciferase gene) into DAOY cells. FA (200 μg/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 FRα 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.

FRα binds to *cis*-regulatory elements of gene promoters. The above studies suggested that FRα translocates to the nucleus and in the presence of FA, it is enriched in the chromatin bound fraction. To determine whether FRα activates *FGFR4*, *FGFR4* promoter-luciferase constructs P-535/+99 from human *FGFR4* promoter[17] 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). FRα 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 FRα activates *FGFR4* promoter by binding to *cis*-regulatory elements.

To establish if FRα activates other FA modulated genes by binding to *cis*-regulatory regions, mouse *Hes1* promoter-luciferase construct[18] was co-transfected with *FRα* expression vector into DAOY cells treated or not treated with FA. *Hes1* is a Pax3 downstream target gene[18], FA increases *Hes1* mRNA and protein levels[14]. *Hes1*-promoter-luciferase reporter activity increased ($p<0.05$) in response to FA. FRα significantly increased ($p<0.001$) *Hes1*-promoter-luciferase without FA (Fig. 2b), with a further increase with FA treatment. These data indicate that FRα transcriptional activation is not limited to *FGFR4*.

To confirm FRα 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. FRα bound to *cis*-regulatory regions of *Hes1* and *Fgfr4* promoters *in vivo* (Fig. 3a).

FRα binds to AANTT consensus sequence on *Hes1* or *FGFR4* promoter. To identify putative FRα binding sequences in *Hes1* and *Fgfr4* promoters, ^{32}P-labeled oligonucleotides were made from appropriate promoter regions and EMSA was performed using affinity-purified GST-FRα fusion protein (Fig. 3b, c). GST-FRα fusion protein bound the *Hes1* oligonucleotide 5′-AAAATTAT-TTTTTTTTTGCGTGAAG-3′ which had AANTT or NAAAAN and/or NTTTTN sequences. When this sequence was mutated as in 5′-AACCCTTATTCCCCTTTGCGTGAAG-3′ there was no shift. Similarly, on the *Fgfr4* promoter the GST-FRα binding site mapped to AANTT or NAAAAN and/or NTTTTN in the oligonucleotide 5′-CAAACAAACAAAAAGAAACAACAAAAA-AACTTTTTA-3′ and NTTTTTN in the oligonucleotide 5′-ATA-AAAGCACAACTTTTTACAAAGTTTAAAGTTTTTT-3′. When the oligonucleotide sequence did not have the AANTT or TTNAA and NAAAAN consensus GST-FRα did not show a shift as in the case of 5′-CGTTCGCGTGCAGTCCGAGATAT-3′.

To further confirm the identity of FRα 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 FRα 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 transcription[14]. 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 FRα and its' subsequent action as a transcription factor. A hypothetical model showing FRα internalization is presented in Fig. 4b. FRα is internalized in a caveolar structure as early endosome[19]. The endosome becomes increasingly acidic[20] and fuses with a lysosome[21]. In the lysosome FA is released[22] and lysosomal GPI-specific phospholipase D[23] cleaves off the GPI anchor on FRα, which is then set free. Free FRα 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 FRα recycling and it is still unclear exactly how FRα translocates into the nucleus.

Binding to *Hes1* and *Fgfr4* promoters suggests FRα involvement in stem cell maintenance and skeletal muscle differentiation, respectively. The list of putative FRα targets shown in Table 1, suggests that FRα may be involved in regulating a plethora of developmental genes involved in myogenesis, skeletonogenesis, cell mobility, neural crest cell migration, cranial and cardiac neural crest formation, hair morphogenesis, oligodendrogenesis, spermatogenesis[24], melanogenesis[25], and epithelial to mesenchymal transformation[26]. A survey of the promoter regions of *c-Met*, *PDGFα*, *TGFβ2*, *MITF*, *N-CAM*, *c-RET*, *MyoD and Tyrp-1* indicates that the FRα binding motif AANTT

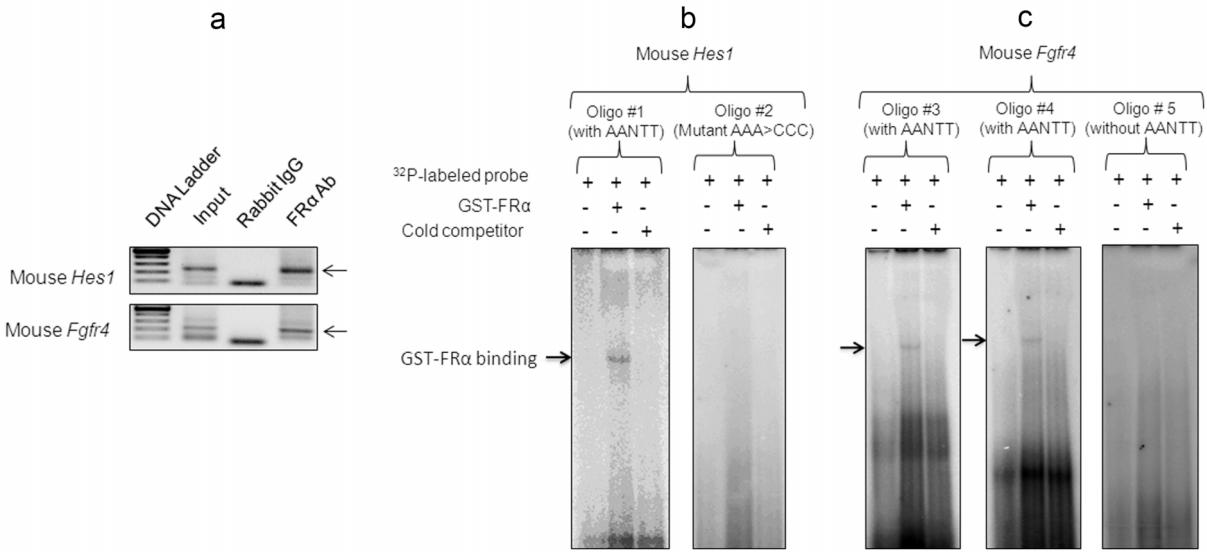

Figure 3 | FRα binds to murine *Hes1* and *Fgfr4* promoter *cis*-regulatory elements. (a) ChIP assays was performed using E10.0 (30 somite) lumbar neural tube. Anti-FRα polyclonal antibody was used to immunoprecipitate (IP) the protein–DNA complex. This antibody is made against epitope corresponding 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-FRα fusion protein and ^{32}P-labeled double-stranded oligonucleotides. Mouse *Hes1* oligo #1 (with AANTT): 5′-AAAAAATTATTTTTTTTTTGCGTGAAG-3′; Mouse *Hes1* oligo #2 (mutant AAA>CCC): 5′-AAACCCTATTTCCCCTTTGCGTGAAG-3′: (c)Mouse *Fgfr4* oligo #3 (with AANTT): 5′-CAAACAAACAAAAAGAAACAACAAAAAAACTTTTTA-3′; Mouse *Fgfr4* oligo #4 (with AANTT): 5′-ATAAAAGCACAACTTTTTACAAAGTTTAAAGTTTTTT-3; Mouse *Fgfr4* oligo #5 (deletion mutant without AANTT) 5′-CGTTCGCGTGCAGTCCGAGATAT-3′. The arrow shows GST-FRα binding to oligonucleotides which have the AANTT sequence.

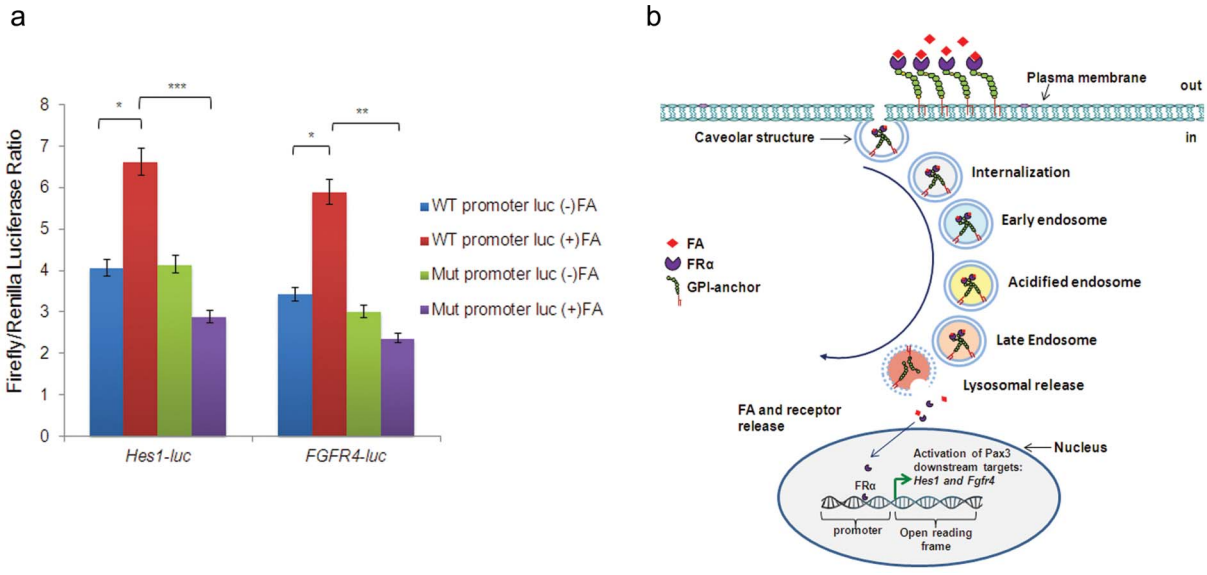

Figure 4 | FA does not activate *Hes1* or *FGFR4* promoter luciferase activity when the FRα consensus sequence (AANTT) is mutated. (a) AA>CC substitution mutations were made at the putative FRα binding sites on *Hes1* or *FGFR4* promoters (mutated sites are shown in the supplemental information). *Hes1* promoter-luciferase containing plasmid or plasmids containing mutated sequences (20 ng) were transiently co-transfected with *FRα-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. *FRα-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 FRα as a transcription factor. FRα, 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 FRα from its GPI-anchor. FRα 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 FRα binding sites in close proximity to each other

Gene Promoter	Accession number	Promoters of Pax3 downstream target genes containing the Pax3 and FRα binding sites in close proximity to each other
Human *MITF*	AF034755.1	1. GTGAAAAATAAAAAGTTATTAACAGTAAAAGAG
		2. GAACGTTTTTTTTTACATGCATAACTAATTAGCTTAGGTTATTATAA
		3. AACATTATTGAACTTCAAATTTTTAGCTTAA
		4. TGAATTAACATTTATTTTTAAACAGAATCTCTTTTCTTTTTAAGTGTAATAGT
		5. CTACTAATGTATAATCGTGTGTTTTCAAAAAATTCTCAATTTTAATTTCTAATATGC
Human *N-CAM*	BE019307; AA364465	1. GATATTTTAGTGTGAAACGTGTTTTTGTCATAGTATTGAAATAAAACTT
Human *c-RET*	NM_020630.4	1. GGATTACAGGTGTGAGCCCTTAGCCTGGCCCCTGAACTTTTTTTTTAACATGGATTTTTAG
		2. CCAGACCTTTTTCCAAGGAGTAAATG
Murine *c-ret*	AY255629.1	1. TCTGCTTTTTAGATTACGTA
		2. AATAGATTAGGAAGCGTCAAAAAATACA
		3. CTCCAAGGTCCTACAGGCGTCTGTTTTTCTTTCCTTCCATTACTAG
		4. CGTCTGTTTTTCTTTCCTTCCATTACTAGTTGCAAGCCATGCTTCAAAAAGCAG
Murine *TGFβ2*	NW_001030678.1	1. CAGGCTAATGAAAAACAACC
Human *PDGFRα*	AJ278993.1	1. CTGCAGTGTCTCTGCTGAAGGATTAAAAAAAAAAAAAT
		2. TGCACCTTTTTATACCCTTGCCCT
		3. TTAACCCCTTGATAATCCCTCAAGGAAAAAAACAAAACAAAAAACCC
		4. CACAAAACAAAAACCCATCATTGTCATATTGGACT
		5. TGATTTTTGGGGGGATCGTTTGTTCCC
Human *TGFβ2*	NM_009367.3	1. TCTTGATTATCTTTTTCTTGGGCT
		2. ACATAATTTTTAGAATTATT
		3. AGAGTTTTTCAATGTGTGATAATGGAAAAAATTCTGTATTCAAGGGA
		4. CCAGTGTAGCAGATCATTTTTCTTACGGGTCTGTTATCC
Human *FGFR4*	Y13901.1	1. AAATAATTACAGCTAGTATTACAAAGCAAGCGCTGCATGCCAGGCTTACACATACTGCTAACATGTCTACTTTTTGCAAC
Murine *Fgfr4*	NT_039589.8	1. GAGTTCCAGGACAGCCAGGGCTATACAGAGAAACCCTGTCTTGAAAAAAACAAAAAACAAAAAACAAAAAACC
Human *HES1*	NM_005524.3	1. CTTTACCTTGTTCCCTCCTTTTTTCAA
		2. AAATATTTTTCAAATGAACTTATTATACA
		3. ACATAATAAACCTTCAACTGCTCAGTAGTTTTTCTT
		4. ATATTTTTTCAAATGAACTTATTATACA
Chicken *MyoD*	L34006.1	1. GCTTAATGTGGAATAATCCAATTCCATATTAAAAAATG
		2. GCTAATCAATAGCTGCTCTCCTTGGGGTGGATCGTTTTTCAG
Murine *Tyrp1*	AF087673.1	1. CTTAATTATAGATATATCCCTAGTTGTCTTTTTGGTTGTTAAATAC
		2. TTTGGTTATTTTATATAATTTTTGTAT
		3. ATCAGTGTGTCTGACCTTTTCTTAAGACTTTAACCATCACAAGGAA
Human *C-MET*	Z26936.1	1. GAAAACTTCTCCACCTAGAAAGTTTCACCTTGTC

or NTTTTN and/or NAAAAN 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 CAGTGT[27]. These observations suggest that FRα and Pax3 might appear as a complex regulating target genes synergistically. However, up-regulation of *Hes1* or *Neurog2* in FA-rescued $Sp^{-/-}$ embryos[14] suggests that FRα may also have a role independent of Pax3. Future work shall test this hypothesis.

The observation that FRα 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 FRα misregulation and for management of human cancers which express FRα 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 FRα. Cazzaniga and colleagues[28] 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 FRα 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 FRα[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 inositol[30]. Grapp and colleagues[29] reported that whereas WT FRα was localized in the plasma membrane, in cerebral folate deficiency FRα mutants were mistargeted to intracellular compartments. The data presented in this paper provides relevant insight to these clinical situations. If FA interaction with FRα 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 FRα responsive genes by FRα and its' role in these multifactorial diseases.

Another clinical role for FRα is in cancer, where it is recognized as a tumor antigen/biomarker[31]. Because of this, diagnostic and therapeutic methods which exploit FRα are being developed for cancer treatment, including the use of folate-drug conjugates[31]. The knowledge that FRα 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 melanoma[32]. 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 FRα binding sites could potentially silence *c-met* and/or *MITF* expression.

In summary, our study shows that FRα is localized in the nucleus, where it binds to *cis*-regulatory elements (AANTT or TTNAA and NTTTTN or NAAAAN) on FA modulated genes and activates their transcription. This novel role of FRα 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, FRα misregulation and cancers which express FRα as a biomarker.

Methods

Antibodies and reagents. pRb antibody-rabbit polyclonal; 1 : 1000, Cell Signaling Technologies: Ser807/811, FRα 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-FRα 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 CO_2 inhalation, and moniliform uterine beads were removed at E10.5. Neural tubes were dissected out as described earlier[33]. 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: 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 FRα. DAOY cells were treated with FA (200 μg/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 μg) were immunoblotted with antibodies against FRα (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-FRα 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 FRα/ICAM-1; FRα/hsp90; FRα/vimentin; FRα/pRB; FRα/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 μg/ml) was added to appropriate wells. The cells were allowed to grow for an additional 30 min. Cells were immunostained with anti-FRα (mouse monoclonal antibody; 1 : 100) and pRB (rabbit polyclonal; 1 : 100). This mouse monoclonal FRα antibody recognizes only the 38 kd band of FRα. 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 earlier[27]. 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 earlier[34]. 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 FRα 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 FRα 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 μg) (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 × 104 cells/60 mm diameter dish in DMEM supplemented with 10% fetal calf serum for 24 h prior to transfection. For co-transfections: human *FRα* cDNA and mouse *Hes1-Luc* promoter or human *FRα* 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 FRα 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 *FRα- pcDNA3* or *pcDNA3* vector control into DAOY cells and luciferase assays were done as described earlier[34] 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-FRα fusion protein. *GST-FRα* fusion plasmid was kindly provided by Dr. Asok Antony. *Escherichia coli* was transformed with *GST-FRα* fusion

plasmid, and the cells were grown overnight in LB medium. GST-FRα 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-FRα 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 earlier[18]. Double-stranded oligonucleotide probes produced, covered the following regions: WT *Hes1* promoter oligo: 5′-AAAAAATTATTTTTTTTTGCGTGAAG-3′ and mutant *Hes1* promoter oligo: 5′-AAACCCTTATTCCCCTTTGCGTGAAG-3′; WT *Fgfr4* promoter oligo #1: 5′-CAAACAAACAAAAAGAAACAACAAAAAAACTTTTA-3′ WT promoter oligo #2: 5′-ATAAAAGCACAACTTTTTACAAAGTTTAAAGTTTTT-3′ and an oligo without AANTT sequence #3: 5′-CGTTCGCGTGCAGTCCGAGATAT-3′. For shift assays 4 μg GST-FRα 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 μg/μl salmon sperm DNA) prior to addition of labeled oligonucleotides. For hot reactions GST-FRα fusion protein and radio-labeled oligonucleotides were added and pre-incubated for 30 min at room temperature. For cold reactions GST-FRα 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× 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-FRα 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

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