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 (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 folate 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)2, proton-coupled folic acid transporter (PCFT), and reduced folic acid carrier (RFC) (see ref. 4 for review). FRα, a GPI-anchored protein1 is critical for embryonic development4. 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 closure6. FRα is one of the components of the caveolae fraction7, which includes EGFR8, caveolin-1 (Cav-1)9, IGF-1 receptor10 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-resistance11. Cav-1 binds to the promoters of cyclin D1 and FRα12, IGF-1 receptor13, BRCA113. This study examines a possible role of another caveolar protein, FRα as a transcription factor for key developmental genes.

Previous data from our lab2,14,15 demonstrated that FA remodels chromatin structures15. A second mechanism of FA action may be through FRα translocating to the nucleus and acting as a transcription factor. Bozard and colleagues16 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 protein2 and increases levels of Hes114.

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...
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) was predominantly present in the non-nuclear membrane fraction as well to the nuclear fraction. 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 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 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 FRα of human origin. This antibody is reported to recognize multiple types of FRα, 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.
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 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 was co-transfected with FRα expression vector into DAOY cells treated or not treated with FA. Hes1 is a Pax3 downstream target gene, FA increases Hes1 mRNA and protein levels. 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, 32P-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’-AAAAATTAT-TTTTTITTGTCGAG-3’ which had AANNTT and/or NTTTNT sequences. When this sequence was mutated as 5’-AACCCCTATTGGCTTTGGTGAAG-3’ there was no shift. Similarly, on the Fgfr4 promoter the GST-FRα binding site mapped to AANNTT or NTTTNT in the oligonucleotide 5’-CAAAACAAAAAGAAACAAAAAA- AACTTITTTTA-3’ and NTTTNTN in the oligonucleotide 5’-ATAAAGACAAACTTTTFAAAAGTTTAAAGTTTITTTT-3’. When the oligonucleotide sequence did not have the AANNTT and AANAAAN consensus GST-FRα did not show a shift as in the case of 5’-CGTTCGGTTGACCTCGGATAT-3’.

To further confirm the identity of FRα binding sites on Hes1 and FGFR4 promoters, AANNTT 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 AANNTT or TTNAA and NTTTNT or NAAAN 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 afflicted gene transcription. 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. The endosome becomes increasingly acidic and fuses with a lysosome. In the lysosome FA is released and lysosomal GPI-specific phospholipase D 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, melanogenesis, and epithelial to mesenchymal transformation. A survey of the promoter regions of c-Met, PDGFs, TGFβ2, MITF, N-CAM, c-RET, MyoD and Tyrp-1 indicates that the FRα binding motif AANNTT
**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 32P-labeled double-stranded oligonucleotides. Mouse *Hes1* oligo #1 (with AANTT): 5'-AAAAAATTATTTTTTTTGCGTGAAG-3'; Mouse *Hes1* oligo #2 (mutant AAAA>CCC): 5'-AAAAAATTATTTTTTTTGCGTGAAG-3'; (c)Mouse *Fgfr4* oligo #3 (with AANTT): 5'-CATAAAACAAAAAAAGAAAACAAAAAATTAAAAAAAATTTTTTA-3'; Mouse *Fgfr4* oligo #4 (with AANTT): 5'-ATAAAAGCACAACTTTTTACAAAGTTTAAAGTTTTTT-3; Mouse *Fgfr4* oligo #5 (deletion mutant without AANTT) 5'-CGTTCGCTGCGATCCGAGAT-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. GTCGAAAAAAATAAAAAGTTTTTTATTACACGAAAAAGAG  
|               |                  | 2. GAACGTTTTTTACATCTAATGAGTTTTGTTACAAATAG  
|               |                  | 3. AACATTTGCTACCCCAAATTCCGTTTA  
|               |                  | 4. TGAAATTTACATTATTTTAAAAAGAATCTCCTATTTTTAAAGTTATAG  
|               |                  | 5. CTATTTATATTATATCTCAATTACATTTATATGCTTCATAT  
| Human N-CAM  | BE019307; AA364465 | 1. GATATTTCTTTTGGTTTTGTTTTTGTCATAGTATGGAAATAAAACTT  
| Human c-RET  | NM_020630.4      | 2. GGATTTACACGCGTGGCCTTTAGGCTGCGCCCTGAACTTTTTTTTTTAACTGCGTTTTTGA  
| Murine c-ret | AY255629.1       | 3. CCACAGCTTTTTGCCAGGAGTTATT  
| Murine TGFβ1 | NW_001030678.1   | 4. TCTCTCTTTTTGAGTTACGTA  
| Human PDGFRα | AJ278993.1       | 5. AATAGATTAGGAAGGCTGACACAAAATACG  
| Human TGFβ1  | NM_009367.3      | 6. ATCTCTCTTTTTTCCCTTTCCATTACTAG  
| Murine TGFβ1 | NW_001030678.1   | 7. CCTCTGTTTTTTTCTTCTTCTCCATTACTAG  
| Human FGFR4  | Y13901.1         | 8. GTGAACCTTTTTACATTCTTTTTTTCAGGG  
| Murine Fgfr4 | NT_039589.8      | 9. CCAGCTTTTTTACATTCTTTTTTTCAGGG  
| Human HES1   | NM_005524.3      | 10. CGGCAAGTTTTTACATTCTTTTTTTCAGGG  
| Chicken MyoD | L34006.1         | 11. GCTTATGCTCTGCTCTTTTTTTTTACATTCTTTTTTTCAGGG  
| Murine TyrP1 | AF087673.1       | 12. GCTTATGCTCTGCTCTTTTTTTTTACATTCTTTTTTTCAGGG  
| Human CMET   | Z26936.1         | 13. GCTTATGCTCTGCTCTTTTTTTTTACATTCTTTTTTTCAGGG  

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or NTTTIN and/or NAAAN 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, GTTGTA, and CAGTTG \(^1\). These observations suggest that FRx and Pax3 might appear as a complex regulating target gene synergistically. However, up-regulation of Hes1 or Neurog2 in FA-rescued \(Sp^+\) embryos \(^1\) suggests that FRx may also have a role independent of Pax3. Future work shall test this hypothesis.

The observation that FRx 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 FRx misregulation and for management of human cancers which express FRx 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 FRx. Cazzaniga and colleagues\(^2\) 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 FRx 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 FRx\(^4\). 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\(^5\). Grapp and colleagues\(^6\) reported that whereas WT FRx was localized in the plasma membrane, in cerebral folate deficiency FRx mutants were mistargeted to intracellular compartments. The data presented in this paper provides relevant insight to these clinical situations. If FA interaction with FRx 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 FRx responsive genes by FRx and its role in these multifactorial diseases.

Another clinical role for FRx is in cancer, where it is recognized as a tumor antigen/biomarker\(^7\). Because of this, diagnostic and therapeutic methods which exploit FRx are being developed for cancer treatment, including the use of folate-drug conjugates\(^8\). The knowledge that FRx 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\(^9\). 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 FRx binding sites could potentially silence c-MET and/or MITF expression.

In summary, our studies show that FRx is localized in the nucleus, where it binds to cis-regulatory elements (AANTT or TTNAA and NTTTTN or NAAAN) on FA modulated genes and activates their transcription. This novel role of FRx as a transcription factor provides insight into developmental mechanisms associated with FA deficiency and FRx misregulation and cancers which express FRx as a biomarker.

### Methods

**Antibodies and reagents.** pRb antibody-rabbit polyclonal; 1:1000, Cell Signaling Technologies; Ser807/811, FRx antibody-rabbit polyclonal; 1: 500, Santa Cruz; sc-28997), NCAM (Santa Cruz; sc-1507), NCADherin (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-FRx antibody from Lifespan biosciences (cat # LS-C25683). 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) and a 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 bars were removed at E10.5. Neural tubes were dissected out as described earlier\(^7\). All animal experiments were approved by IACUC – Children’s Hospital of Chicago Research Center, Chicago (Approval # 12-09-03) and the committee for Animal Studies (Assurance ID: 13-0010.09) and all experiments were performed in accordance with institutional guidelines and regulations.

### Statistical analysis.

Values given are means. SEM. Probabilities (p) were calculated with GraphPad'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 FRx.

DAOY cells were treated with FA (200 \(\mu\)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 \(\mu\)g) were immunoblotted with antibodies against FRx (rabbit polyclonal, 1:500), NCAM (1:500); NCADherin (1:1000); I-CAM-1 and Rhodamine (1:1000); vimentin (1:10,000); pRB (1:500) and Histone H3 (1:500). This rabbit polyclonal antibody is made against the FRx antibody corresponding to amino acids 1-257 representing full length FRx of human origin. This antibody is reported to recognize multiple types of FR, \(\alpha\), \(\beta\) and \(\gamma\). The average band intensity of FRx/ICAM-1; FRx/hsp90; FRx/vimentin; FRx/pRB; FRx/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 \(\mu\)g/ml) was added to appropriate wells. The cells were allowed to grow for a additional 30 min. Cells were immunostained with anti-FRx (mouse monoclonal antibody; 1:100) and pRB (rabbit polyclonal; 1:100). This mouse monoclonal FRx antibody recognizes only the 38 kd band of FRx. 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\(^7\). 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\(^7\). PCR was performed with primers for murine Hes1 and Fgf4 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 FRx binding to cis-regulatory elements.

### Analysis of Hes1 and Fgf4 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 FGF4 promoter constructs were provided by Dr Shereen Ezzat, Departments of Medicine, Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada. The FRx expression construct in pcDNA3 was kindly provided by Dr Asok Antony, Indiana University Medical School Indianapolis, IN, USA. *Remilla* luciferase plasmid, pRL-null (0.5 ng) (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 x 104 cells/60 mm diameter dish in DMEM supplemented with 10% fetal calf serum for 24 h prior to transfection. For co-transfections: human FRx cDNA and mouse Hes1-Luc promoter or human FRx CDNA and human FGF4-Luc promoter were transfected into the cells using MegaTran 1.0 from Origen; 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 FRx binding sites on murine Hes1 or human FGF4 promoters were made with the QuickChangeXL site-directed mutagenesis kit (Stratagene). FA-mutant luciferase (pRL-null) plasmid or plasmids containing the mutated sequences (20 ng) were transiently co-transfected with FRx- pcDNA3 or pcDNA3 vector control into DAOY cells and luciferase assays were done as described earlier\(^7\) using the Dual Luciferase kit from Promega. *Remilla* luciferase plasmid, pRL-null (5 ng/well) (Dual Luciferase System Promega), was simultaneously transfected as an insertional control for transfection efficiency.

### Purification of GST-FRxs fusion protein.

GST-FRxs fusion plasmid was kindly provided by Dr. Asok Antony. *Escherichia coli* was transformed with GST-FRxs fusion
plasmid, and the cells were grown overnight in LB medium. GST-FRξ 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 pH 7.8 buffer. Supernatant containing GST-FRξ fusion protein among other proteins was loaded onto glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). Unbound proteins were washed with 50 mM Tris-HCI 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-32P] ATP by T4 polynucleotide kinase. EMSA was done as described earlier19. Double-stranded oligonucleotide probes produced, covered the following regions: WT Hes1 promoter oligo: 5'-AAAAAATTATTTTTTTTATCGTGAAAG-3' and mutant Hes1 promoter oligo: 5'-AAACCTCTCTCTCTCTTTGTTGAGGA-3'. WT Fgfr4 promoter oligo: 1'- 5'-CAAAAAACAAAAAAAGAACAAACAAATAATTTTTT-3' WT promoter oligo #2: 5'-ATATGAGACATTTTTCATACATTATAAAGTTTTT-3' and an oligo without AANNT sequence #3: 5'-GCCCTGGCGTGTACCTGCGAGATA-3'. For shift assays 4 μg GST-FRξ fusion protein was pre-incubated at room temperature for 30 min with 40 μg area and EMSA reaction buffer (100 mM Tris-HCl pH 7.5, 500 mM KCl, 6.5% glycerol, 50 mM pyrophosphate, 25 mM DTT in 2.5% Tween 20, 1 μg/ml salmon sperm DNA) prior to addition of labeled oligonucleotides. For hot reactions GST-FRξ fusion protein and radio-labeled oligonucleotides were added and pre-incubated for 30 min at room temperature. For cold reactions GST-FRξ fusion protein and unlabeled oligonucleotides were added and labeled oligonucleotides were added 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 0.25X 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.

1. Wodarczyk, B. J., Tang, L. S., Tripplet, A., Aleman, F. & Finnell, R. H. Spontaneous neural tube defects in splotch mice supplemented with selected micronutrients. *Toxicol. Appl. Pharmacol.* 213, 55–63 (2005).
2. Mayanil, C. S. & Ichi, S. Maternal intake of folic acid and neural crest stem cells. *Vitam. Horm.* 87, 143–173 (2013).
3. Zhu, H. et al. Differentially expressed genes in embryonic cardiac tissues of mice lacking Foll1 gene activity. *BMC Dev. Biol.* 7, 128 (2007).
4. Zhao, R., Diop-Bove, N., Visentin, M. & Goldman, I. D. Mechanisms of membrane transport of folates into cells and across epithelia. *Annu. Rev. Nutr.* 31, 177–201 (2011).
5. Ratnam, M., Marquardt, H., Duhring, J. L. & Freisheim, J. H. Homologous membrane folate binding proteins in human placenta: Cloning and sequence of a new human folate receptor isoform. *Exp. Cell Res.* 231, 253–262 (1997).
6. Smart, E. J., Ying, Y. S., Mineo, C. & Anderson, R. G. A detergent-free method for isolation of membrane folate receptors. *Kidney Int.* 72, 362–368 (2011).
7. Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W. & Schneider, M. A. Cholesterol and atherosclerosis. *Am. J. Physiol.* 278, C623–C632 (2000).
8. Grapp, M. et al. Molecular characterization of folate receptor 1 mutations delineates cerebral folate transport deficiency. *Brain* 135, 2022–2031 (2012).
9. Dill, P. et al. Pyridoxal phosphate-responsive seizures in a patient with cerebral folate deficiency (CFD) and congenital deafness with leukemia, aplasia, microtia and microdontia (LAMM). *Mol. Genet. Metab.* 104, 362–368 (2011).
10. Vladov, I. R. & Leamon, C. P. Engineering Folate-Drug Conjugates to Target Cancer: from Chemistry to Clinic. *Bioconjug. Chem.* 23, 1357–1369 (2012).
11. Macarensa, J. B. et al. PAX3 and SOX10 activate MET receptor expression in melanoma. *Pigment Cell Melanoma Res.* 23, 225–237 (2010).
12. Ichi, S. et al. Role of PAX3 acetylation in the regulation of Hes1 and Neurog2. *Mol. Biol. Cell* 22, 503–512 (2011).
13. Mayanil, C. S. et al. Regulation of murine TGFβ2 by PAX3 during early embryonic development. *J. Biol. Chem.* 281, 24544–24552 (2006).

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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-FRξ 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**

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