Folate receptor alpha is necessary for neural plate cell apical constriction during *Xenopus* neural tube formation

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Summary statement
Folate supplementation reduces the incidence of neural tube defects by unclear mechanisms. Here, we find that folate receptor promotes changes in neural plate cell shape necessary for neural tube formation.
Abstract
Folate supplementation prevents up to 70% of neural tube defects (NTDs), which result from a failure of neural tube closure during embryogenesis. The elucidation of the mechanisms underlying folate action has been challenging. This study introduces *Xenopus laevis* as a model to determine the cellular and molecular mechanisms involved in folate action during neural tube formation. We show that knockdown of folate receptor-α (FRα) impairs neural tube formation and leads to NTDs. FRα knockdown in neural plate cells only is necessary and sufficient to induce NTDs. FRα-deficient neural plate cells fail to constrict, resulting in widening of the neural plate midline and defective neural tube closure. Pharmacological inhibition of folate action by methotrexate during neurulation induces NTDs by inhibiting folate interaction with its uptake systems. Our findings support a model for folate receptor interacting with cell adhesion molecules, thus regulating apical cell membrane remodeling and cytoskeletal dynamics necessary for neural plate folding. Further studies in this organism may unveil novel cellular and molecular events mediated by folate and lead to new means for preventing NTDs.
Introduction

Neural tube defects (NTDs) are among the most common serious morphological defects diagnosed in human fetuses and newborns with combined incidence of ~1/1,000. They result from the failure of the neural tube to close leading to exencephaly or spina bifida (Wallingford et al., 2013). Currently, there is no effective treatment for NTDs once the neural tube has failed to close and, since closure is completed by day 28 of gestation in humans, preventive therapy must be targeted to early pregnancy.

A vast number of clinical studies have established that folate is a prominent environmental factor needed for appropriate neural tube closure (Detrait et al., 2005; MRC, 1991; Pitkin, 2007). Low blood folate levels in pregnant women are correlated with higher risk of NTDs in their offspring (Detrait et al., 2005; Smithells et al., 1976) and periconceptional supplementation of folate decreases the recurrence and occurrence of NTDs (MRC, 1991).

Folate belongs to the vitamin B family. Folate-derived metabolites are required for DNA, protein and lipid methylation. In particular, folate participates in thymidine and purine synthesis, thus is specially needed for DNA replication and cell division, processes characteristic of rapid growth. Uptake of folate by mammalian cells is mediated by three membrane proteins, reduced-folate carrier, folate receptor (FR, also called FOLR or folate binding protein (folbp)), and the proton-coupled folate transporter (Antony, 1992; Antony, 1996; Sirotnak and Tolner, 1999).

The mechanisms by which folate promotes neural tube formation are unclear. The importance of folate uptake systems for the formation of the neural tube becomes apparent from studies in humans and mice. Significant associations between single-nucleotide polymorphisms (SNPs) in different human folate transporters and myelomeningocele compared to the ethnically-paired healthy population have been found, including one SNP in folr1, 2 in folr2, 5 in folr3 and 2 in the reduced folate carrier (O'Byrne et al., 2010). In mice, knockout of folr1, Folbp1-/-, results in open neural tube by E9.5, when heterozygotes or wild-type littermate embryos show a closed neural tube. This phenotype leads to death of homozygous embryos in utero (Finnell et al., 2002; Piedrahita et al., 1999; Spiegelstein et al., 2004; Wallingford et al., 2013). The specific cellular mechanisms dependent on FRα (FOLR1) during mouse neural tube formation are unclear.

Whether folate participation in methylation reactions to promote rapid growth is the aspect of folate action necessary for neural tube formation is not completely understood.
Certain mutant variant of the methylenetetrahydrofolate reductase, one of the enzymes involved in folate metabolism, has been shown to increase the risk of incidence of NTDs in homozygous infants as well as in homozygous mothers’ offspring (Blom et al., 2006; van der Put et al., 1995; van der Put et al., 1996), although not every ethnic group studied exhibit this association (Koch et al., 1998; Mornet et al., 1997; Papapetrou et al., 1996; Speer et al., 1997; Wilcken and Wang, 1996). In addition, deficiencies in methylation disturb chick embryo neurulation (Afman et al., 2005; Afman et al., 2003). However, mice in which several enzymes involved in folate metabolism are disrupted do not show NTDs (Chen et al., 2001; Swanson et al., 2001; Watanabe et al., 1995). Moreover, screenings made in humans showed that there are cases with innate errors in folate metabolism but in whom NTDs are not over-represented (Blom et al., 2006). Altogether, these studies suggest that the identification of relevant aspects of folate action that influence neural tube formation demands further investigation.

Additional progress in the prevention of NTDs remains on hold due to the lack of understanding of the basic cellular and molecular mechanisms underlying folate action. *Xenopus laevis* animal model is especially suited for investigating events occurring during embryogenesis, because of the ready access to observation and manipulation of early developmental stages. Indeed, it has been the pioneer model system from which principles of the process of neurulation have been established (Davidson and Keller, 1999; Haigo et al., 2003; Kee et al., 2008; Keller et al., 1992).

Here we use the *Xenopus laevis* model system to demonstrate that folate promotes neural tube formation by facilitating changes in neural plate cell shape required during neurulation, through a folate receptor-dependent action. The use of this animal model may lead to the understanding of the mechanisms of folate action during neural tube formation and hence, may help devising more effective therapies for the prevention of NTDs.
Results

Folate receptor-α is expressed in *Xenopus* embryos during neurulation.

The human FRα and its murine homologue Folbp1 are primarily found in the placenta, the choroid plexus, and the brush border membrane in the kidney (Antony, 1992; Elwood, 1989; Holm et al., 1991; Prasad et al., 1994; Sellhub and Franklin, 1984). In the developing mouse embryo, Folbp1 is highly expressed in the yolk sac, neural folds, and neural tube (Barber et al., 1999; Saitsu et al., 2003). *Xenopus laevis* frl1 is cloned (accession numbers: BC074206.1 (Klein et al., 2002)) and shows 80% homology with the human folr1 isoform. Western blot assays with a custom-made antibody against the *Xenopus* protein reveal that FRα is readily expressed in mature oocytes and its presence persists throughout development (Fig. 1A,B). Specificity of the antibody is demonstrated by the detection of overexpressed *Xenopus* FRα both in immunostained sections from xFRα-mRNA unilaterally injected embryos (Fig. S1A,B) and in Western blot assays from xFRα-flag mRNA-injected embryos (Fig. S1C). Folate is also present in neural plate stage *Xenopus laevis* embryos (stages 13-17, Fig. 1C). Immunostaining of transverse sections from neurulating embryos shows that xFRα is expressed in the developing neural plate (Fig. 1D,E). FRα is enriched in the apical surface of the neural plate, along with C-cadherin (Fig. 1E), in the interectodermal cleft of the two-cell-layered neural plate, and at the boundaries between neural plate and mesoderm (Fig. 1D). In non-neural tissue FRα is present in the myocoele and in the notochord lumen (Fig. 1D). These results demonstrate the presence of both FRα and folate in *Xenopus* embryos during neural plate folding and neural tube formation.

Molecular knockdown of folate receptor hinders neural tube closure.

To determine whether FRα plays a role in *Xenopus* neural tube formation, we used two non-overlapping translation-blocking morpholinos against *Xenopus laevis* FRα sequence (FRα-MO1 and FRα-MO2, see Materials and Methods for details) to knock down its expression. Both morpholinos similarly disrupt neural plate folding (Fig. 2A). Results using FRα-MO1 (hereby referred as FRα-MO) are presented in the remaining of the study. Specificity of FRα-MO is demonstrated by Western blot assays from embryos expressing FRα mRNA sensitive and insensitive to morpholino inhibition, sense-flag-FRα and flag-FRα, respectively (see Materials and Methods and Fig. 2B). Results show that FRα-MO does not affect expression of morpholino-insensitive flag-FRα mRNA (Fig. 2C, lanes 3 and 4) while reproducibly blocks expression of morpholino-sensitive (sense-flag-FRα) mRNA (Fig. 2C, lanes 5 and 6).
These results show that downregulation of exogenous FRα expression occurs when FRα-MO targets the sense sequence. However, Western blot assay from whole neurulating embryos does not reveal differences in the level of FRα expression between control morpholino (CMO) and FRα-MO-injected groups (Fig. 2D, lanes 1 and 2). This is possibly due to substantial maternal contribution of FRα (Fig. 1B, first lane, oocyte), which may make impractical the detection of the reduction in FRα level from downregulation of de novo expressed protein in whole-embryo lysates. Nevertheless, a reproducible and significant reduction in FRα immunolabeling is apparent in the apical surface of the neural plate when endogenous FRα expression is knocked down by FRα-MO (Fig. 2E,G), suggesting that de novo synthesis results in localization of the receptor at the apical neural plate cell surface. In contrast, unilateral injection of control morpholino (CMO) does not affect FRα expression (Fig. 2F,G). Additionally, knockdown of FRα does not result in a significant change in folate levels during neurulation (Fig. 1C), suggesting that FRα is not crucial for folate homeostasis during neural tube formation.

We find that knockdown of FRα expression in developing Xenopus embryos (Fig. 2) impairs neural tube formation and induces NTDs (Fig. 3). The penetrance of the NTD phenotype is approximately 90% (Fig. 3A) and is specific to neurulation since defects in gastrulation are not significantly represented in the FRα-MO-injected group; there is no delay during gastrulation stages in the FRα-MO-injected group (mean stage ± SD: FRα-MO 11.3±0.3, n:131; CMO 10.3±0.1, n:66; FRα-MO+FRα-mRNA 11.4±0.2, n:65), there is no overrepresentation of apparent gross morphology defects in gastrulating embryos (defective embryos during gastrulation: FRα-MO 14%, n:131; CMO 26%, n:66; FRα-MO+FRα-mRNA 17%, n:65) and there is no significant change in embryo viability during gastrulation (dead embryos: FRα-MO 11%, n:131; CMO 9%, n:66; FRα-MO+FRα-mRNA 7%, n:65). When 20 pmol FRα-MO are injected the prevalent phenotype (4 out of 6 experiments) is severe, the neural tube is open throughout the anterior-posterior axis, and there is degeneration of neural tissue, apparent as amorphous white tissue protruding from the flanking pigmented non-neural ectoderm in the midline region (Fig. 3A, top middle and bottom left). To rescue FRα-MO phenotype we designed xFRα mRNA resistant to FRα-MO inhibition (Fig. 2B and Materials and Methods) that in addition renders higher level of FRα protein expression compared to wild type mRNA when injected in developing embryos (Fig. 2D, lanes 5 and 6). Western blot assays demonstrate that FRα-MO downregulates wild type FRα (Fig. 2D, lanes 3 and 5) but does not affect resistant FRα mRNA (Fig. 2D, lanes 4 and 6). The severe phenotype is rescued by restoring xFRα expression through the injection of resistant FRα
mRNA (Fig. 3A). In contrast, incubating FRα-MO-injected embryos with 150 μM folinic acid fails to rescue the severe NTD phenotype (Fig. 3A) or Embryos exhibiting severe NTD phenotype do not resist histological procedures due to neuroectoderm degeneration (stage 19), and 100% of them die by the time neural tube closure is complete (stage 21) in sibling control-morpholino-injected embryos. However, in 2 out of 6 experiments the majority of embryos did not exhibit the severe phenotype and they survived past stage 19 (40 FRα-MO; 30 CMO; 44 FRα-MO+folinic acid). To assess the histological features of the neural tissue due to FRα deficiency we sectioned FRα-MO-injected embryos that exhibit this moderate phenotype upon completion of neural tube closure (stage 20-21) in control groups (Fig. 3B). The moderate phenotype consists of incomplete convergence of neural folds in the midline without pronounced degeneration of neural tissue (Fig. 3B, left), which makes these embryos amenable to sectioning and immunohistological procedures. The histological assessment of those experiments with prevailing moderate phenotype shows that most embryos despite their mildly abnormal overall appearance (Fig. 3B, left) present a defective, spread out and flattened, neural tissue with no lumen, even in cases where the non-neural ectoderm covers up the neural tissue (Fig. 3B). This phenotype is partially rescued by incubating FRα-MO-injected embryos with folinic acid (Fig. 3B) unlike the embryos exhibiting the severe phenotype that were only rescued by restoring FRα expression (Fig. 3A). These results identify FRα as a relevant molecule in the process of *Xenopus* neurulation, in agreement with what has been reported in other model systems (Spiegelstein et al., 2004), and suggest that the interaction between folate and its receptor is necessary for neural tube formation. We ruled out the possibility that FRα-MO was inducing neural plate cell death by assessing number of apoptotic cells during neural plate folding, a developmental period in which apoptosis is negligible in the neural tissue (Hensey and Gautier, 1998; Sugimoto et al., 2007). Results show that there are no apoptotic cells in FRα-MO-containing or contralateral neural plate (Fig. S2A).

**Folate receptor localized in the superficial neural plate is necessary for the apical constriction of neural plate cells and required for neural plate folding.**

To assess whether folate effect is tissue specific, FRα-MO was targeted to neural plate cells by injecting 1.8-3 pmol FRα-MO into the dorsal medial and dorsal lateral animal blastomeres of 16-cell-stage embryos (Wallingford and Harland, 2002). Results show that knocking down FRα in neural tissue disrupts neural tube formation in 100% of the embryos while injection of CMO does not affect neurulation in most embryos and only induces a mild phenotype in 15%
of them (Fig. 3C). Transverse sections of FRα-MO unilaterally injected embryos in target tissues reveal that downregulation of FRα expression in the neural plate perturbs its folding (Fig. 2E). In contrast, knockdown of FRα in non-neural tissues like the mesoderm and non-neural ectoderm (Fig. S3) or injection of CMO in neural tissue (Fig. 2F) does not affect neural plate folding, indicating that FRα expression in the neural plate is necessary to promote neural tube formation and that FRα knockdown in neural plate only is sufficient to induce NTDs.

Injection of the 16-cell-stage dorsal medial or dorsal lateral animal blastomeres renders a neural plate with primarily medial or lateral affected cells, respectively (Wallingford and Harland, 2002). Embryos with FRα-MO-containing medial neural plate cells exhibit 100% incidence of NTD phenotype (Fig. 3C). Moreover, results show that apical constriction of cells from the superficial layer of the medial neural plate is deficient in morphant cells compared to cells in the contralateral wild type side (Fig. 4A-C). This leads to wider apical surface at the midline that hinders folding of the neural plate (Fig. 4A-C). In contrast, cellular defects when lateral neural plate cells are targeted are not apparent and the overall morphogenesis of the neural tube is not compromised (Fig. 4D). To measure quantitatively and dynamically the impairment of apical constriction of medial neural plate cells resulting from knocking down FRα we time-lapse imaged the apical surface of the neural plate during stages of neural plate folding (from stage 15-15.5 to stage 16.5-17) in FRα-MO- and CMO-unilaterally-injected live embryos expressing membrane-GFP. Data show that FRα-MO-containing cells fail to reduce their apical surface over time (change in apical surface: 17±12 \( \mu \text{m}^2/\text{h} \), mean±SEM, n:30 cells) while CMO-containing and contralateral wild-type cells in CMO or FRα-MO embryos exhibit a rate of surface reduction of 84±9, 105±10, 103±13 \( \mu \text{m}^2/\text{h} \), mean±SEM, n:30 cells from 5 embryos/group, respectively, during neural plate folding (Fig. 5, Movies 1 and 2). We did not include dividing apical neural plate cells (Movie 3) in the apical surface measurements to exclude the effect of cell division on cell surface reduction during neural plate folding. Folic acid deficiency is known to inhibit cell proliferation. Hence, it may be argued that FRα knockdown may induce neural tube defects by impairing cell division. However, in *Xenopus laevis* neural tube formation proceeds even when cell division is inhibited (Harris and Hartenstein, 1991). In agreement with this, our data show that only a small number of neuroepithelial apical cells undergo division (wild-type, WT: 2±0.5% per 1 h of neurulation), while all neuroepithelial cells in the superficial layer exhibit apical constriction (Fig. 5, Movie 2). Nevertheless, similar numbers of dividing cells are apparent in WT, FRα-MO- and CMO-containing apical neural plate (FRα-MO:
1±0.5%, CMO: 0.9±0.6% mean±SEM percent dividing cells/h). These results suggest that expression and function of FRα in medial neural plate cells during neurulation is necessary for medial cell apical constriction.

To determine the early targets of FRα deficiency leading to impaired apical constriction we examined actin dynamics in the superficial neural plate during initial constriction. We failed to find any significant differences between actin dynamics in superficial medial neural plate cells containing FRα-MO and wild type counterparts (Fig. S4A). Similarly, localization of apical proteins like the tight junction component ZO-1 is comparable in wild type and FRα-MO-containing cells (Fig. S4B). These results suggest that regulating actin dynamics or establishment of overall apicobasal polarity are not initial effectors of FRα action.

Remodeling of adherens junctions between cells is crucial for tissue morphogenesis (Nishimura and Takeichi, 2009; West and Harris, 2016). C-cadherin is an important component of adherens junctions in X. laevis neural plate (Nandadasa et al., 2009). Apical FRα in the neural plate partially colocalizes with the adherens junction component C-cadherin and its molecular partner β-catenin (Fig. 6A). Moreover, both C-cadherin and β-catenin coimmunoprecipitate with FRα (Fig. 6B). Altogether, these results indicate molecular interaction between FRα and prominent cell adhesion and cytoskeleton organizer molecules in the apical surface of medial neural plate cells. C-cadherin endocytosis is required for morphogenic movements in gastrulating Xenopus embryo (Jarrett et al., 2002). We find that neural plate cells undergoing apical constriction contain C-cadherin-immunopositive puncta associated with early endosomes (EEA1-immunopositive vesicles (Mu et al., 1995), Fig. S5), which indicates that C-cadherin endocytosis occurs during apical constriction. Indeed, reduction of apical surface in constricting cells (Fig. 5) requires internalization of apically localized C-cadherin. FRα knockdown in the apical medial neural plate is accompanied by a decrease in the number of C-cadherin-containing endosomes (Fig. 6C). This effect does not appear to be an overall disruption of endocytosis but specific to C-cadherin compartmentalization, since total number of EEA1-immunopositive vesicles is comparable in FRα-MO and CMO-containing medial neural plate cells. In contrast, total C-cadherin-containing internal vesicles are markedly reduced in FRα deficient cells (Fig. 6C). Altogether these results suggest that FRα interacts with cell adhesion and cytoskeletal machinery responsible for medial neural plate cell apical constriction. This interaction appears to be necessary for regulating trafficking of cadherins.
**Pharmacological inhibition of folate uptake systems induces neural tube defects.**

Pharmacological inhibition of folate action in developing *Xenopus* embryos allows for better temporal resolution of the perturbation and assessment of neural tube formation dynamics. Results show that incubation of *Xenopus* embryos with methotrexate (MTX), a widely used folic acid uptake inhibitor (Jolivet et al., 1983), from the moment neurulation begins, perturbs neural tube formation. This effect is dose-dependent with a threshold at 10 μM (Fig. 7). MTX effect is due to inhibiting folic acid action because preincubation of embryos with 1-10 mM folic or folinic acid rescues MTX-induced phenotype (Fig. 7). We ruled out the possibility that MTX treatment during the process of neurulation has a cytotoxic effect on neural plate cells by assessing number of apoptotic cells. Results show that there are no apoptotic neural plate cells during neurulation in control or MTX-treated embryos (Fig. S2B).

We assessed the effect of 3 lipophilic methotrexate analogs that also inhibit dihydrofolate reductase (DHFR) but unlike MTX, do not affect folate interaction with the folate receptor or carrier (Grem et al., 1994; Hook et al., 1986; Zimmerman et al., 1987). Chemotherapeutic efficacy and DHFR inhibitory effect of the drugs have been demonstrated *in vitro*, in human colon and Chinese hamster ovary cancer cells and in mouse leukemia cells, and *in vivo*, in mice inoculated with leukemia cells and in rat intestine (Grem et al., 1994; Hook et al., 1986; Zimmerman et al., 1987). Results show that none of the lipophilic DHFR inhibitors used in saturating doses induce NTDs (Fig. 7). Our findings suggest that MTX-induced NTDs are due to interfering with the interaction between folate and its uptake systems and not with folate metabolism.
Discussion
This study introduces *Xenopus laevis* as an instrumental system for investigating the mechanisms of folate action during neurulation. Our findings identify a novel role of folate and its receptor in regulating changes in neural plate cell morphology, which are necessary for appropriate neural tube formation. The fact that folate action is relevant for neural tube formation in human, mouse, chicken, zebrafish and frog argues for both a conserved function of this molecule and conserved mechanisms of neurulation. Although folate is known as an essential cofactor that supports rapid growth by enabling DNA and amino acid synthesis, in *Xenopus laevis*, unlike mammals and birds, cell proliferation is not necessary for neural tube formation; *Xenopus* embryos undergo neurulation even when DNA synthesis is blocked (Harris and Hartenstein, 1991). Nevertheless, interfering with folate action induces NTDs in this species arguing for a previously unidentified role of folate during neural tube closure. Hence, this model system provides the opportunity to determine novel mechanisms triggered by folate action. Indeed, we find that inhibiting one of the most important enzymes of folate metabolism by analogs of methotrexate that bypass the uptake systems due to their lipophilic nature (Grem et al., 1994; Hook et al., 1986; Zimmerman et al., 1987), does not induce NTDs, unlike methotrexate, suggesting that folate interaction with its receptor is relevant for the formation of the neural tube besides its action as a vitamin and growth promoting factor.

Despite the fact that apoptosis is not apparent during neural plate folding in embryos deficient in folate receptor, in the majority of FRα-MO-injected embryos strong neural tissue degeneration occurs at later neurulation stages, indicating that ultimately neural cell death takes place as a result of FRα downregulation. Although the mechanisms responsible for neural tissue degeneration remain to be elucidated, one possibility is that interfering with folate receptor action results in the observed disruption of trafficking of apical cell adhesion proteins like C-cadherin, major cadherin present during *Xenopus laevis* early neurulation (Nandadasa et al., 2009), disturbing the necessary dynamics of cell-cell attachment and impeding neural plate cell apical constriction, as shown in this study. In this context, it is possible that prolonged exposure of embryonic neural tissue to the external saline may cause degeneration. This scenario resembles the two-hit-pathogenesis model for human neural tube defects where the open neural tube is the first hit followed by *in utero* acquired neural tissue destruction as the second hit (Meuli and Moehrlein, 2014).

This study points out to the neural tissue as the main target of folate action during neural tube formation. Neural plate cells are prominently engaged during neurulation and their change in cell morphology is crucial for shaping the neural plate and closing of the tube.
We find FRα colocalized at the apical membrane of superficial neural plate cells with C-cadherin and β-catenin. Apically localized molecules play critical roles during neural plate folding by scaffolding proteins that participate in the apical constriction of cells (Eom et al., 2011; Haigo et al., 2003; Hildebrand and Soriano, 1999; Morita et al., 2010; Nandadasa et al., 2009; Nishimura and Takeichi, 2008; Ossipova et al., 2014). For example, cadherins coordinate cytoskeletal dynamics and cell-cell interactions necessary for tissue bending and morphogenesis (Nishimura and Takeichi, 2009; West and Harris, 2016).

We discovered a molecular interaction among FRα, C-cadherin and β-catenin. Apical constriction requires a spatiotemporal regulation of apical membrane remodeling, that in turn depends on timely endocytosis of apical membrane components (Lee and Harland, 2010). The polarized localization of FR-α in neural plate cells during neural tube formation is critical to neural tube morphogenic process by enabling medial neural plate cell apical constriction, potentially through the regulation of C-cadherin trafficking. The results from this study focus the research field on the neural tissue and the process of neural tube formation as specific targets of folate action and argue for considering more specific roles of the vitamin folate on nervous system development and function. Intriguingly, it has been shown that folate improves axonal regeneration after spinal cord and sciatic nerve injury by an upregulation of FRα expression (Iskandar et al., 2010) and supplementation of pregnant rats with high folate diet alters synaptic transmission and seizure susceptibility in offspring (Girotto et al., 2013). A recent study shows that presence of folate receptor blocking antibodies during gestation and pre-weaning in rats disturbs adult social behavior of offspring (Sequeira et al., 2016), in agreement with the association between folate receptor autoantibodies found in children suffering from the infantile-onset cerebral folate deficiency, who exhibit neuropsychiatric and neurologic manifestations (Ramaekers et al., 2005).

Future studies are needed to further elucidate the cellular and molecular mechanisms underlying folate action during nervous system development, which will contribute to devising effective therapies to prevent neural tube defects.
Materials and Methods

Frog embryos. Fertilized oocytes were kept in 10% MMR saline, containing (in mM): 10 NaCl, 0.2 KCl, 0.1 MgSO₄, 0.5 Hepes, 5 EDTA and 0.2 CaCl₂. Developmental stages were recorded according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Animals were handled according to the IACUC guidelines.

Western blot assays. Crude membrane fraction was obtained from non-fertilized eggs, stage-10, -17 and -20 embryos, 10 embryos for each group to assess endogenous expression of folate receptor-α (FRα). PVDF membrane was probed with anti-FRα rabbit polyclonal affinity purified antibody raised against the peptide KHQKVDPGPEDDLHC (custom made by GenScript), 1:500 in 5% milk followed by incubation with HRP-conjugated secondary antibodies (Jackson ImmunoResearch) 1:10,000 (cat# NEL103E001, PerkinElmer). To assess exogenous flag-tagged xFRα expression PVDF membranes were immunoblotted with primary anti-flag tag mouse antibody (cat#MA1-91878 Thermo Scientific). See Supplementary Materials and Methods for details.

Coimmunoprecipitation. Fifteen neurulating embryos were homogenized in extraction buffer containing 1% Triton X-100, 150 mM NaCl, 25 mM Tris pH 7.4, 5 mM EDTA, protease inhibitor cocktail (Thermo Scientific, cat. # 78415) on ice. Insoluble proteins were pelleted by centrifugation at 16,100 rcf for 10 min. FRα was immunoprecipitated from the soluble fraction overnight at 4°C through mild rocking by anti-FRα rabbit polyclonal antibody (GenScript, custom-made) chemically cross-linked to protein G-agarose beads (Roche, cat. # 11719416001). Beads were resuspended in 2X protein loading buffer containing 10 mM DTT and incubated for 10 min at 80°C. Samples were processed for Western blot assays with anti-C-cadherin (DSHB, 1:100 cat. # 6B6), anti-β-catenin (Sicgen, 1:500 cat. # AB0095-200) and anti-FRα antibodies. See Supplementary Materials and Methods for details.

Measurement of folate levels by ELISA assay. Stage-12.5-25 embryos were homogenized with 0.5% Triton X-100. Samples were centrifuged at 13,000 rcf and supernatants were loaded onto ELISA plates. Detection of folate level was performed according to instructions of the folate receptor binding-based Enzyme Immunoassay Kit for quantitative determination of folate in biological samples based on the capacity of folate to bind to folate receptor (Cat# 7525-300, Monobind, Inc.).

Immunohistochemistry. Stage-14-18 and -21-23 embryos were fixed for 30 min at 23 °C with freshly made 2% trichloroacetic acid (TCA) or 4% PFA and processed for immunostaining as
previously described (Belgacem and Borodinsky, 2011; Belgacem and Borodinsky, 2015; Borodinsky et al., 2004; Swapna and Borodinsky, 2012) with modifications and by using standard protocols of paraffin embedding and sectioning (Tu and Borodinsky, 2014). Tween-20 was used in blocking, antibody and washing solutions for FRα immunostaining since stronger detergents have been reported to result in artificial redistribution of glycosylphosphatidyl-inositol-anchored proteins (Heffer-Lauc et al., 2007). Primary antibodies used were: anti-xFRα or anti-γ-cadherin 6B6 1:50 (Developmental Studies Hybridoma Bank), anti-β-tubulin E7 1:300 (Developmental Studies Hybridoma Bank), anti-E-cadherin 5D3 1:100 (Developmental Studies Hybridoma Bank), anti-GFP 1:500 (cat# GFP-1020), anti-Sox2 1:300 (Cat # AF2018, R&D Systems). See Supplementary Materials and Methods for details.

**FRα knockdown.** Two-cell-stage embryos were bilaterally injected with 4-10 nl of 1 mM FRα-morpholinos, FRα-MO1 and FRα-MO2 (MO oligo sequence written from 5’ to 3’ and complementary to folate receptor: GGCCCCCGTAACATGGTTACAAGC (FRα-MO1), AATATGGCACGAGTCGCAACCCACA (FRα-MO2)). Controls were sibling embryos injected with standard control morpholino (CMO: CCTCTTACCTCAGTTACAATTTATA). Morpholinos were injected along with dextran-Alexa-Fluor conjugates or GFP mRNA to assure permanency of MO reporter after TCA fixation.

Rescue experiments were implemented by expressing FRα-MO1-resistant *Xenopus laevis* FRα mRNA, lacking 3’ UTR, 5’ UTR substituted for gcc acc sequence (Kozak, 1987a; Kozak, 1987b) and with a number of wobble mutations in coding region (MO-resistant FRα-RNA: ... gcc acc atg ctt aga gga gct ctc; wt-FRα-RNA: ...g ctt gta acc atg tta cgg ggg gcc... ) or by incubating embryos with 150 μM folinic acid. mRNA was synthesized as previously described (Belgacem and Borodinsky, 2011; Borodinsky et al., 2004; Swapna and Borodinsky, 2012) using the *Xenopus laevis* FRα template (XGC African clawed frog FRα cDNA, Clone ID: 7012141, Open Biosystems, Thermo Fisher Scientific, Inc.), subcloned into pCS2+ vector. Mutations were done using the site-directed mutagenesis kit (Ambion; (Swapna and Borodinsky, 2012)) and PCR reaction to render FRα-MO1-resistant mRNA. Two hundred and fifty pg xFRα mRNA were bilaterally microinjected in 2-cell-stage embryos or coinjected with specific MOs. We also designed morpholino-insensitive or front-tagged FRα (flag-FRα) and morpholino-sensitive or back-tagged FRα (FRα-flag) constructs by subcloning *Xenopus laevis* FRα into flag-pCS2+ plasmid (a gift from Peter Klein, Addgene plasmid #16331). Another morpholino-sensitive construct was obtained by
annealing of synthesized FRα-MOI anti-sense and sense 5’GCTTGTAACCATGTACGGGGGCC3’ oligonucleotides inserted in front of the flag-FRα (sense-flag-FRα). Seven hundred pg of FRα-MOI-resistant *Xenopus laevis* FRα mRNA were injected in one blastomere of 2-cell-stage embryos to unilaterally overexpress *Xenopus* FRα.

Quantitative assessment of FRα knockdown by FRα-MO was done by measuring the area labeled by FRα immunostaining in medial neural plate cell apical surface in sectioned stage-14.5-17 embryos unilaterally injected with FRα-MO or CMO after uniform thresholding of labeled images with NIS Elements software (Nikon, Inc.). Measurements were done in the apical surface of 3 consecutive wild type and 3 paired FRα-MO- or CMO-containing medial neural plate cells from the midline in 10 embryos per group in 42 sections.

Quantitative assessment of C-cadherin-containing vesicles (>0.5 μm) and endosomes (EEA1-immunopositive, 0.2−3 μm) were done by thresholding immunolabeled transverse sections of the medial neural plate followed by counting stained vesicles with NIS Elements software (Nikon, Inc.). Samples were from stage-15.5-16 embryos unilaterally injected with FRα-MO and CMO in single blastomeres at 2-cell stage. Measurements were done in 3 consecutive FRα-MO- and 3 paired CMO-containing medial neural plate cells from the midline in 18 sections from 5 embryos.

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*Measurement of neural plate cell apical constriction and cell divisions in live embryos.* Two-cell-stage embryos were bilaterally injected with membrane-GFP (pCAG-mGFP was a gift from Connie Cepko, Addgene plasmid # 14757 (Matsuda and Cepko, 2007)) and unilaterally injected with 10 pmol FRα-MO or CMO per cell along with Alexa 594-conjugated dextran.
Rate of apical constriction was analyzed in time-lapse recordings of neurulating embryos over 1 h period (stage 15-15.5 to 16.5-17) with an acquisition rate of 1 frame/5 min. Surface of medial neural plate cells was measured over time in CMO, FRα-MO-containing and contralateral wild type cells by creating a region of interest outlining cell boundaries and using NIS-Elements software (Nikon, Inc.). Cells analyzed were 113 and 115 in CMO- and FRα-MO-injected embryos, respectively, n:5 embryos for each group.

**Measurement of actin dynamics.** Two-cell-stage embryos were bilaterally injected with 800 pg mCherry tagged utropin mRNA (mCherry-UtrCH was a gift from William Bement, Addgene plasmid # 26740 (Burkel et al., 2007)) and unilaterally injected with 10 pmol FRα-MO per cell along with Alexa 647-conjugated dextran. Medial neural plates of embryos at stage 14-14.5 were imaged for 15 min at 30-55-s interval using 60x objective and Z-stack confocal imaging (Sweptfield confocal, Nikon). Fluorescence intensity profiles across cell-cell borders were measured at each time frame on maximum intensity projections using 2.3-3.5 μm stripe (NIS Elements software, Nikon). Intensity profile peaks were fitted with Gauss function in R software following background subtraction. Average Gaussian’s width at 50% height was used as a parameter describing F-actin distribution at cell-cell borders in FR-MO-containing and wild type medial neural plate cells. Standard deviation of Gaussian’s amplitude normalized to amplitude median during 15-min recoding was used to measure dynamics in F-actin accumulation in cell-cell borders. Cell borders analyzed were 28 in each group, wild-type and FRα-MO1-containing cells, n:7 embryos.

**Pharmacological inhibition of folate action.** Neural plate stage embryos (from stage 13 to stage 20) were incubated with 0.1-1500 μM methotrexate (MTX), 0.1-300 μM MTX-ester, 0.1-100 μM pyrimethamine and 100-500 μM trimetrexate or vehicle only (control, 0.3% DMSO or saline). Rescue experiments were done by preincubating embryos with folic or folinic acid (metabolically active form of folic acid) starting at 2-4-cell stage. MTX was added at stage 13, corresponding to the beginning of neural plate folding and neural tube formation.

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**Data collection and Statistics.** Numbers of samples analyzed are indicated for each set of experiments. Significance was evaluated using Student t-test or Mann-Whitney U-test.
(Wilcoxon rank-sum test) and ANOVA. Differences were considered significant when $p<0.05$.

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

**Author contributions.** OAB, OV and LNB designed the experiments, OAB and OV performed the experiments and analyzed the data, OAB and LNB wrote the manuscript.

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Fig. 1. Folate receptor-α expression during *Xenopus laevis* neural tube formation. (A) Time course (in hours post fertilization, hpf) of *Xenopus laevis* neurulation. (B) Western blot assays from homogenates of wild-type or *Xenopus laevis* folate receptor-α (xFRα)-overexpressing embryos at the indicated developmental stages. (C) Folate is present during *Xenopus* neural tube formation and folate levels do not change upon FRα knockdown. Data shows folate levels measured by ELISA assay in homogenates from stage-13-17 (14.5-19 Development • Advance article
hpf) wild-type (WT), folate receptor-alpha morpholino 1 (FRα-MO1)- or standard control morpholino (CMO)-injected embryos, mean±SEM, n: 8 independent measurements from n>40 embryos/group, average embryo weight: 2±0.4 mg. (D,E) Neural plate stage embryos were processed for xFRα (green) and β-tubulin (D, red) or C-cadherin (E, red) immunostaining. Shown are representative transverse sections of immunostained samples at neural plate stages 15 (D) and 17 (E). (D) Arrow on top panel indicates xFRα localization at the apical neural plate surface and arrowheads indicate localization in extracellular spaces. Panels on the bottom correspond to the outlined area on the panel above and illustrate xFRα localization to extracellular spaces. Not: notochord, L: notochord lumen. (E) White arrow indicates that xFRα localizes to apical neural plate surface along with adherens junction protein C-cadherin. Dashed box corresponds to the zoomed region below. Scale bars, 20 μm.
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Fig. 2. Efficacy and specificity of folate receptor-α knockdown by targeted translation-blocking morpholino. Two- (A,C,D) or sixteen- (E-G) cell-stage embryos were unilaterally (A,E-G) or bilaterally (C,D) microinjected with standard control morpholino (CMO), morpholino against xFRα (FRα-MO) and/or with 600 pg FRα-MO-sensitive (wt-xFRα mRNA or sense-flag-xFRα mRNA) or insensitive xFRα mRNA (flag-xFRα mRNA or resistant-xFRα mRNA). (A) Two non-overlapping xFRα translation blocking morpholinos (10 pmol/blastomere) elicit similar defects in neural plate folding. Shown are representative examples of unilaterally injected CMO, FRα-MO1 and FRα-MO2 embryos, red indicates morpholino-containing tissue. (B) Schematics of construct design for FRα-mRNA sensitive and resistant to FRα-MO. (C,D) Specificity of FRα-MO. Representative Western blot assays from neural plate stage embryos bilaterally injected (10 pmol MO/blastomere) with the indicated constructs and probed with anti-flag (C) or anti-FRα (D). GAPDH and Na+/K+ ATPase α1 subunit were used as loading controls. (E,F) Representative examples of transverse sections from neurulating embryo unilaterally microinjected at the 16-cell stage with 1.8 pmol FRα-MO (E) or CMO (F) per blastomere along with GFP-mRNA in the dorsal medial and lateral animal blastomeres, as indicated in drawing on the right, sectioned and processed for xFRα (green), GFP (red) and β-tubulin (white) immunostaining. Red indicates FRα-MO- (E) or CMO- (F) containing cells. Scale bars, 20 µm. (E) FRα-MO downregulates expression of endogenous xFRα in the neural plate apical surface. Brackets indicate same number of FRα-MO-containing (red) and wild type (white) apical neural plate cells. (F) Presence of CMO in medial neural plate cells does not affect FRα expression. (G) Graph shows area labeled by apical FRα immunostaining in 3 wild type and morpholino-containing medial neural plate cells (indicated with brackets in (E,F)), mean±SEM, n=42 sections from 10 FRα-MO and CMO unilaterally-injected embryos, ****p<0.0001, ns: not significant, paired and unpaired Student t-tests.
**Fig. 3. Folate receptor-α knockdown in medial neural plate induces neural tube defects.**

(A-B) Two-cell-stage embryos were bilaterally microinjected with standard control morpholino (CMO) or morpholino against xFRα (FRα-MO). Rescue experiments were done by either microinjecting along with FRα-MO, 250 pg xFRα mRNA resistant to FRα-MO (resistant-xFRα mRNA) or by incubating FRα-MO injected embryos with 150 μM folinic acid (FA). (A) Representative examples of control and experimental sibling embryos during neural tube closure (20 hpf) from 4 out of 6 experiments in which the severe NTD phenotype prevails in FRα-MO-injected embryos. Arrowheads indicate open neural tube. Graph shows incidence of severe and none severe defective neural tube, mean±SEM, (n of embryos): 20 pmol CMO (89), 20 pmol FRα-MO (68), 20 pmol FRα-MO+150 μM FA (54), FRα-MO+xFRα mRNA (46), ****p<0.0001, two-tail Mann-Whitney U-test (Wilcoxon rank-sum test). (B) FRα-MO-induced moderate NTD phenotype results in open neural tube. Shown are representative examples of whole embryos at stage 21 and transverse sections of the neural tissue from FRα-MO-injected embryos exhibiting moderate NTD phenotype (arrowheads, 2 out of 6 experiments) and their siblings injected with CMO or FRα-MO and FA. Embryos were then processed for β-tubulin (green) and E-cadherin (red) immunostaining and nuclear labeling (blue, DAPI). Graph shows percent of embryos with open neural tube, (n of embryos): CMO (21), FRα-MO (21), FRα-MO+FA (20), ****p<0.0001, ***p<0.0005, *p<0.01, Mann-Whitney two-tail U-test (Wilcoxon rank-sum test). (C) Dorsal medial and lateral (medial+lateral neural plate, NP) or only dorsal medial (medial neural plate, Med NP) animal blastomeres from 16-cell-stage embryos were microinjected with 3 pmol/blastomere CMO or FRα-MO along with Alexa 594-dextran conjugate (in red). Embryos were fixed and photomicrographed under a macroscope. Red indicates CMO- or FRα-MO-containing tissue. Embryos with severe neural tube defects out of total in each group were: 0/33 CMO-NP, 42/42 FRα-MO-NP and 44/44 FRα-MO-Med NP.
Fig. 4. Folate receptor-α in medial neural plate cells is necessary for changes in cell morphology necessary for neural plate folding. Representative examples of neurulating embryos unilaterally microinjected at the 16-cell stage with 1.8 pmol FRα-MO along with GFP-mRNA in the dorsal medial (A-C) or dorsal lateral (D) animal blastomeres, as indicated in drawings on the right, sectioned and processed for Sox2, neural progenitor marker (blue), FRα (green), GFP (red) and β-tubulin (white) immunostaining. Red indicates FRα-MO-containing cells. (A) FRα knockdown in medial neural plate (Med NP) cells impedes neural plate folding. Asterisks indicate same number of medial cells in wild type (blue) and FRα-deficient (pink) sides of the neural plate counted from the midline to represent the impact to the extent of the medial apical surface (white and red double arrows). Curves indicate the bending of the neural plate in wild type (white) and FRα-deficient (red) tissues. Arrows in (A) indicate cells shown in (B) (white, wild type) and (C) (red, FRα-deficient). (B) Wild type apically constricted (bracket) cell (2nd from the midline) from the superficial layer of the medial neural plate. Arrowhead indicates apically localized FRα. (C) Cell (2nd from the midline) from the superficial layer of the FRα-deficient side (red) of the medial neural plate fails to constrict (bracket). Arrowhead indicates lack of apically localized FRα. (D) FRα knockdown in lateral neural plate (Lat NP) cells does not induce any apparent morphogenic phenotype. Scale bars, 20 µm.
Fig. 5. Medial neural plate cells deficient in folate receptor α fail to constrict apically during neural plate folding. Two-cell-stage embryos were unilaterally microinjected with 10 pmol morpholino against xFRα (FRα-MO) or control morpholino (CMO) along with Alexa Fluor 594-dextran conjugate and bilaterally injected with membrane-GFP. Apical neural plate was time-lapse imaged from whole embryos at a rate of 0.2 min⁻¹. Regions of interest were selected to contour cells close to the midline that remain visible during 1-h recording and did not divide during this period. (A) Shown are representative examples for
indicated time points of FRα-MO- and CMO-unilaterally-injected and imaged embryos. Outlined is one wild type (dashed) and MO-containing (solid) cell for which the apical surface was measured over time. Numbers indicate apical cell surface for the same outlined cells at 0 and 60 min of recording. Scale bar, 10 μm. (B) Graph shows apical cell surface (in %) at the indicated time points relative to the same cell surface at time 0 (100%), mean±SEM, n: 30 FRα-MO-, 28 CMO-containing cells, and 30 contralateral wild type (WT) cells per group, ****p<0.0001, ns: not significant, 2-way ANOVA.
Fig. 6. Folate receptor interacts with C-cadherin and is necessary for its endocytosis in medial neural plate cells during neural plate folding. (A) Neural plate stage embryos were processed for xFRα (green), C-cadherin (red) and β-catenin (blue) immunostaining. Shown is a representative transverse single z-section of immunostained stage-15 medial neural plate cell with orthogonal view through the indicated plane to demonstrate partial apical colocalization of FRα, C-cadherin and β-catenin in the neural plate. Scale bar, 5 µm. (B) C-cadherin and β-catenin coimmunoprecipitate with FRα. Lysates from wild-type neural plate-stage embryos were incubated with anti-FRα-crosslinked beads or naked beads. Coimmunoprecipitated proteins were dissociated from beads and run in SDS-PAGE gels for Western blot assays with anti-FRα, C-cadherin and β-catenin antibodies. Shown are representative Western blot assays performed 5 times with similar results. (C) The number of C-cadherin-containing vesicles including C-cadherin-containing early endosomes is reduced in FRα-deficient medial neural plate cells. Two-cell-stage embryos were unilaterally microinjected with CMO and FRα-MO in contralateral blastomeres. Shown is a representative example of transverse section from neurulating embryo immunostained for C-cadherin (green) and EEA1 (red). Schematic indicates the cells from which number of immunopositive vesicles were counted. Yellow arrow points to a C-cadherin+/EEA1+ vesicle and white arrow points to a C-cadherin+/EEA1− vesicle. Scale bar, 10 µm. Graph shows mean ± SEM C-cadherin and EEA1 labeled vesicles/cell measured in 54 CMO- and 54 FRα-MO-containing medial neural plate cells in 18 sections from 5 embryos, **p<0.005, paired Student t-test.
Fig. 7. Pharmacological inhibition of folate uptake systems affects *Xenopus laevis* neural tube formation. Embryos at stage 13 (14.75 hpf) were incubated with 100 nM-1.5 mM methotrexate (MTX), 1 nM-300 μM methotrexate ester, 1 nM-100 μM pyrimethamine, 100-500 μM trimetrexate or vehicle (control, 0.3% DMSO or saline). Embryos were fixed and photographed under a macroscope when controls reached neural tube closure, at stage 20 (22 hpf). Shown are representative examples. Arrowheads indicate defective neural tube closure. Rescue of 1.5 mM MTX-induced NTD phenotype was performed by preincubating 2-4-cell-stage embryos with 10 mM folinic acid (FA). Graph shows mean ± SEM percent defective embryos, n≥6 independent experiments with n≥10 embryos per group, per experiment, *p<0.01, 2-way ANOVA.
Materials and Methods

*In vitro fertilization.* Mature oocytes were collected in a dish from a previously hCG injected female frog and a small piece of testis was minced. This is considered time 0 of fertilization. Fertilized oocytes were kept in 10% MMR saline, containing (in mM): 10 NaCl, 0.2 KCl, 0.1 MgSO₄, 0.5 Hepes, 5 EDTA and 0.2 CaCl₂. Dejellying of embryos was done by briefly swirling fertilized eggs in 2% cysteine solution, pH 8. Developmental stages were recorded according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Animals were handled according to the IACUC guidelines using humane procedures to prevent animal suffering.

*Western blot assays.* Crude membrane fraction was obtained from non-fertilized eggs, stage-10, -17 and -20 embryos, 10 embryos for each group to assess endogenous expression of folate receptor-α (FRα). Briefly, embryos were homogenized in 20 mM Hepes pH 7.4, 1 mM EDTA, 1 mM EGTA, protease inhibitors cocktail (784115, Thermo Scientific) on ice and centrifuged for 10 min at 1,000 rcf. Supernatant was then centrifuged at 16,100 rcf for 30 min. Pellets of crude membrane fraction were resuspended in 2x protein loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% (w/v) glycerol, 0.005% bromophenol blue). PVDF membrane was probed with anti-FRα rabbit polyclonal affinity purified antibody raised against the peptide KHQKVDPGPEDDLHC (custom made by GenScript), 1:500 in 5% milk followed by incubation with HRP-conjugated secondary antibodies (Jackson ImmunoResearch) 1:10,000 and visualized by Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate (cat# NEL103E001, PerkinElmer). PVDF membranes were stripped in 0.2 M glycine HCL buffer, pH 2.5, 0.05% Tween for 20 min and reprobed with anti-α1 subunit of Na⁺/K⁺ ATPase antibody (Abcam, cat#ab767), plasma membrane marker, 1:1000 in 5% BSA. To assess exogenous flag-tagged xFRα expression, 8 stage-17 embryos injected with 600 pg flag-xFRα mRNA at 2-cell stage were homogenized in extraction buffer containing 1% Triton X-100, 150 mM NaCl, 25 mM Tris pH 7.4, 1mM EDTA, 1mM EGTA and protease inhibitors cocktail. Samples were centrifuged at 16,100 rcf for 10 min and pellet discarded. Supernatant was processed as described above and immunoblotted with primary anti-flag tag mouse antibody (cat#MA1-91878 Thermo Scientific), 1:500, then
reprobed with GAPDH antibody, 1:20,000 (cat# sc-47724 Santa Cruz Biotechnology) as protein loading control.

**Coimmunoprecipitation.** Fifteen neurulating embryos were homogenized in extraction buffer containing 1% Triton X-100, 150 mM NaCl, 25 mM Tris pH 7.4, 5 mM EDTA, protease inhibitor cocktail (Thermo Scientific, cat. # 78415) on ice. Insoluble proteins were pelleted by centrifugation at 16,100 rcf for 10 min. FRα was immunoprecipitated from the soluble fraction overnight at 4°C through mild rocking by anti-FRα rabbit polyclonal antibody (GenScript, custom-made) chemically cross-linked to protein G-agarose beads (Roche, cat. # 11719416001). Beads were resuspended in 2X protein loading buffer containing 10 mM DTT and incubated for 10 min at 80°C. Samples were separated on 10% polyacrylamide gels and immunoblotted with anti-C-cadherin (DSHB, 1:100 cat. # 6B6), anti-β-catenin (Sicgen, 1:500 cat. # AB0095-200) and anti-FRα antibodies followed by HRP-conjugated anti-mouse and anti-goat secondary antibodies incubation (Jackson ImmunoResearch, cat. #s 515-035-003 and 705-035-003, respectively) and visualization with enhanced chemiluminescence (ECL2, Pierce, cat. # 80196).

**Measurement of folate levels by ELISA assay.** Stage-12.5-25 embryos were partially dejellied by cysteine treatment and samples were weighed, followed by cell lysis and homogenization with 0.5% Triton X-100. Samples were centrifuged at 13,000 rcf and supernatants were loaded onto ELISA plates. Detection of folate level was performed according to the instructions of the folate receptor binding-based Enzyme Immunoassay Kit for the quantitative determination of folate in biological samples (Cat# 7525-300, Monobind, Inc.). This kit is designed to measure folate levels based on the capacity of folate to bind to the folate receptor.

**Immunohistochemistry.** Stage-14-18 and -21-23 embryos were fixed for 30 min at 23°C with freshly made 2% trichloroacetic acid (TCA) or 4% PFA and processed for immunostaining as previously described (Belgacem and Borodinsky, 2011; Belgacem and Borodinsky, 2015; Borodinsky et al., 2004; Swapna and Borodinsky, 2012) with modifications and by using standard protocols of paraffin embedding and sectioning (Tu and Borodinsky, 2014) excluding the permeabilization step with Triton-X100. Tween-20
was used in blocking, antibody and washing solutions for FRα immunostaining since stronger detergents have been reported to result in artificial redistribution of glycosylphosphatidyl-inositol-anchored proteins (Heffer-Lauc et al., 2007). Incubations with primary and secondary antibodies were carried out overnight at 4°C and for 2 h at 23°C, respectively. Primary antibodies used were: anti-xFRα 1:400 (custom made by GenScript), anti-C-cadherin 6B6 1:50 (Developmental Studies Hybridoma Bank), anti-β-tubulin E7 1:300 (Developmental Studies Hybridoma Bank), anti-E-cadherin 5D3 1:100 (Developmental Studies Hybridoma Bank), anti-GFP 1:500 (cat# GFP-1020), anti-Sox2 1:300 (Cat # AF2018, R&D Systems). Antigen retrieval was performed by microwaving samples in 0.05% citraconic anhydride, pH 7.4 (Namimatsu et al., 2005). Briefly, rack with slides was placed in 200 ml glass container covered with plastic wrap, boiled for 15 sec at maximum power followed by 3 min wait in hot buffer. Slides were washed 2 times for 5 min with PBS. Further processing starting with 1% BSA blocking step was done using SNAP i.d. 2.0 System for immunohistochemistry (Millipore).

**FRα knockdown.** Two-cell-stage embryos were bilaterally injected with 4-10 nl of 1 mM FRα-morpholinos, FRα-MO1 and FRα-MO2 (MO oligo sequence written from 5' to 3' and complementary to folate receptor: GGCCCCCCGTAACATGGTTACAAGC (FRα-MO1), AATATGGCACGAGTCGCAACCCACA (FRα-MO2)). Controls were sibling embryos injected with standard control morpholino (CMO: CCTCTTACCTCAGTTACAATTTATA). Morpholinos were injected along with dextran-Alexa-Fluor conjugates or along with GFP mRNA to assure permanency of MO reporter after TCA fixation.

Rescue experiments were implemented by expressing FRα-MO1-resistant *Xenopus laevis* FRα mRNA, lacking 3' UTR, 5' UTR substituted for gcc acc sequence (Kozak, 1987a; Kozak, 1987b) and with a number of wobble mutations in coding region (MO-resistant FRα-RNA: ... gcc acc atg ctt aga gga gct ctc; wt-FRα-RNA: ...g ctt gta acc atg tta cgg ggg gcc...) or by incubating embryos with 150 µM folinic acid. mRNA was synthesized as previously described (Belgacem and Borodinsky, 2011; Borodinsky et al., 2004; Swapna and Borodinsky, 2012) using the *Xenopus laevis* FRα template (XGC African clawed frog FRα cDNA, Clone ID: 7012141, Open Biosystems, Thermo Fisher Scientific, Inc.), subcloned into pCS2+ vector. Mutations were done using the site-directed mutagenesis
kit (Ambion; (Swapna and Borodinsky, 2012)) and PCR reaction to render FRα-MO1-resistant mRNA. Two hundred and fifty pg xFRα mRNA were bilaterally microinjected in 2-cell-stage embryos or coinjected with specific MOs. We also designed morpholino-insensitive or front-tagged FRα (flag-FRα) and morpholino-sensitive or back-tagged FRα (FRα-flag) constructs by subcloning *Xenopus laevis* FRα into flag-pCS2+ plasmid (a gift from Peter Klein, Addgene plasmid #16331). Another morpholino-sensitive construct was obtained by annealing of synthesized FRα-MO1 anti-sense and sense 5’GCTTGTAAACCATGTACGGGGGCC3’ oligonucleotides inserted in front of the flag-FRα (sense-flag-FRα). Seven hundred pg of FRα-MO1-resistant *Xenopus laevis* FRα mRNA were injected in one blastomere of 2-cell-stage embryos to unilaterally overexpress *Xenopus* FRα.

Quantitative assessment of FRα knockdown by FRα-MO was done by measuring the area labeled by FRα immunostaining in medial neural plate cell apical surface in sectioned stage-14.5-17 embryos unilaterally injected with FRα-MO or CMO after uniform thresholding of labeled images with NIS Elements software (Nikon, Inc.). Measurements were done in the apical surface of 3 consecutive wild type and 3 paired FRα-MO- or CMO-containing medial neural plate cells from the midline in 10 embryos per group in 42 sections.

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**Data collection and Statistics.** Numbers of samples analyzed are indicated for each set of experiments. Significance was evaluated using Student t-test or Mann-Whitney U-test (Wilcoxon rank-sum test) and ANOVA. Differences were considered significant when p<0.05.


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Movie 1. Impaired apical constriction in FRα-deficient neural plate cells. Two-cell-stage embryos were bilaterally injected with membrane-GFP (in green) and unilaterally with 10 pmol FRα-MO + AlexaFluor 594-dextran conjugate (in red). Confocal images of the apical neural plate in live embryos at stage 15-15.5 were taken every 5 min. Shown are z-projections of the sequence of time frames during 1 h recording. Note decrease in cell surface, folding of the neural plate and appearance of non-neural ectoderm (relatively big hexagonal cells) towards the end of the movie in the wild-type side (top, green), while neural plate cells containing FRα-MO fail to constrict and the neural plate does not fold (bottom, yellow).
Movie 2. Apical constriction is not affected in CMO-containing neural plate cells.

Two-cell-stage embryos were bilaterally injected with membrane-GFP (in green) and unilaterally with 10 pmol CMO + AlexaFluor 594-dextran conjugate (in red). Confocal images of the apical neural plate in live embryos at stage 15-15.5 were taken every 5 min. Shown are z-projections of the sequence of time frames during 1 h recording. Sample realignment was done after 30-min recording to recenter the field of view on the neural plate midline. Note decrease in cell surface and folding of the neural plate in the wild-type (top, green) and CMO-containing neural plate (bottom, yellow).
Movie 3. Apical neural plate cell division. Two-cell-stage embryos were bilaterally injected with membrane-GFP (in green) and unilaterally with 10 pmol FRα-MO + AlexaFluor 594-dextran conjugate (in red). Confocal images of the apical neural plate in live embryos at stage 15-15.5 were taken every 5 min. Shown are z-projections of the sequence of time frames during 25 min recording. Asterisks indicate dividing and daughter cells. Dividing cells were excluded from the measurements of changes in apical cell surface during neural plate folding (Fig. 6). The proportion of dividing apical cells is small during neural plate folding and is similar in wild type and FRα-deficient cells (see Results for details).
Fig. S1. Specificity of Xenopus folate receptor-α antibody. (A,B) Two-cell-stage embryos were unilaterally injected with 700 pg xFRα mRNA+GFP mRNA (A) or Alexa Fluor 594-dextran conjugate (B), paraffin-sectioned and processed for xFRα immunostaining with (A) or without (B) FRα primary antibody added. Scale bar: 20 μm. (C) Schematic of construct to demonstrate specificity of xFRα antibody and representative Western blot from whole cell homogenates from embryos injected at the 2-cell stage with the indicated constructs. xFRα: Xenopus laevis folate receptor-α, FRα-MO: xFRα-targeted translation blocking-morpholino.
Fig. S2. FRα-MO or methotrexate does not induce apoptosis of neural plate cells during neurulation. (A) Two-cell-stage embryos were unilaterally injected with 10 pmol folate receptor-α morpholino (FRα-MO) and Alexa 594-conjugated dextran and grown until neural plate stage 17 (18.75 hpf). (B) Early neural plate stage embryos (stage 13, 14.75 hpf) were incubated in the absence (Control) or presence of 1-1.5 mM methotrexate (MTX) until closure of the neural tube in controls (stage 20, 21.75 hpf). (A,B) Embryos were then processed for TUNEL assay, immunostaining for β-tubulin and nuclear labeling with DAPI. Shown are representative transverse sections of neural tissue from aforementioned embryos. Positive control is a sample treated with DNase; negative control is a sample in which no labeling enzyme (Terminal Deoxynuclotidyl Transferase) was added. Scale bars: 20 μm.
Fig. S3. Knockdown of folate receptor $\alpha$ in mesoderm or non-neural ectoderm does not induce neural tube defects. Targeted blastomeres of 16-cell-stage embryos were unilaterally microinjected with 3 pmol/cell morpholino against the Xenopus laevis FR$\alpha$ (FR$\alpha$-MO) along with Alexa 594-dextran conjugate in indicated blastomeres (right). Neural plate stage embryos were then sectioned and processed for immunostaining with anti-Î³-tubulin (grayscale) and Sox2 (green). Red indicates MO-containing cells. Scale bars: 20 μm.
Fig. S4. **FRα knockdown does not induce changes in actin dynamics or in overall apicobasal polarity in the folding neural plate.** (A) Two-cell-stage embryos were unilaterally microinjected with FRα-MO along with Alexa Fluor 594-dextran conjugate (red) and bilaterally injected with mCherry-UtropinCH (F-actin reporter, green). Superficial medial neural plate was time-lapse imaged from whole embryos at a rate of 1-2 min⁻¹. Fluorescence intensity profiles (arrows) across cell-cell borders were measured among wild-type (WT, 1) and FRα-MO (2) containing cells during 15 min imaging. Scale bar, 10 μm. Intensity profiles were fitted with Gauss function using R software after background substraction. Bar graph shows mean±SEM peak width at 50% maximum intensity (left) and standard deviation (SD) of normalized maximum intensity during 15 min recording (right), n: 28 cell-cell borders per group, t-test. (B) Unilateral FRα-MO-containing neural plate stage embryos were sectioned and processed for immunostaining with anti-ZO-1 (green). Scale bar, 20 μm.
Fig. S5. Neural plate cell apical constriction is concurrent with endocytosis of C-cadherin. Neural plate stage embryos were sectioned and processed for immunostaining with anti-C-cadherin (green) and anti-EEA1 (red). Scale bar, 20 µm. Arrow points to a C-cadherin-containing endosome.