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Emerging roles for folate receptor FOLR1 in signaling and cancer

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Abstract

Folates are B vitamins that function in one-carbon metabolism. Folate receptors are one of three major types of folate transporters. The folate receptors FOLR1 and FOLR2 are overexpressed in multiple cancers. The overexpression of FOLR1 is often associated with increased cancer progression and poor patient prognosis. There is emerging evidence that FOLR1 is involved in signaling pathways that are independent of one-carbon metabolism. Recent publications implicate a direct role of FOLR1 in three signaling pathways: JAK–STAT3; ERK1/2; and as a transcription factor. Six other signaling pathways have been proposed to include FOLR1, but these currently lack sufficient data to infer a direct signaling role for FOLR1. We discuss the data that support non-canonical roles for FOLR1, and its limitations.

Keywords

folate; cancer; cell signaling; JAK-STAT; ERK; folate receptor alpha

Introduction

Folate (vitamin B_9) is an essential nutrient that is required for one-carbon metabolism. The folate receptors FOLR1 and FOLR2 have restricted tissue expression, but many cancers overexpress one of the two receptors. We will review the overexpression of FOLR1 and FOLR2 in different cancers and the impact on cancer progression. Recent publications suggest that FOLR1 functions in a surprisingly large number of intracellular signaling pathways. We will critically appraise the data that implicate FOLR1 in non-metabolic signaling pathways, many of which impact cancer cells.

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Declaration of Interests

The authors declare no conflicts of interest.

Folates and one-carbon metabolism

Folates have a canonical role in one-carbon metabolism, where they donate and receive one-carbon unit. One-carbon metabolism functions in the synthesis of nucleotides (purines and deoxythymidine monophosphate, dTMP), the amino acid methionine (which is required to generate the methyl-donor S-adenosyl methionine), and the interconversion of glycine and serine [1].

Folates are a family of molecules that are comprised of a pteridine ring, para-aminobenzoic acid (PABA), and one or more glutamates (Glu) [1]. Folates differ in their pteridine rings. The folate in serum that mediates systemic distribution in animals is 5-methyl-tetrahydrafolate (5-methyl-THF) with a single Glu [1].

Folate transporters

Mammals utilize three types of folate transporters: the reduced folate carrier (RFC); the proton-coupled folate transporter (PCFT); and folate receptors (FOLRs). RFC is a low-affinity, high-throughput transporter that is ubiquitously expressed in mammalian cells and mediates the bulk of reduced folate transport, predominantly the serum folate 5-methyl-THF, into the majority of mammalian cells [2] (Fig. 1, left). PCFT absorbs dietary folates in the small intestine [3] (Fig. 1, middle).

FOLR are high-affinity, low-throughput transporters [2]. There are four FOLR genes, *FOLR1–4* (aka, folate receptors α, β, γ, and δ; and the historical name folate binding protein, FBP). FOLR2 is expressed in a subset of macrophages and monocytes [4-6]. FOLR3 is expressed in neutrophil granulocytes and monocytes and is a secreted protein that binds folate in the bloodstream [7,8]. FOLR4 is expressed in regulatory T cells and mammalian eggs, but is unable to bind folate [9,10]. In the egg, FOLR4 binds the sperm protein Izumo to allow fertilization [9]. FOLR1 and FOLR2 bind folates on the cell surface (Fig. 1, right), and FOLR bound to folate are brought into the cell by endocytosis [2]. After endocytosis, folates bound to FOLR1 are released from acidified endosomes into the cytoplasm by PCFT [11].

FOLR1 has limited tissue expression that is generally restricted to the luminal (apical) surface of polarized epithelia, including proximal kidney tubules, type 1 and 2 pneumocytes in the lungs, choroid plexus, ovary, fallopian tube, uterus, cervix, epididymis, submandibular salivary gland, bronchial glands, and trophoblasts in the placenta [12]. In several tissues, FOLR1 functions in the transcytosis of folates across cellular barriers. In the choroid plexus, FOLR1 transports folates from the basolateral to the apical membrane of the choroid plexus, and is then transported in exosomes across the blood brain barrier [13]. In the placenta, FOLR1 transports folates from the mother to the fetus [14]. And in the kidney, FOLR1 reabsorbs folates from pre-urine for transport back into the body [15].

Non-canonical signaling roles of the folate receptor in non-mammals

Caenorhabditis elegans folate signaling through FOLR-1

In the roundworm *C. elegans*, the folate 10-formyl-THF acts as a signal to increase the rate of germ stem cell proliferation [16]. Three lines of evidence indicate that 10-formyl-THF acts as a signal, rather than through metabolism. First, the stimulation of germ cell proliferation by 10-formyl-THF requires FOLR-1, but FOLR-1 is not required to provide folates for metabolism (which is instead the role of RFC). Second, other folates can rescue folate deficiency better than 10-formyl-THF but are unable to stimulate increased germ cell proliferation above the basal rate, thereby decoupling metabolism from the stimulatory role. Third, a ligand that cannot function in metabolism (a pteroate, structurally similar to folates but lacking Glu) can stimulate the FOLR-1-dependent pathway.

A potential non-canonical role for FOLR1 in Xenopus

In embryos of the frog *Xenopus laevis*, the apical surface of the neural plate constricts to induce the curvature that shapes the neural tube. FOLR1 is localized to the apical surface and is required for the constriction of the neural plate [17]. FOLR1 physically interacts with C-cadherin and β-catenin, which are components of adherens junctions [17]. FOLR1 is required for the endocytosis of C-cadherin from the apical surface, which facilitates the constriction [17]. The physical interaction of FOLR1 with C-cadherin suggests that FOLR1 may directly mediate the endocytosis of C-cadherin endocytosis, which would be a non-metabolic role in regulating adherens junctions.

FOLR1 overexpression and linkage to cancer progression

FOLR1 is overexpressed in many cancers of epithelial origin (Table 1). Among the highest levels of FOLR1 overexpression are in cancers of the female reproductive tissues – the ovary and uterus (Table 1). Significant overexpression of FOLR1 is also observed in brain carcinomas [18]. Additionally, FOLR1 overexpression is associated with metastatic pancreatic carcinomas and lymphomas [18].

Multiple studies have been conducted to determine the association between FOLR1 and cancer progression or reduced patient survival. A meta-analysis was performed on breast, lung, endometrial, and ovarian cancer that assessed the effect of FOLR1 overexpression on patient survival [19]. It was reported that breast cancers with high FOLR1 expression had a **hazard ratio (HR)** (see Glossary) of 2.66 for **disease-free survival (DFS)** and a HR of 1.97 for **overall survival (OS)** [19]. This indicates that FOLR1 overexpression in breast cancer results in a 97% higher likelihood of patient death. Uterine endometrial cancer had a HR of 1.3 for DFS [19]. There was no statistical increase in DFS or OS for ovarian and lung cancers [19]. However, a caveat is that because such a high percentage of ovarian cancers express FOLR1 (Table 1) comparisons between FOLR1-expressing and non-expressing cells are not as relevant.

Despite the lack of correlation of FOLR1 expression with patient survival for all types of ovarian cancer [19-22], higher expression of FOLR1 is observed in ovarian cancers with higher histologic grades, more advanced stages, increased aneuploidy, a higher percentage

of S phase cells, and higher resistance to chemotherapy – all markers of cancer progression [23-26]. Patients with serous ovarian carcinomas with higher FOLR1 expression levels had increased HRs for DFS (2.45) and OS (3.60) [24]. Additionally, inhibition of FOLR1 in ovarian cancer cells in vitro reduced the cancer attributes of cell proliferation, growth in soft agar, and cell migration and invasiveness [25,27].

FOLR2 is overexpressed in 50% of myelogenous leukemias [28,29]. Because FOLR2 is expressed in tissue-resident macrophages, FOLR2-positive signals often increase near solid tumors due to macrophages in the stroma [4]. Careful analysis that distinguished FOLR2 signal in the stroma vs. in cancers found that nearly 25% of cancer samples express FOLR2, primarily malignancies of the lung, liver, skin, kidney, and soft tissue [30] (Table 1). However, the expression of FOLR2 does not correlate with the stage of cancer, size of tumors, or involvement of lymph nodes [30].

Inactivation of FOLR2 has been shown to reduce ART, mTOR, and ribosomal S6 kinase signaling in non-small cell lung cancer cell lines [31]. However, that study did not provide evidence for a direct role of FOLR2 in signaling vs. an indirect role in metabolism.

Indirect evidence that FOLR1 functions independently of metabolism

FOLR1 has been proposed to promote cancer by providing increased levels of folates for one-carbon metabolism [32]. However, even when FOLR1 is overexpressed, it generally transports less folate into cells than RFC [33]. In five ovarian cancer cell lines (including SKOV-3 cells), FOLR1 only contributes ~20% of the uptake of the serum folate 5-methyl-THF, while RFC is responsible for ~70% of the uptake [33].

In SKOV-3 cells, FOLR1 *knockdown* reduced cell proliferation, migration, and invasiveness, implying that FOLR1 expression promotes these cancer attributes [25]. Surprisingly, the *overexpression* of RFC had a similar effect in reducing cell proliferation, migration, and invasiveness. That the overexpression of RFC and FOLR1 produced contrasting outcomes of inhibiting and promoting cancer attributes suggests that the cancer attributes do not arise simply from increasing folate uptake. A caveat is that the study was carried out with folic acid (FA) at 12–60 nM as the sole source of folate [25]. RFC transports the fully-oxidized FA much less efficiently than reduced folates [34]. When FA is provided at low, physiological levels, FOLR1 may transport the majority of the FA. Hence, it is possible that the reduction of cancer attributes associated with FOLR1 knockdown resulted from a lack of folates for metabolism.

Additional evidence that suggests FOLR1 acts independently of metabolism is that the proliferation of erythropoietic cells is significantly increased by incubation with anti-FOLR1 antibodies [35]. The binding of antibodies to cell surface receptors can, in many instances, activate signaling independently of ligand binding [36]. This particular anti-FOLR1 antibody prevents folate from binding to FOLR1 [35] suggesting that the stimulation of erythropoiesis was independent of folate transport.

Criteria for a direct FOLR1 signaling role

Our assessment of whether the data supports a non-canonical role for FOLR1 in signaling relies on three criteria: 1) demonstrating that inactivating FOLR1 reduces aspects of the signaling; 2) physical interaction of FOLR1 with signaling components; and 3) that signaling events occur rapidly after folate stimulation.

Short time frames for the activation of intracellular signaling events (e.g., within 2–10 minutes) are consistent with a direct effect on signaling. We cannot formally rule out the possibility that the rapid signaling events arise from changes in one-carbon metabolism. We are not aware of published studies that determine how quickly cells alter the levels of cellular readouts of one-carbon metabolism, such as nucleosides or methionine, in response to the addition of external folate. Thus, the rapidity of changes in one-carbon metabolism are not known. However, there are currently no known mechanisms by which changes in one-carbon metabolism would rapidly alter intracellular signaling events (such as protein phosphorylation).

Physical interaction of FOLR1 with signaling components suggests a direct role in the signaling pathway. In the analyzed papers, physical interaction is analyzed by co-immunoprecipitation (co-IP). Notably, co-IP indicates physical association but does not imply direct protein-protein interaction, as proteins can physically associate with each other via binding to intermediary proteins.

When assessing experimental results, it is important to note the type of folate that is used. Many studies state that they are using “folate”. However, “folate” can refer to the reduced folates that take part in one-carbon metabolism or to FA, which is a synthetic, non-natural folate that is fully oxidized [37]. FA must undergo two rounds of reduction by dihydrofolate reductase (DHFR) in order to be used in one-carbon metabolism [37]. Surprisingly, FOLR1 has a much higher affinity for the non-natural FA than for naturally-occurring reduced folates [38]. For example, the affinity of FOLR1 for FA is 14-fold higher than for the serum folate 5-methyl-THF [38]. In contrast, RFC transports FA one-to-two orders of magnitude less efficiently than reduced folates [34]. Therefore, the use of FA shifts cellular responses toward FOLR1 and away from RFC.

The concentration of folates used to stimulate cells is important. Certain studies use excessive, supraphysiological folate concentrations to stimulate cells (see Table 2). Excessive folate levels can potentially induce non-physiological cellular responses. We are mindful that when trying to detect rapid readouts of intracellular signaling pathways (e.g., phosphorylation of signaling proteins), it is often helpful to transiently pulse with a high ligand concentration. Ideally, this would be combined with experiments using physiological concentrations to follow downstream events that are regulated by the pathways.

Additionally, it is important to consider the basal level of folate in the cell culture medium prior to the addition of folate. Tissue culture media contain a wide range of FA concentrations, such as, no folate (for folate-free RPMI-1640), 2.3 μM FA (for RPMI-1640), and 9.1 μM FA (for DMEM). A 9.1 μM FA concentration is 325 to 758-fold higher than the median levels of serum folate (which was 0.012 μM in the US population pre-fortification

and 0.028 μM post-fortification [39]). FOLR1 has a K_D of only ~0.0001 μM for FA [38], which implies that FOLR1 is near-maximally stimulated in most cell-culture media prior to adding extra folate.

Several optimal experimental strategies for folate levels can be envisioned. To determine the role of FOLR1 when analyzing FOLR1 knockout cells it would be helpful to provide physiological levels of 5-methy-THF so that the uptake of folate through RFC occurs normally. To assess rapid cellular responses, cells can be starved for all folate by using folate-free medium supplemented with fetal bovine serum that has been charcoal-treated or dialyzed to remove folate present in FBS [25,40]. The levels of FA added can be at physiological levels to activate the FOLR1 response, given its very low K_D for binding FA [38].

Signaling pathways linked to FOLR1

In this section, we describe three signaling pathways for which the evidence suggests a direct signaling role for FOLR1. There are six other reported signaling pathways for which there is insufficient evidence to infer a direct signaling role for FOLR1 that will not be discussed in detail. These include proposed roles of FOLR1 in pathways for: Wnt and ERK (where adding 0.1 μM FA increases the expression of active beta-catenin, phospho-ERK, and Wnt and ERK-regulated genes within 12-48 hrs, with FOLR1 required for the increased gene expression) [41]; Wnt and TGFβ ($FOLR1^{-/-}$ mouse embryonic fibroblasts have increased Wnt activity and reduced TGFβ activity in response to 40 μM FA) [42]; NOTCH3 and FGFR1 (overexpression of FOLR1 upregulates *NOTCH3* and *FGFR1* mRNA) [43]; and LYN tyrosine kinase and the G-protein coupled receptor component $G\alpha_{i\text{-}3}$ (FOLR1 physically interacts with both proteins in an ovarian cancer cell line, but no data on whether FOLR1 impacts those signaling pathways) [44].

JAK–STAT3 signaling

JAK–STAT3 signaling is often activated in epithelium-derived cancers, where it promotes proliferation and is associated with poor patient prognosis [45]. Three laboratories have reported that FA induces the activation of JAK–STAT3 signaling [40,46,47] (Table 2) (Fig. 2). Addition of FA induces the activating phosphorylation of the STAT3 transcription factor within 5 min in HeLa cells [40], and within 30 min in mouse neural precursor cells [46]. The fast activation suggests a non-metabolic pathway. FOLR1 co-IPs with the gp130 co-receptor that binds JAK, suggesting a direct linkage between FOLR1 and the signaling pathway [40,46] (Fig. 2).

Issues of concern for JAK–STAT3 signaling include the very high levels of FA (453–1360 μM) used to induce phospho-STAT3 [40,46,47]. These FA levels are >16,000-fold higher than the median concentration of serum folate in the fortified US population (0.028 μM) [39]. In neural precursor cells, stimulation with 1134 μM FA produced substantially more phospho-STAT3 than stimulation with 453 μM, suggesting that the pathway is not saturated at 453 μM [46]. This does not match the expectation of FOLR1 initiating the signaling because it would be expected that FOLR1 binding to FA would be saturated at 453 μM (given the K_D for FOLR1 binding FA is ~0.0001 μM [38]), and so a further increase in FA

levels should not increase FOLR1 activity. This leads to the question of whether FOLR1 is required for the induction of phospho-STAT3. Two FOLR1 knockdown experiments showed either a normal increase of phospho-STAT3 in response to FA (despite FOLR1 knockdown to undetectable levels) or a reduced but still significant increase of phospho-STAT3 [40]. This suggests that FOLR1 may not be essential for the activation of the JAK–STAT3 pathway in response to FA.

ERK1/2 signaling

There are multiple papers from three laboratories that describe FOLR1 promoting signaling that involves the serine/threonine kinases ERK1 and ERK2 (MAP kinase) (Table 2).

FOLR1/PGR/SRC/ERK/IκB-α/NF-κB/p53/p21 & p27—Several papers from the Lee laboratory describe a signaling pathway for FOLR1 that includes the progesterone receptor (PGR) and the tyrosine kinase SRC [48-51] (Fig. 3A). FOLR1 is shown to physically associate with PGR in the COLO-205 colon cancer cell line, and PGR independently interacts with SRC [49]. The data infers a downstream pathway of activated phospho-SRC inducing the phospho-activation of ERK1/2. ERK1/2 phosphorylates IκB-α to release the NF-κB transcription factor, which translocates to the nucleus where it induces the expression of the tumor suppressor p53 transcription factor. p53 then induces the expression of the CDK-inhibitors $p21^{CIP1}$ and $p27^{KIP1}$, which bind cyclin-dependent kinases (CDKs) to inhibit the cell cycle [48,49,50] (Fig. 3A). FOLR1 is required for the FA-induced increase in phospho-SRC and p53 levels [48].

The main features of this pathway are observed in human umbilical venous endothelial cells, except that the folate receptor expressed in those cells is FOLR3 rather than FOLR1 [52,53]. The physical interaction of FOLR3 and phospho-SRC was shown to increase in the cytosol upon adding FA [52].

A similar pathway is present in breast cancer cell lines, where FOLR1, PGR, and SRC all co-IP together [51]. In the breast cancer cell lines, FA promotes cell proliferation and migration. The promotion of cancer attributes in breast cancer cells by FOLR1–ERK signaling is consistent with FOLR1 overexpression worsening breast cancer outcomes [19]. The contrasting FOLR1–ERK-mediated inhibition of cancer attributes in colon cancer cells is consistent with FOLR1 protecting against colon cancer in a mouse model [54]. Intracellular signaling pathways often produce different cellular outcomes in different tissues as a consequence of the differential expression of downstream effectors [55].

FOLR1/MEK/ERK/TSLC1—Z. Liu et al., 2017, described a different FOLR1 and ERK1/2 pathway in a nasopharyngeal cancer cell line [56] (Fig. 3B). FA at 10 μM induces an increase in phospho-MEK and phospho-ERK1/2 levels in 10 min [56]. FOLR1 is required for the FA-induced increase in phospho-ERK1/2 levels, and the inhibition of cell proliferation, migration, and invasiveness [56]. The pathway is associated with increased transcription of the tumor suppressor TSLC1 [56], which is downregulated or inactivated in 87% of nasopharyngeal tumors [57].

An analysis of cervical cancer tissue samples showed a correlation between the severity of the lesions and increased levels of FOLR1, phospho-ERK1/2, and phospho-FOS and phospho-JUN transcription factors [58]. FOS and JUN can be phospho-activated by ERK1/2 [59] to promote cancer and cell proliferation [59].

Issues of concern for ERK1/2 signaling focus on the higher than physiological level of FA used to activate the signaling pathway. 10 μM FA was used to induce the FOLR1-dependent phosphorylation of SRC or ERK1/2 within 2–10 min [48,56]. While this concentration of FA is substantially lower than in the JAK–STAT3 studies, it is still elevated. Both groups report using RPMI-1640 as the base (90%) culture medium, which has 2.3 μM FA [48,56]. Thus, the added 10 μM FA is higher level than what is in the culture media. It would be worthwhile to analyze these pathways in response to lower folate concentrations added to folate-free media.

FOLR1 as a transcription factor

The Mayanil laboratory published three papers where they provide data suggesting that FOLR1 functions as a transcription factor that translocates from the cytoplasm to the nucleus, where it directly regulates gene expression [60-62] (Table 2) (Fig. 4). Two FOLR1 isoforms of 42 and 38 kDa are initially observed primarily in the cytosol [60]. The 38 kDa isoform undergoes nuclear localization 15–30 min after stimulation with 453 μM FA in the human medulloblastoma cell line DAOY, and with 22 nM of 5-methyl-THF in the mouse neural crest cell line O9-1 [60,62].

FOLR1 has previously been reported to be localized to the nuclear envelope in murine Muller retinal cells [63]. TEM images in that publication suggest that FOLR1 is also in the nucleoplasm (see their Fig. 5C) [63].

The Mayanil laboratory used a candidate gene approach to identify six genes whose transcription is increased by FA in an FOLR1-dependent manner [60,61]. FOLR1 was shown (using chromatin-immunoprecipitation) to physically associate with the regulatory regions of the *HES1, FGFR4, OCT4, SOX2*, and *KLF4* genes within 15–30 min of stimulation with 223 nM FA [60,61]. FOLR1 is proposed to bind AANTT DNA sequences based on in vitro binding to oligonucleotides and electrophoretic mobility shift assay [60,61]. AANTT has too little information to direct specific binding for a monomeric FOLR1. Most transcription factors increase DNA-binding specificity either by homomeric association or interaction with other DNA-binding proteins [64], and presumably FOLR1 would employ a similar strategy.

The Mayanil laboratory reported that the 38 kDa isoform of FOLR1 physically bound to chromatin assembly factor 1 subunit A (CHAF1A, CAF-1) and the microRNA processing Drosha–DGCR8 complex [61,62]. FOLR1 was proposed to act as an inhibitor of both CAF-1 and Drosha–DGCR8, but this was not directly tested.

Issues of concern for FOLR1 as a transcription factor include that only a single laboratory has reported this pathway, and so reproducibility between laboratories has not been established. Several conclusions in the papers are not well supported, including the DNA

sequence bound by FOLR1 and the functional relevance of binding Drosha–DGCR8 and CAF-1. Another issue is the nature of the culture media used for experiments. Of the three papers, only the middle chronological paper mentions using folate-depleted media, but it was not described how the media was folate-depleted [61]. There is no mention of folate-depleted media in the third chronological paper, where a physiological level of 22 nM 5-methyl-THF was reported to induce FOLR1 nuclear localization [62]. The base medium for culturing the cells in that paper was reported to be NeuroBasal, which has 9.1 μM FA [62]. A 22 nM concentration of 5-methyl-THF would presumably require a medium largely devoid of FA to have an effect.

Assessment of evidence for non-metabolic signaling

A central question is whether FOLR1 functions directly in signaling vs. through changes in one-carbon metabolism. The studies on FOLR1 functioning as a transcription factor imply non-metabolic functions, as FOLR1 is reported to physically associate with the DNA regulatory regions of genes whose expression is regulated in an FOLR1-dependent manner [61]. For the other pathways, the current data cannot conclusively rule out that the FA-induced changes in the signaling pathways are not due to one-carbon metabolism. The rapid timing of signaling events cannot be used to distinguish this because there is a lack of data on how quickly the outputs of one-carbon metabolism are altered after the addition of FA. And while there are no known mechanisms by which changes in the rate of one-carbon metabolism would induce the rapid signaling events, this cannot formally be ruled out.

An ideal approach to distinguish the role of FOLR1 in signaling vs. metabolism would be to use a non-metabolic ligand for FOLR1. Pteroates linked to alternate molecules (rather than Glu) can bind to FOLR1 at high-affinity [65] and are presumably non-metabolic. Demonstrating that FOLR1-dependent signaling can be activated by a non-metabolic ligand would provide definitive evidence that the signaling is independent of one-carbon metabolism.

Concluding Remarks

FOLR1 has been associated with nine potential signaling pathways. Current evidence is not sufficient to infer that FOLR1 directly regulates the following pathways: Wnt; TGFβ; NOTCH3; FGFR1; LYN; and $G\alpha_{i\text{-}3}$. For the remaining three pathways, several aspects of these pathways are not determined or are of potential concern (see Outstanding Questions). The current data suggests that the JAK-STAT3 pathway requires high, non-physiological concentrations of folate and the importance of FOLR1 for the response is not well established. The data on FOLR1 functioning as a transcription factor would benefit from replication by a second laboratory. Currently, the most compelling evidence for FOLR1 signaling is via the activation of ERK1/2, which has been demonstrated by three laboratories to occur with lower, although still non-physiologically-high, levels of FA. Secondary effects of changes in metabolism cannot be fully ruled out for the JAK–STAT3 and ERK1/2 pathways. But this could be addressed by determining if these pathways can be activated by a non-metabolic ligand for FOLR1.

FOLR1 is often overexpressed in epithelium-derived cancers and is associated with neoplastic progression and poor prognosis in a subset of those cancers. FOLR1 has the highest affinity for FA [38], and thus would be expected to be particularly activated by the presence of unmetabolized FA (UMFA). The fortification of grains in the US led to a consumption of 215–240 μg/day of FA [66], which is below the level expected to generate UMFA (≥ 300 μg/day) [67]. However, the use of FA supplements increases the likelihood of UMFA [67]. A study of fasting post-menopausal women in the US found that 78% had serum UMFA [68]; and nearly all pregnant women taking FA supplements in a Canadian study had serum UMFA [69]. Thus, if FOLR1 signaling is established, it would be useful to determine its role in normal physiology and cancer progression for populations with or without UMFA.

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Glossary

Disease-free survival (DFS)
the length of time after primary treatment during which the patient does not show symptoms of cancer.

Hazard ratio (HR)
a ratio of how often a particular deleterious event happens in one group compared to another group (generally the control group) over time. For example, a HR above 1 for DFS means that the analyzed group had less DFS (with less DFS being the deleterious event) than the control group.

Overall survival (OS)
the length of time from either the date of diagnosis or the start of treatment that patients remain alive.

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Outstanding Questions

- What is the normal (non-cancer) physiological roles of the proposed FOLR1 signaling pathways, and in which tissues do they function?
- What is the physiological relevance of JAK–STAT3 signaling at normal serum folate levels (rather than the supraphysiological concentrations tested), and is FOLR1 required for the signaling pathway?
- How does folate binding to FOLR1 mechanistically initiate the ERK1/2 signaling pathways?
- What is the functional relevance (if any) of the physical interaction of FOLR1 with LYN and $G\alpha_{i\text{-}3}$?
- For FOLR1 functioning as a transcription factor, what is the structure of the 38 kDa FOLR1 isoform that translocates to the nucleus?
- How does FOLR1 localize to the cytoplasm (for both ERK1/2 signaling and FOLR1 acting as a transcription factor), and does the cytoplasmic form of FOLR1 have a GPI-anchor?
- Are any of the observed signaling pathway activations secondary consequences of activating different upstream signaling pathways?
- To what extent does UMFA from the ingestion of FA supplements activate signaling in normal human physiology or cancer? Would the use of a different folate that can function in one-carbon metabolism but with a lower affinity for FOLR1, such as folinic acid (125-fold lower affinity), reduce this signaling? Given the proposed non-canonical role of FOLR1 in *Xenopus* neural tube closure, would the use of folinic acid as a supplement (rather than FA) provide the same reduction in neural tube defects (as it might not activate FOLR1 to the same extent)?

Highlights

The folate receptors FOLR1 and FOLR2 have restricted normal tissue expression but are overexpressed in multiple cancers. The overexpression of FOLR1 in several epithelium-derived cancers promotes cancer progression.

Physical association of FOLR1 with signaling components and the rapid induction of signaling events upon addition of folate suggests that FOLR1 can function directly in intracellular signaling pathways.

There are multiple proposed FOLR1-linked signaling pathways. Three of the signaling pathways have stronger evidence for the direct involvement of FOLR1. Limitations of the data do not allow a definitive assessment of the physiological relevance of the proposed pathways. Nevertheless, the overall data suggests the likelihood that FOLR1 has non-canonical role(s) in signaling.

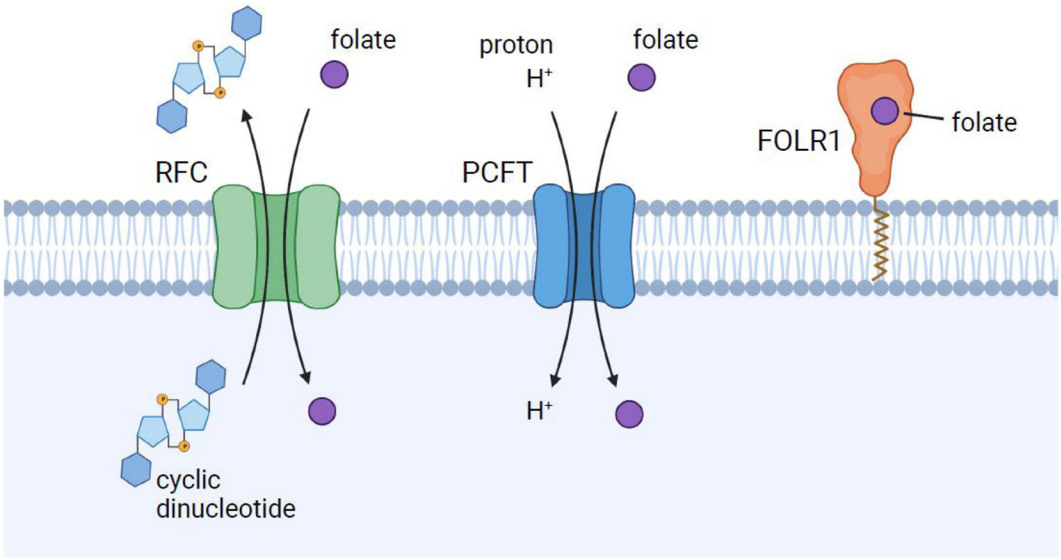

Figure 1. The three types of folate transporters.

Left) RFC (aka, the solute transport carrier SLC19A1) is an anion antiporter that uses a gradient of higher organic phosphate in the cell to transport folate into the cell while transporting organic phosphate out of the cell. The term "organic phosphate" refers to phosphate linked to organic molecules, and these can be relatively large molecules such as cyclic dinucleotides [70]. **Middle)** PCFT (aka, SLC46A1) couples the transport of folates with protons (H^+) along the pH gradient from the lower pH environment of the lumen of the intestine to the higher pH within intestinal cells. PCFT also releases folate from acidified endosomes after the endocytosis of FOLRs. **Right)** FOLR1 and FOLR2 are high-affinity, low throughput transporters of folate. Each FOLR only binds one folate and brings the folate into cells via endocytosis of the FOLR–folate complex. The reliance on endocytosis to bring in a singly-bound folate ensures that this is a relatively slow, low-throughput transport mechanism. FOLR1 and 2 are linked to the plasma membrane by a carboxy terminal glycosylphosphatidylinositol (GPI) anchor. Figure created with BioRender.com.

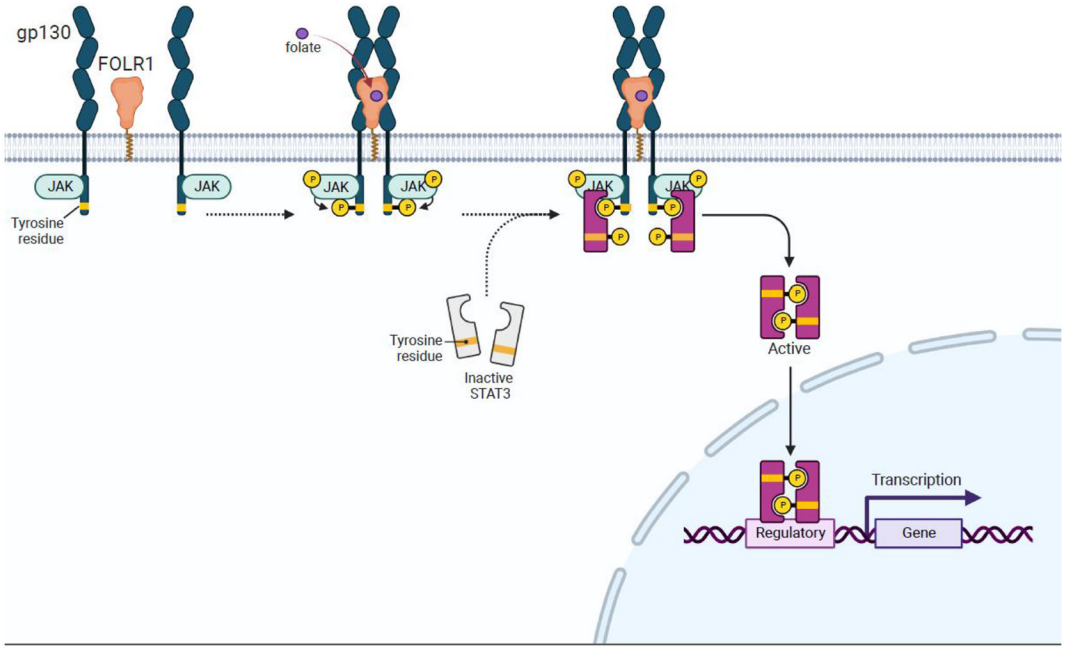

Figure 2. Proposed role for FOLR1 in JAK–STAT3 signaling.

JAK is the Janus kinase, a tyrosine kinase that associates with the intracellular domains of specific receptors [45]. In JAK–STAT signaling, ligand binding brings together two receptors (or co-receptors), the associated JAK then cross-phosphorylate the receptors, which allows two STAT3 proteins to bind the tyrosine-phosphorylated receptors, the STAT3 are subsequently phosphorylated by JAK. The two phospho-STAT3 dimerize and enter the nucleus where they function as transcription factors. In the proposed signaling pathway, the gp130 co-receptor, which is associated with JAK, binds to FOLR1. FOLR1 binding to FA (which is the folate used in the experiments) brings two gp130 together to initiate the JAK–STAT3 pathway to generate phospho-STAT3 dimers, which act as transcription factors. In the diagrams of proposed pathways, dashed lines denote uncertainty in how a step is accomplished or the structure of a protein complex. Here, the dashed lines indicate that it is not known if the physical interaction between FOLR1 and gp130 is dependent on FOLR1 binding FA. Figure created with BioRender.com.

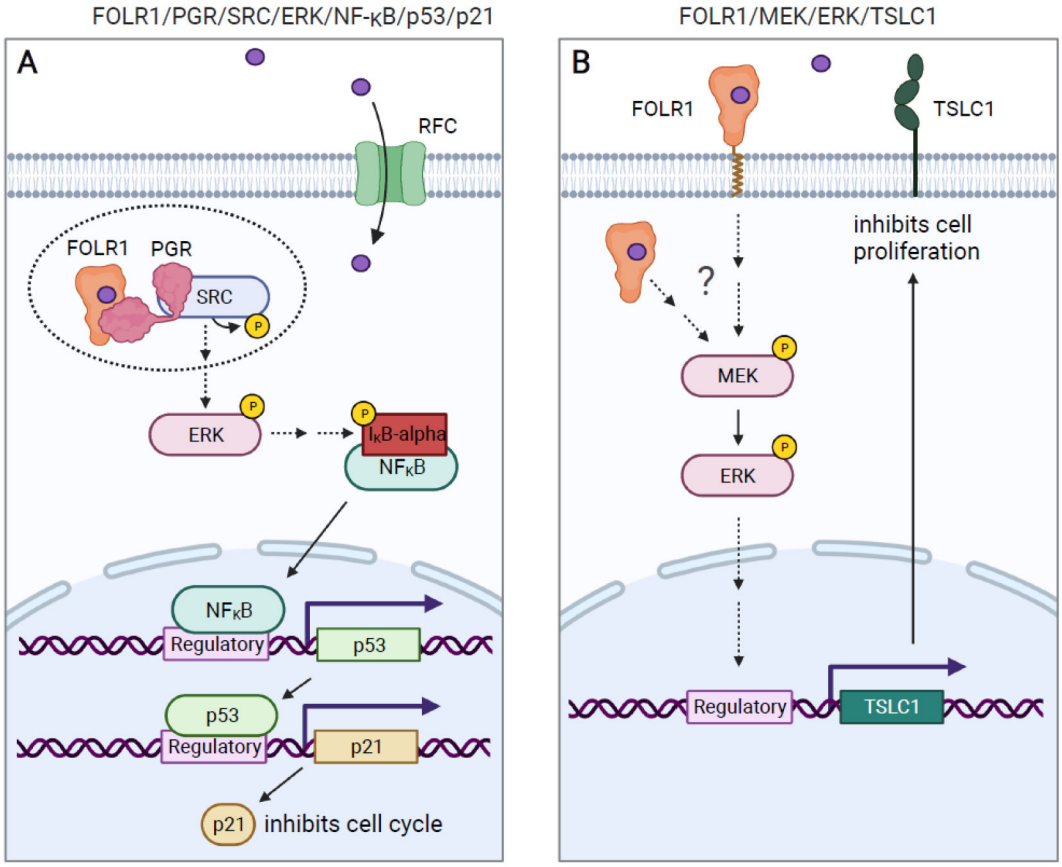

Figure 3. Proposed roles for FOLR1 in ERK1/2 signaling.

A) Signaling through FOLR1–PGR–SRC. Cytoplasm-localized FOLR1 (which may or may not have a GPI anchor, shown without) binds to FA (which may be brought into cells through RFC or FOLR1). The FOLR1–FA complex physically interacts with PGR. PGR interacts with both FOLR1 and SRC, either separately [49] or in a trimeric complex [51]. The dashed circle signifies that the exact interactions among the three proteins are not known. SRC autophosphorylates to activate itself and promotes the phospho-activation of ERK1/2. Activated ERK1/2 phosphorylates IκB-α, releasing NF-κB to translocate to the nucleus and induce the transcription of p53, which induces the transcription of $p21^{CIP1}$. $p21^{CIP1}$ inhibits CDK–cyclin complexes to block cell cycle progression. **B)** FOLR1 signaling through MEK–ERK. It is not known if FOLR1 bound to FA initiates this pathway from the plasma membrane or intracellularly. MEK is phospho-activated, and phospho–activates ERK1/2 [56]. Activated ERK1/2 induces transcription of *TSLC1* (aka cell adhesion molecule 1, *CADM1*), which acts at the plasma membrane to prevent cell proliferation. Figure created with BioRender.com.

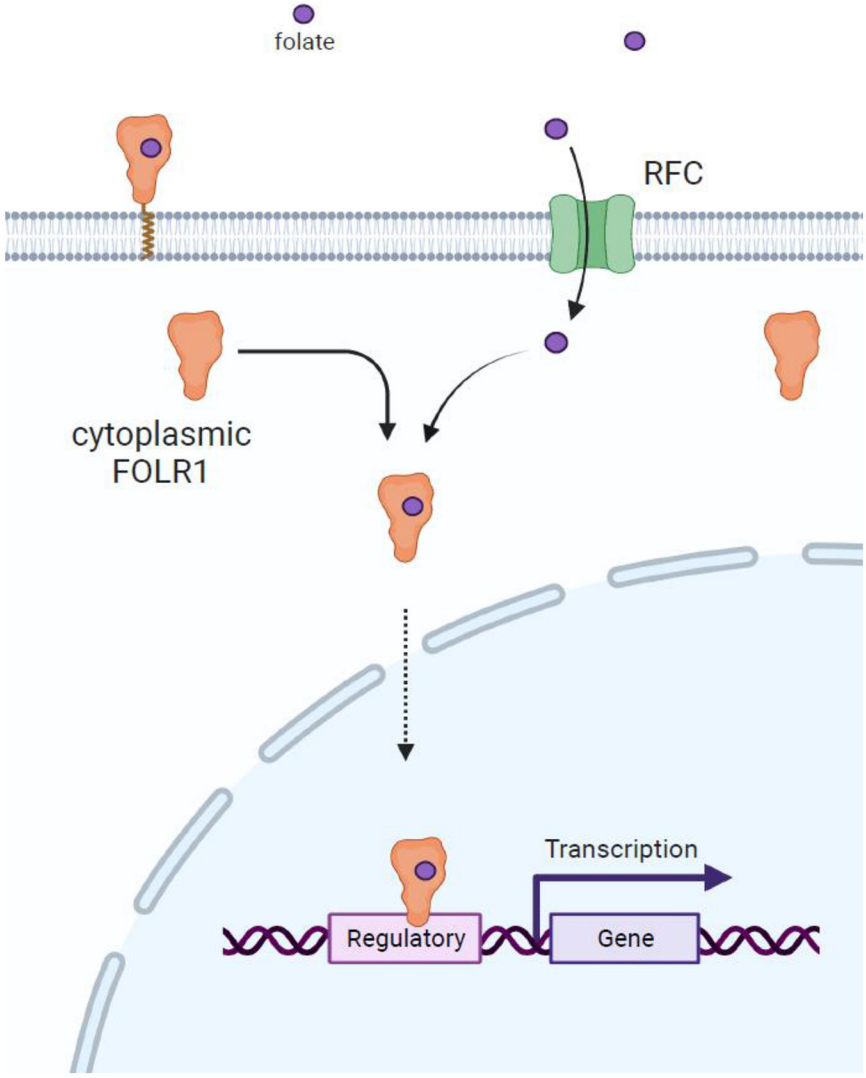

Figure 4. Proposed role of FOLR1 as a transcription factor.

Folate (FA or 5-methyl-THF) are brought into the cytoplasm either through the action of RFC or membrane-localized FOLR1. Cytoplasmic FOLR1 (which may or may not have the GPI anchor, shown without) binds folate and then translocates to the nucleus, where it functions as a transcription factor, directly binding gene regulatory regions. The mechanism by which FOLR1 translocates to the nucleus is not known, and hence the translocation arrow is denoted by a dashed line. FOLR1 does not have a canonical nuclear localization sequence (data not shown). The FOLR1 isoform that translocates to the nucleus is a smaller 38 kDa (relative to a larger 42 kDa isoform that does not translocate) [60]. The 38 kDa isoform is acetylated on lysine residues, which promotes its nuclear translocation [62]. Figure created with BioRender.com.

Table 1.

Expression of FOLR1 and FOLR2 in different cancers

Tissue and cancer	Express high FOLR1 (%)	Ratio cancer/normal FOLR1 protein [18]	Express FOLR2 (%) [30]
Ovary			
Primary tumor	72% [20,71]		
Serous carcinoma	82%, 100% [18,20,71]	22.3	50%
Endometrioid carcinoma	22%, 67%, 100% [18,20,71]	10.2	
Mucinous carcinoma	0%, 22%, 55% [18,20,71]	1.2	14%
Clear cell carcinoma	63%, 80% [20,71]		
Metastatic	90%, 100% [18,20]	30.1	
Papillary serous cystadenocarcinoma			8%
Normal	0% [18]	1.0	0%
Uterus			
Endometrial Primary carcinoma	20% [18]	9.8	
Endometrial Metastatic	100% [18]	8.6	0%
Uterine serous carcinoma	69% [72]		
Squamous cell carcinoma			20%
Endometrial adenocarcinoma			5%
Normal	0% [18]	1.0	0%
Brain			
Primary carcinoma	25% [18]	14.1	
Glioblastoma			18%
Astrocytoma			10%
Normal	0% [18]	1.0	0%
Pancreas			
Primary carcinoma	10% [18]	1.5	
Metastatic	100% [18]	3.6	
pancreatic ductal adenocarcinoma	80% [73]		5%
Normal	0% [18]	1.0	0%
Lymphoma			
Primary	0% [18]	1.0	
Metastatic	50% [18]	2.1	75%
Normal			0%
Breast			
Primary carcinoma	43% [18]	1.9	0%
Triple-negative breast cancer	67% [75]		
- Metastatic	50% [75]		
Infiltrating ductal carcinoma			20%
Invasive ductal carcinoma			3%
Normal	20% [18]	1.0	0%
Lung			

Tissue and cancer	Express high FOLR1 (%)	Ratio cancer/normal FOLR1 protein [18]	Express FOLR2 (%) [30]
Primary carcinoma	36% [18]	0.8	
Adenocarcinoma	60%, 72%, 74% [74,76,77]	0.9	56%
Mesothelioma	67% [18]	1.0	
Metastatic	50% [18]	0.8	
Squamous cell carcinoma	13%, 33%, 51% [74,76,77]		14%
Non-small cell carcinoma			100%
Normal	75% [18]	1.0	
Kidney			
Primary carcinoma	86% [18]	0.9	
Metastatic	50% [18]	0.3	
Renal cell carcinoma			100%
Clear cell carcinoma			15%
Normal	100% [18]	1.0	
Liver			
Primary carcinoma	0% [18]	1.3	
Hepatocellular carcinoma			53%
Normal	0% [18]	1.0	0%
Prostate			
Primary carcinoma	0% [18]	1.2	
Normal	0% [18]	1.0	
Colorectal			
Primary carcinoma	33% [78]	0.1	
Metastatic	44% [78]	0.8	
Normal	7% [78]	1.0	0%
Bladder			
Primary carcinoma	20% [18]	0.7	
Transitional cell carcinoma			9%
Normal	50% [18]	1.0	
Hematopoietic			
Acute Myelogenous Leukemia			68%
Head and Neck			
Squamous cell carcinoma			0%
- primary tumors	45% [79]		
- lymph node metastases (LNM)	40% [79]		
Normal	0% [79]		0%
Cervix			
Squamous cell carcinoma	41% [58]		5%
Normal	12% [58]		
Soft tissue			
Spindle cell type liposarcoma			100%

Tissue and cancer	Express high FOLR1 (%)	Ratio cancer/normal FOLR1 protein [18]	Express FOLR2 (%) [30]
Fibrosarcoma			70%
Mucinous liposarcoma			25%
Liposarcoma			20%
Skin			
Malignant melanoma			40%
Thyroid			
Papillary carcinoma			30%
Follicular carcinoma			22%
Normal			0%
Stomach			
Adenocarcinoma			11%
Normal			0%
Esophagus			
Squamous cell carcinoma			10%
Normal			0%
Testis			
Seminoma			7%
Normal			0%

Table 2:

Papers that propose a direct role for FOLR1 in signaling.

Cells	Folate added (μM)	Increase in markers (time post folate) *requires FOLR1	FOLR1 physical interaction	Notes	Imply direct FOLR1	Ref.
JAK–STAT signaling						
HeLa (cervical cancer cell)	FA (567–1360)	- p-STAT3 (5 min)	- co-IP gp130 with FOLR1	- STAT3-regulated genes increase with FA (FA)	YES	[40]
mouse neural precursor cells	FA (453–1134)	- p-STAT3 (30 min)	- co-IP FOLR1 with gp130	- FOLR1 promotes astrocyte differentiation	YES	[46]
mouse embryonic stem cells (ESC)	FA (1000)	- nuclear p-STAT3 (steady state)		- no increase in p-STAT3 but translocation to nucleus	NO	[47]
mouse mammary epithelium tumors	5-fold increase FA in diet			- FA induced breast cancers in mice that express FOLR1 and p-STAT3	NO	[80]
ERK1/2 signaling						
FOLR1/PGR/SRC/ERK/IκB-α/NF-κB/p53/p21 & p27						
COLO-205 (colon cancer cell line)	FA (1–10)	p-SRC (2 min)* - p-ERK1/2 (2 min) - nuclear NF-κB - p53 (12 hrs)* - p21 & p27 (12 hrs)*		- inhibition of cell proliferation starts at 0.1 μM FA (max at 10 μM)	YES	[48]
COLO-205	FA (10)	- p-SRC (2 min) - p-ERK (2 min) - p53 (18 hrs) - p21 & p27 (18–20 hrs)	- co-IP SRC with PGR - co-IP FOLR1 with PGR	- PGR required for increase in FA-induced signaling markers	YES	[49]
COLO-205	FA (10)	- p-SRC (2 min) - p-p21 (18-20 hrs) - p(Ser32)-IκB-α - RhoA increased cytosol, decreased membrane frac.		- inhibition of cell migration starts at 0.1 μM FA (max at 10 μM)	YES	[50]
T47D and MCF-7 (breast cancer cell lines)	FA (1)	- p-SRC (5 min) - RhoA increase membrane fraction, decrease cytosol	- co-IP FOLR1, PGR with SRC - co-IP FOLR1, SRC with PGR	- increased proliferation and migration starts at 1 μM FA (max 10 μM)	YES	[51]
FOLR1/MEK/ERK/TSLC1						
HONE1 (nasopharyngeal cell line)	FA (10)	- p-MEK (10 min) - p-ERK1/2 (10 min)* - TSLC1*		- FOLR1 and TSLC1 are required for FA to inhibit proliferation, migration, invasiveness	YES	[56]
FOLR1/ERK/FOS–JUN						
HeLa		- steady-state levels of p-ERK, p-FOS, p-JUN*		- FOLR1 p-ERK, p-FOS, p-JUN levels correlated - severity of squamous cervical carcinomas	NO	[58]
FOLR1 interaction with LYN and $G\alpha_{i-3}$						

Cells	Folate added (μM)	Increase in markers (time post folate) *requires FOLR1	FOLR1 physical interaction	Notes	Imply direct FOLR1	Ref.
IGROV1 (ovarian cancer cell line)			- co-IP FOLR1, $G\alpha_{i-3}$ with LYN - co-IP LYN, $G\alpha_{i-3}$ with FOLR1	FOLR1, LYN, and $G\alpha_{i-3}$ in detergent-resistant membrane - no data on FOLR1 importance for LYN or $G\alpha_{i-3}$ signaling	YES	[44]
FOLR1 as a transcription factor						
DAOY (medulloblastoma cell line)	FA (453)	- nuclear localization of FOLR1 38 kD isoform (15-30 min)		- before FA stimulation, FOLR1 48 and 38 kD isoforms in cytosol fraction	YES	[60]
O9-1 (neural crest cell line)	FA (0.23)	- FOLR1 ChIP of *Oct4, Sox2, Klf4* regul. DNA (15-30 min) - *Oct4, Sox2, Klf4, Trim71* expression (12-24 hrs)* - decrease miR-let-7, miR-138 (6 hrs)*	- co-IP of DGCR8, Drosha with FOLR1	- FA- and FOLR1-induced genes promote pluripotency	YES	[61]
O9-1 (differentiated into glial cells)	5-m-THF (0.022)	- nuclear localization of FOLR1 38 kD isoform (30 min)	- co-IP of FOLR1 with CAF-1	- FOLR1 is required for 5-m-THF to dedifferentiate glial cells	YES	[62]

The information in this Table is not comprehensive, many aspects of the signaling pathways are not included.