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Novel folate binding protein-1 interactions in embryonic orofacial tissue

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ABSTRACT

Aim: To identify proteins with which FolBp1 may interact within lipid rafts in tissue derived from embryonic orofacial tissue.
Methods: A yeast two-hybrid screen of a cDNA library, derived from orofacial tissue from gestational day 11 to 13 mouse embryos, was conducted.
Key findings: Using the full-length FolBp1 protein as bait, two proteins that bind FolBp1 were identified, Bat2d, and a fibronectin type III-containing domain protein. Results were confirmed by glutathione S-transferase pull-down assays.
Significance: As a component of membrane lipid raft protein complexes, these binding proteins may represent "helper" or chaperone proteins that associate with FolBp1 in order to facilitate the transport of folate across the plasma membrane. The protein–protein interactions detected, while limited in number, may be critical in mediating the role of FolBp1 in folate transport, particularly in the developing embryo.

Introduction

Folic acid is an essential vitamin involved in both one-carbon metabolism and the biosynthesis of many endogenous compounds, e.g. nucleic acids and amino acids. It plays a major role in normal development of the mammalian craniofacial complex and central nervous system (Barber et al. 1999b; Finnell et al. 1998). Moreover, deletion of the *FolBp1* gene in mice results in embryonic lethality (Piedrahita et al. 1999). Periconceptional folate supplementation prevents several congenital anomalies of the orofacial region (Badovinac et al. 2007; Loffredo et al. 2001; Shaw et al. 1995; Tolarova 1982; Tolarova and Harris 1995; Werler et al. 1999), neural tube (Antony and Hansen 2000; De Marco et al. 2000; Finnell et al. 1998) and cardiovascular system (Botto and Yang 2000; Scanlon et al. 1998). Uptake of extracellular folate by mammalian cells involves utilization of both the reduced folate carrier (RFC1), an energy dependent, integral transmembrane protein with high affinity for folate, and the folate binding proteins (FolBp1 or FolR1 and 2 in the mouse) (Barber et al. 1999a; Brigle et al. 1994, 1991), which are bound to the plasma membrane by glycosyl–phosphatidyl–inositol links (Lacey et al. 1989). Folate enters cells via receptor-mediated endocytosis as 5-methyltetrahydrofolic acid, which then donates a 5'methyl group to homocysteine during methionine synthesis. The folate receptors are then recycled back to the cell surface by endosomal trafficking (Anderson et al. 1992; Birn et al. 1993; Kamen et al. 1988). Since cells of the developing embryo are entirely dependent on maternal folate to support their high level of proliferative activity, folate receptors are crucial for transplacental maternal-to-fetal folate transport (Henderson et al. 1995), as well as embryonic utilization of folate (Piedrahita et al. 1999).

GPI-anchored proteins, such as FolBp1, are located in the plasma membrane as lipid rafts which often serve as platforms for signal transduction events and protein recycling (Miotti et al. 2000; Wang et al. 2002). Data from our laboratory indicates that these receptors are expressed by embryonic orofacial tissue during midgestation (Mukhopadhyay et al. 2004).

As outlined above, uptake of extracellular folate, via binding to FolBp1, is requisite for normal development of the mammalian craniofacial complex and CNS since folate deficiency, as for example in the case of inactivation of folate binding protein-1, results in orofacial and CNS defects. How folate ameliorates the adverse developmental effects of folate deficiency is entirely unknown! FolBp1 has been reported to associate with other cell surface proteins such as megalin (Birn et al. 2005) and lyn (a src-family non-receptor kinase) and G (alpha)(i-3) (Miotti et al. 2000). Clearly, such interactions do not represent the entire repertoire of FolBp1 binding partners. Thus, our rationale for performing a screen to identify other proteins with which FolBp1 may interact within lipid rafts, was to potentially identify novel interactions between FolBp1 and other binding partners. This, in turn, would help identify downstream signaling pathways that could help clarify the mechanism(s) by which folate protects against folate-deficiency induced developmental defects. In the current study, we screened for FolBp1-mediated protein

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interactions rather than FolBp2 because FolBp2-deficient mice are viable with no visible phenotype (Piedrahita et al. 1999). This suggests that interactions between FolBp2 and other proteins have little effect upon embryonic development. Inactivation of folate binding protein-1, however, significantly and adversely impacts murine embryonic development (Piedrahita et al. 1999). A two-hybrid screen of murine orofacial tissue was thus performed using a FolBp1-bait construct, in order to identify proteins with which FolBp1 may interact within lipid rafts.

Materials and methods

Yeast two-hybrid embryonic orofacial tissue expression library and bait constructs

A cDNA expression library was prepared by Research Genetics (Huntsville, AL) using RNA isolated from murine embryonic maxillofacial tissue on gestation days 11–13. The library was cloned into pGADT7 (Clontech, Palo Alto, CA), modified by destroying an existing *Not*I site at nucleotide 4520, and insertion of a unique *Not*I site within the multi-cloning site. The cDNA library was inserted into the *Eco*RI–*Not*I site to create the final expression library. To generate the yeast two-hybrid bait plasmid, the full length FolBp1 reading frame was RT-PCR amplified, subcloned into pCRII-TOPO and verified by sequencing. FolBp1 was subcloned into the pGBKT7 vector as an *Sfi*I-*Pst*I fragment to generate the bait plasmid, pGBKT7-FolBp1.

Yeast Two-Hybrid Assay

The Matchmaker Yeast Two-Hybrid System 3 (Clontech) was used to screen for proteins expressed in embryonic maxillofacial tissue that interact with FolBp1. The bait plasmid pGBKT7-FolBp1 described above was transformed into *Saccharomyces cerevisiae* (strain AH109) along with the expression library in pGADT7. In order to initially detect library protein-FolBp1 interactions, transformants were plated on yeast medium in the absence of histidine, leucine, tryptophan, adenine, and supplemented with 2.5 mM 3-amino-1,2,4-triazole in order to select for expression of yeast markers for leucine, tryptophan, adenine and histidine synthesis.

Approximately 5×10^5 yeast transformants were screened, yielding >65 $Ade^+/His^+/Leu^+/Trp^+$ colonies. These were replated onto selective media as above, but which was also supplemented with 20 μg/ml X-α-gal. Blue colonies bearing the $His^+/Leu^+/Trp^+/Ade^+$ phenotype were cultured and plasmid DNA isolated using standard protocols (Ausubel et al. 1994). Purified plasmids from positive colonies were re-introduced into *S. cerevisiae* (AH109) along with pGBKT7 or pGBKT7-FolBp1. Control plasmids pGADT7-T antigen and pGBKT7-lamin C, or pGADT7-T antigen and pGBKT7-p53 were co-

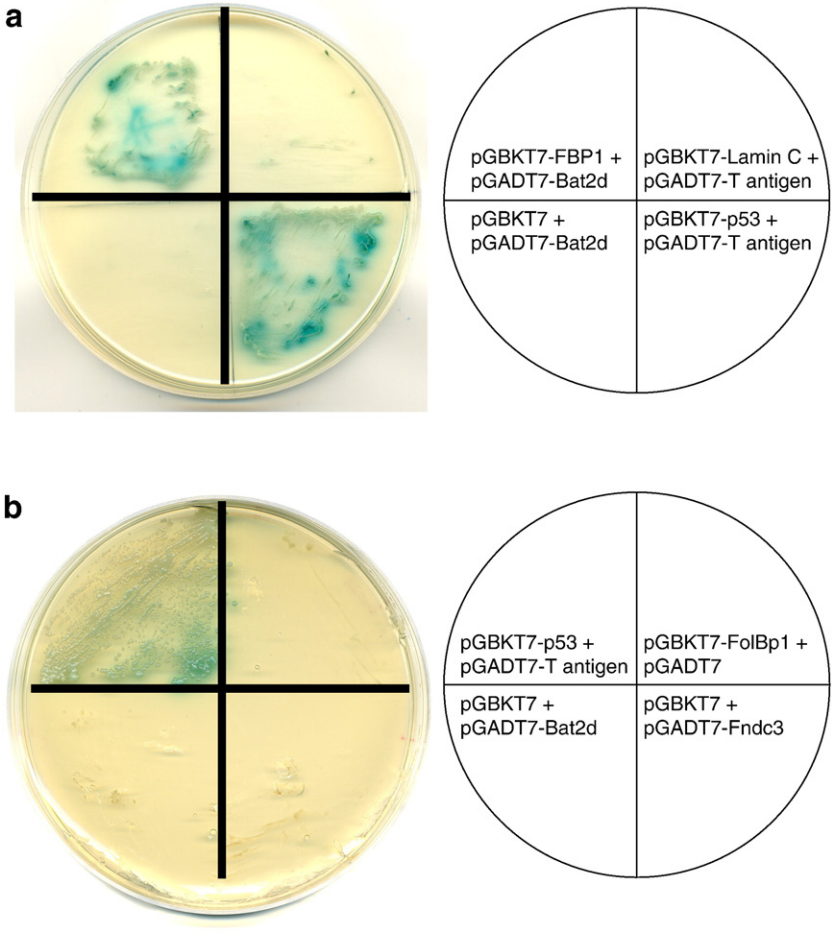

Fig. 1. Representative images from yeast two-hybrid assays demonstrating interaction of FolBp1 with Bat2d. Yeast plasmids, pGADT7 and pGBKT7 that contained the indicated cDNA, were co-transformed into *S. cerevisiae*, AH109, and plated onto high stringency media (media lacking histidine, tryptophan, leucine, and adenine) in the presence of 20 μg/ml X-α-gal. a. No growth was observed in yeast transformed with only the FolBp1 cDNA (pGBKT7-FolBp1 plus empty pGADT7), Bat2D, or Fndc3 cDNAs (pGADT7-Bat2d or pGADT7-Fndc3 plus empty pGBKT7) indicating that the cDNAs did not self-activate in the assay. The pGBKT7-p53 and pGADT7-T antigen plasmids produced yeast growth due to the interaction between p53 and T antigen. b. Yeast growth occurred when pGBKT7-FolBp1 and pGADT7-Bat2d were co-transformed, but not when Bat2d was expressed with empty vector pGBKT7. The combinations of pGADT7-Bat2d or pGADT7-Fndc3 with empty vector pGBKT7, or pGBKT7-FolBp1 with empty vector pGADT7 produced no growth. pGBKT7-lamin C and pGADT7-T antigen produced no growth (negative control) while pGBKT7-p53 and pGADT7-T antigen produced yeast growth.

Table 1
Novel Folbp1 binding proteins identified in a yeast two-hybrid screen.

Protein	Other names	Function	GenBank Accession no.	Reference
Bat2d	HBxAg, KIAA1096	Unknown surface-expressed protein	AK122441	(Banerji et al. 1990)
Fibronectin type III domain containing 3a	Fndc3a	Component of extracellular matrix proteins	NM_207636	(Calaycay et al. 1985)

transformed into *S. cerevisiae* as negative and positive controls, respectively. Clones maintaining the $Ade^+/His^+/Leu^+/Trp^+/X\text{-}\alpha\text{-}gal^+$ phenotype were sequenced in order to determine the identity of potential FolBp1-interacting proteins.

Glutathione S-transferase pull-down assay

To assay for in vitro interaction between folate binding protein 1 and the identified yeast two-hybrid cDNA clones, GST pull-down assays using purified GST–FolBp1 fusion protein and *in vitro* translated, [^{35}S]methionine-labeled protein were performed. Identity of fusion proteins was verified by DNA sequencing. pGST-FolBp1 was then transformed into *E. coli* BL21 cells and recombinant protein expression was induced for 4 h with 0.2 mM isopropyl-thio-b-D-galactopyranoside. Preparation of cleared lysates and purification of GST-fusion proteins were as previously described (Ausubel et al. 1994). The presence of the GST-FolBp1 fusion protein in an 8 M urea supernatant was confirmed by SDS-PAGE. [^{35}S]Methionine-labeled *in vitro* translated proteins were prepared with the TNT T7 coupled transcription/translation system (Promega, Madison, WI) and 5 µl was mixed with approximately 1 µg of the purified GST–FolBp1 bound to glutathione–Sepharose. The Sepharose: protein complex was washed and bound material eluted by the addition of 3x Laemmli sample loading buffer (Ausubel et al. 1994; Laemmli 1970), boiled and separated on an 8–16% polyacrylamide gel under reducing/denaturing conditions. Gels were fixed and exposed to Kodak X-Omat film for 7 days at −80 °C. Following autoradiography, dried gels were rehydrated and stained with Coomassie blue to determine loading efficiency for each sample.

Mouse E13.5 embryo palatal dissections

In order to localize Bat2d, Fndc3 and FolBp1 expression to the developing palate, palatal tissue from E13.5 embryos was dissected in cold PBS and fixed overnight in 4% paraformaldehyde in PBS. Tissue was stored in methanol and processed for *in situ* hybridization (Warner et al. 2007).

in situ hybridization

The cloned *Bat2d* fragment was excised from the library plasmid pGAD-Bat2d by *Bam*HI-*Eco*RI double digestion and subcloned into pSPT19 (Roche Diagnostics, Indianapolis, IN) to generate pSPT-Bat2d. The plasmid pSPT-Bat2d was linearized by *Nco*I restriction digestion to produce an antisense transcript of ~630 nucleotides corresponding to nucleotides 5188–5821 of the Bat2d cDNA sequence (GenBank accession number AK122441). A ~650 bp *Eco*RI-*Hin*dIII fragment of Fndc3 corresponding to nucleotides 3596-4241 of the cDNA sequence (GenBank accession number NM_207636) was subcloned into pSPT19 (generating pSPT-Fndc3). Antisense and sense anti-digoxigenin-labeled probes were generated using a DIG RNA labeling kit (Roche Diagnostics, Indianapolis, IN). Bound probes were detected by an alkaline phosphatase conjugated anti-digoxigenin antibody at a 1:2000 dilution (Anti-Digoxigenin-AP Fab Fragments, Roche Diagnostics) in 1% sheep serum (overnight, 4 °C). Bound signals were visualized with 2% (vol/vol) NBT/BCIP in NTMT substrate buffer (Roche Diagnostics) for 2-3 hours. Company, Cincinnati, OH).

Quantitative real-time PCR (QRT-PCR)

cDNA was synthesized from amplified cRNA prepared from freshly dissected E13.5 embryonic palatal tissue using random hexamer primers and Superscript II reverse transcriptase (Invitrogen Life Technologies, Inc., Carlsbad, CA). Quantitative real-time PCR (QRT-PCR) analysis was performed on a TaqMan ABI Prism 7000 Sequence Detector System (Applied Biosystems, Foster City, CA). FolBp1 and Gapdh (glyceraldehyde phosphate dehydrogenase) primers were designed by Applied Biosystems' "Assay by Design" custom service and both forward and reverse primers were used at a concentration of 900 nmol/l while the concentration of the probe was 250 nmol/l. For each reaction, a parallel reaction lacking template was performed as a negative control. Raw data were acquired and processed with ABI Sequence Detector System software, version 1.0 (Applied Biosystems, UK). The glyceraldehyde phosphate dehydrogenase gene was used as an amplification control.

Results

Yeast two-hybrid screen

Yeast colonies grew and turned blue when individual plasmids were cotransformed with pGBKT7-FolBp1, but not with the empty vector pGBKT7 (Fig. 1a). Blue colonies were also generated by cotransformation of pGBKT7-p53 and pGADT7-T antigen, but not by cotransformation with pGBKT7-lamin C and pGADT7-T antigen (Fig. 1). Two potential FolBp1 binding proteins (Table 1), Bat2d

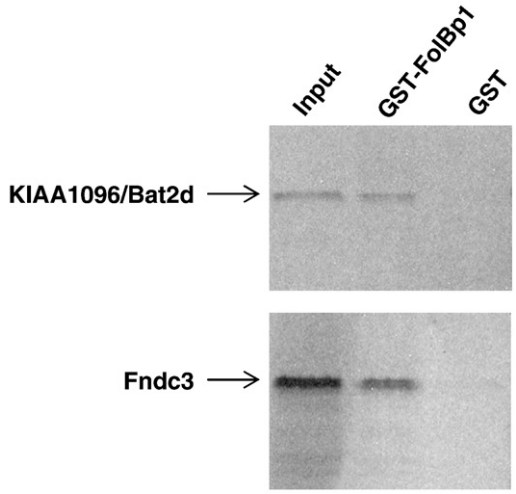

Fig. 2. Autoradiogram demonstrating *in vitro* binding between GST-FolBp1 and Bat2d or Fndc3a. GST-FolBp1 fusion proteins were expressed in, and purified from, *E. coli*. GST-FolBp1 (1 µg) bound to glutathione–agarose, was mixed with [^{35}S]methionine-labeled, *in vitro* translated proteins, and analyzed by SDS-PAGE followed by autoradiography. In each panel, the left lane demonstrates the expected size of either the Bat2d or Fndc3 band from the input protein lysate. The center lane shows an identically sized protein that was isolated by an interaction with FolBp1-GST-agarose. The absence of protein bands in the right lanes shows that neither Bat2d nor Fndc3 bound non-specifically to GST-agarose. The input lane demonstrates the signal from ~10% of the amount of *in vitro* translated protein present in each sample.

(*Bat2d*) (Banerji et al. 1990) and fibronectin domain type III-containing protein (*Fndc3*) (Calaycay et al. 1985) were identified based on positive interactions in the initial yeast two-hybrid screen with FolBp1. Neither cDNA (Bat2d, or Fndc3) resulted in a positive signal (i.e. growth on nutritionally restrictive media) when expressed alone, demonstrating that FolBp1 is required for a positive response in the yeast two-hybrid assay. pGBKT7-FolBp1 also did not confer a positive response when transformed singly, or with the empty pGADT7 vector (Fig. 1a), confirming that the Gal4-FolBp1 fusion protein does not activate the yeast reporter genes alone.

GST-pull-down assay

To confirm protein interactions revealed with the yeast two-hybrid assay, *in vitro* GST pull-down assays were performed for Bat2d and Fndc3a. The results presented in Fig. 2 confirm that recombinant GST-FolBp1 protein interacts with both Bat2d and Fndc3a. Neither protein exhibited any non-specific interaction with recombinant GST alone. Since *in vitro*-translated proteins were used in the pull-down assay, the confirmed interactions were direct and are unlikely to have involved any other intermediate proteins. These data are consistent with, and confirm the data derived from, the yeast two-hybrid screen.

Expression of binding proteins in the embryonic palate

Expression of *Fndc3a* and *Bat2d* mRNAs localized to the inner edges (flanking the nasal septum) of the E13.5 palatal shelves (Fig. 3). Both transcripts exhibited similar expression profiles along the anterior to posterior axis of the palatal shelves. Quantitative real time PCR performed on RNA extracted from dissected E13.5 palatal shelves confirmed expression of FolBp1 in this tissue.

Discussion

The primary function of the plasma membrane associated FolBp1 is the binding and transport of folate and related molecules into cells by endocytosis. Since FolBp1 is not directly linked to signaling cascades, the limited number of proteins shown to interact with FolBp1 was not unexpected. The proteins that *were* identified, Bat2d and the fibronectin type III domain-containing protein, also localize to the cell surface (Banerji et al. 1990; Calaycay et al. 1985). Bat2d (HLA-B-associated transcript) is a 1909 amino acid residue protein of ~295 kDa. The clone identified by the screen contained a 2000 bp fragment of the three-prime 2000 bp of the Bat2d gene, which has also been designated HBxAG or KIAA1096 (Banerji et al. 1990; Huang et al. 2002; Sun et al. 2003). A protein homology analysis using the

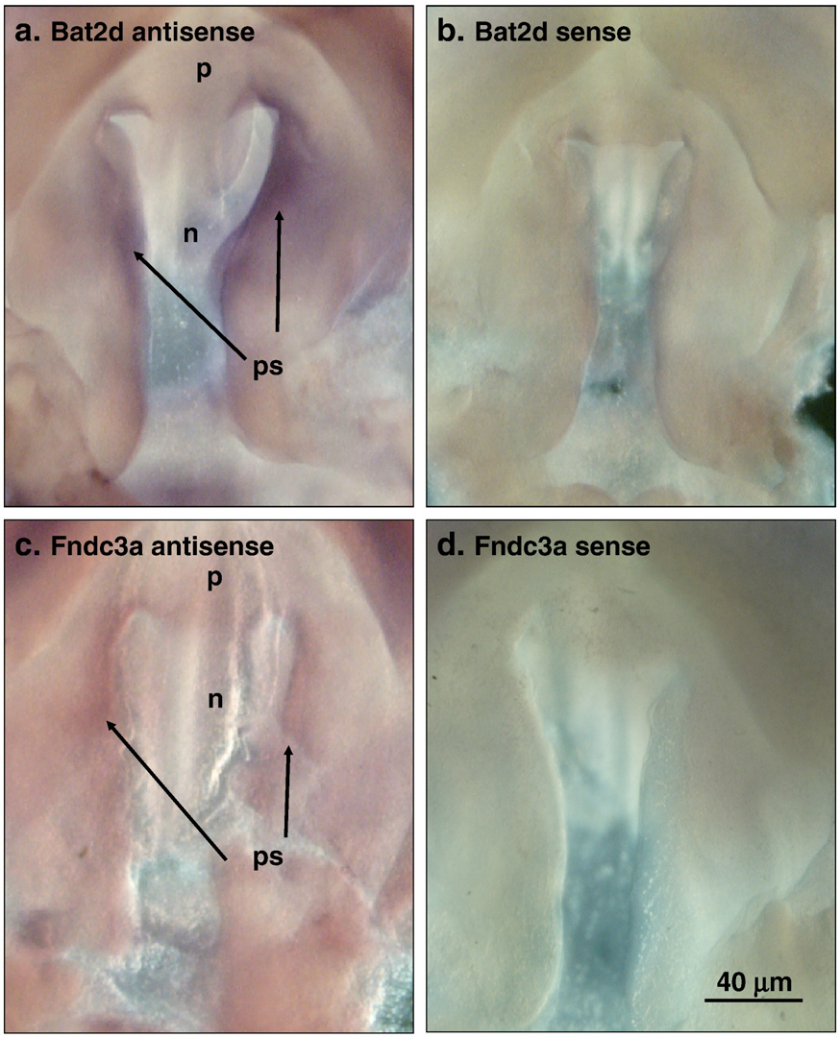

Fig. 3. Distribution of expression of Bat2d and Fndc3a to mouse E13.5 palatal tissue. Expression of *Bat2d* and *Fndc3a* (panels a and c) was determined by *in situ* hybridization. Expression of both transcripts was localized to the edges of the palatal shelves (indicated by the arrows). Hybridization was also conducted with the corresponding Bat2d and Fndc3a sense probes (negative controls) (panels b and d). Oro-maxillary tissue is shown with the anterior/nasal side uppermost. The lower jaw and tongue were removed during dissection to facilitate visibility of palatal tissue. In each panel, the nasal septum (n), primary palate (p) and the palatal shelves (ps) are indicated.

BLASTp program (http://www.ncbi.nlm.nih.gov/BLAST/) detected 100% identity between amino acids 1637–1836 of the full-length Bat2d protein. Analysis of the Bat2d/KIAA1096 protein sequence (GenBank accession number AK122441) by Pfam (http://www.sanger.ac.uk/Software/Pfam) detected a 200 amino acid residue, proline rich domain at the N-terminus that is shared by extracellular matrix proteins such as collagens and elastins (Banerji et al. 1990) (Fig. 4).

The Bat2d/HBxAG gene has been found to be up-regulated in a cell line in response to metal toxicity (Sun et al. 2003) and in human bladder cancer and other invasive cancers (Huang et al. 2002). No published data regarding the biochemical function of the Bat2d protein exist. Nevertheless, the up-regulation of Bat2d in response to heavy metals allows conjecture that this protein may be involved in the transport of small molecules such as heavy metal ions out of the cell, in order to effect cellular detoxification. Rapidly proliferating invasive tumor cells possess increased requirements for folate in support of DNA synthesis. Bat2d may be a plasma–membrane-associated "helper" or chaperone protein that associates with FolBp1 in order to facilitate the transport of folate across the plasma membrane. Although FolBp1 functions to transport folate into the cell by endocytosis, Bat2d may complex with folate-bound FolBp1 for stability.

Previous findings had reported interactions between FolBp1 and the proteins megalin, lyn and G(alpha)(i-3) (Birn et al. 2005; Miotti et al. 2000), but these interactions were not detected in this study. This could be accounted for by the possibility that these proteins may not be expressed in the embryonic palate or, that their associations with FolBp1 may occur within lipid rafts and do not involve physical binding.

An interaction was also detected between FolBp1 and a partial clone of the fibronectin type 3 domain (Fndc3a). The Fndc3a domain, one of three types of internal repeats found in the extracellular matrix protein fibronectin, is also present in membrane spanning cytokine receptors, growth hormone receptors, tyrosine phosphatase receptors and adhesion molecules such as integrins (Geerts et al. 1999; Yang et al. 2005; Zabeau et al. 2005). The clone identified by our two-hybrid screen contains an ~800 bp reading frame that aligns with the three C-terminal repeats of the fibronectin type 3 domain (Fig. 4). FolBp1 may interact with either fibronectin itself or with cell surface integrins that contain the Fndc3a domain, both types of proteins playing important roles in cell migration and tissue remodeling (Armstrong and Armstrong 2000; Miyamoto et al. 1998; Newgreen and Thiery 1980).

Both *Bat2d* and *Fndc3a* mRNA were localized to the palatal shelves in the orofacial regions of E13.5 embryos. While *in situ* hybridization lacked the sensitivity to detect expression of FolBp1 in the same location, QRT-PCR did detect expression in this tissue implying that both Bat2d and Fndc3a co-localize with FolBp1 in the embryonic palatal shelves. Current understanding of craniofacial morphogenesis, including that of palatal shelf elevation has recently been reviewed (Chai and Maxson 2006). Palatal shelf elevation is thought to be brought about by a number of interacting processes including mesenchymal proliferation and growth factor-induced ECM synthesis. Fibronectin, one extracellular matrix molecule, exhibits a homogeneous distribution throughout the embryonic palate mesenchyme at all stages of palatal development (Singh et al. 1998). How, or if, FolBp1 and any of its binding partners contribute to palatal shelf elevation must remain speculative. However, our observation that extracellular matrix protein fibronectin is a potential binding partner for FolBp1, raises the possibility that this cell surface receptor may play a functional role in contributing to ECM-mediated morphogenesis of the secondary palate. Considered together with *in vitro* binding assays that demonstrated direct binding, co-localization with FolBp1 further support putative interactions between both Bat2d and Fndc3a with FolBp1.

Folate receptors have been localized to lipid rafts in plasma membranes (Wang et al. 2002). The glycosyl–phosphatidyl–inositol-linked protein FolBP1 appears to physically interact with cell surface proteins or extracellular matrix proteins. This property is intriguing considering the expression of FolBP1 by neural crest cells in the developing embryo (manuscript in preparation). Congenital anomalies associated with perturbation of neural crest cell function often involve a failure in cell migration and/or tissue remodeling. Since cell migration requires interactions between cell surface receptors, such as integrins, and extracellular matrix proteins, such as fibronectin and collagens, localization within lipid rafts would bring the FolBP1 protein into association with other cell surface receptors that are involved in signaling pathways that control migration.

Folate plays an essential role in embryonic development being both a donor and acceptor of single-carbon groups in thymidine,

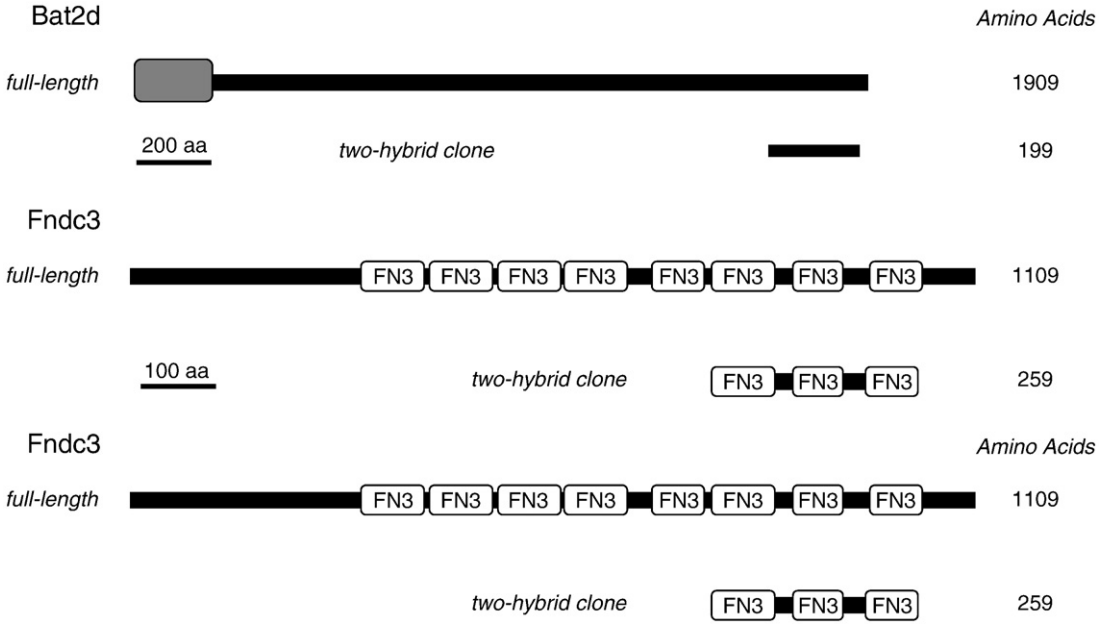

Fig. 4. Schematic illustrating the position of the Bat2d and Fndc3 clones isolated in the yeast two-hybrid screen in relation to their full-length counterparts. The domain organization of full-length Bat2d/KIAA1096 was obtained using Pfam (http://www.sanger.ac.uk/ Software/Pfam) while the fibronectin type III domain (FN3) organization in the full-length Fndc3 protein and in the corresponding two-hybrid clone was obtained using the BLASTp program (http://www.ncbi.nlm.nih.gov/BLAST/).

purine and methionine synthesis (Wagner 1995). Folate also contributes to the synthesis and recycling of s-adenosylmethionine, a molecule responsible for transmethylation, polyamine synthesis and trans-sulfuration reactions. These biochemical processes are of such fundamental importance to the growth of cells, that any perturbation mediated, for example, by a reduction in the levels of intracellular folate would have profound effects upon embryonic cell growth and proliferation.

Conclusions

Using the full-length FolBp1 protein as bait, two proteins that bind FolBp1 were identified, Bat2d, and a fibronectin type III-containing domain protein. Results were confirmed by glutathione S-transferase pull-down assays. These proteins localize to the cell surface where Bat2d may be a plasma–membrane-associated "helper" or chaperone protein that associates with FolBp1 in order to facilitate the transport of folate across the plasma membrane. The fibronectin type III-containing domain protein is discussed in terms of a possible role in cell migration and tissue remodeling. Both *Bat2d* and *Fndc3a* mRNA were localized to the palatal shelves in the orofacial regions of E13.5 embryos. Considered together with *in vitro* binding assays that demonstrated direct binding, co-localization with FolBp1 support putative interactions between both Bat2d and Fndc3a with FolBp1.

Acknowledgements

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