

DIP2 disco-interacting protein 2 homolog A (*Drosophila*) is a candidate receptor for follistatin-related protein/follistatin-like 1 – analysis of their binding with TGF- β superfamily proteins

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Keywords

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Follistatin-related protein (FRP)/follistatin-like 1 (FSTL1) is a member of the follistatin protein family, all of which share a characteristic structure unit found in follistatin, called the FS domain. Developmental studies have suggested that FRP regulates organ tissue formation in embryos. Immunological studies showed that FRP modifies joint inflammation in arthritic disease, and modulates allograft tolerance. However, the principle physiological function of FRP is currently unknown. To address this issue, we cloned four FRP-associated proteins using a two-hybrid cloning method: disco-interacting protein 2 homolog A from *Drosophila* (DIP2A), CD14, glypican 1 and titin. Only DIP2A was expected to be a membrane receptor protein with intracellular regions. Over-expression of FLAG epitope-tagged DIP2A augmented the suppressive effect of FRP on FBJ murine osteosarcoma viral oncogene homolog (FOS) expression, and the Fab fragment of IgG to FLAG blocked this effect. Knockdown of Dip2a led to Fos gene up-regulation, and this was not affected by exogenous FRP. These *in vitro* experiments confirmed that DIP2A could be a cell-surface receptor protein and mediate a FOS down-regulation signal of FRP. Moreover, molecular interaction analyses using Biacore demonstrated that FRP bound to DIP2A and CD14, and also with proteins of the TGF- β superfamily, i.e. activin, TGF- β , bone morphogenetic protein 2/4 (BMP-2/4), their receptors and follistatin. FRP binding to DIP2A was blocked by CD14, follistatin, activin and BMP-2. FRP blocked the ligand–receptor binding of activin and BMP-2, but integrated itself with that of BMP-4. This multi-specific binding may reflect the broad physiological activity of FRP.

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Abbreviations

ActR-IIB, activin receptor type IIB; Akt1, V-akt murine thymoma viral oncogene homolog 1; BMP-2/4, bone morphogenetic protein 2/4; BMPRI1A, bone morphogenetic protein receptor type IA; DIP2A, DIP2 disco-interacting protein 2 homolog A; Dox, doxycycline; FLRG, follistatin-related gene protein; FOS, FBJ murine osteosarcoma viral oncogene homolog; FRP, follistatin-related protein; FSTL1, follistatin-like 1; Hpdt, hypoxanthine guanine phosphoribosyl transferase; Mmp3, matrix metalloproteinase 3; SPARC, secreted protein acidic and rich in cysteine; TGF- β , transforming growth factor β ; TGF- β RII, transforming growth factor β type II receptor; X- α -Gal, 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside.

Introduction

The gene for follistatin-related protein (FRP) was originally cloned as TSC-36, one of the genes that is up-regulated by TGF- β 1 in a mouse osteoblastic cell line [1]. Subsequently, we and other groups in various fields ranging from clinical immunology to basic neurology independently cloned FRP, and therefore FRP has been given various names. Zwijsen *et al.* [2] isolated rat and human FRP from glioma cell lines by heparin affinity chromatography, with the aim of discovering novel regulatory proteins secreted by glial cells. We cloned FRP as one of the target proteins of auto-antibodies from patients with rheumatoid arthritis, and confirmed its arthritis-modifying functions *in vitro* and *in vivo* [3–5]. Tochitani *et al.* cloned the FRP gene, which they designated *occl1*, as one of the marker molecules preferentially expressed in the primary visual cortex of the adult monkey brain, and demonstrated that its expression is dependent on neuron activity and subject to developmental regulation [6–8]. In addition, they also observed its characteristic expression in the adult monkey hippocampus [9].

FRP is named after follistatin, an inhibitor of activin, as its amino acid sequence is similar to that of a characteristic structural unit in follistatin, called the FS domain [2]. FRP is a secreted protein with a molecular weight of 50–55 kDa, and is expressed in almost all organs [3]. In addition to follistatin and FRP, other FS domain-bearing proteins, such as secreted protein acidic and rich in cysteine (SPARC), SPARC-like 1/hevin, testican, and follistatin-related gene protein (FLRG)/follistatin-like 3, have been cloned, and together they comprise the follistatin family [10]. FLRG is a translated product of the follistatin-related gene (*FLRG*), and was isolated from a B-cell chronic lymphocytic leukemia cell line [11]. As the expression ‘follistatin-related’ may cause confusion of FRP with FLRG, they have been given other names, FSTL1 and FSTL3 respectively, in the Online Mendelian Inheritance in Man database (OMIM; Johns Hopkins University, <http://www.ncbi.nlm.nih.gov/omim>). FLRG shows binding activity for activin and BMP-2, and inhibits their signaling [12]. Recently, the tick *Haemaphysalis longicornis* homolog of FRP was reported to have binding activity for activin and BMP-2 [13]. SPARC, the best-studied protein of the group, is known to regulate cell–matrix interactions and thereby regulate tissue remodeling and homeostasis [10].

Developmental studies have suggested that FRP regulates organ tissue formation in embryos. In the early stages of development, FRP is expressed ubiquitously. However, in later stages, its expression

becomes regionally restricted, and is mostly found in the mesenchymal tissue [14]. The function of FRP has been partially elucidated in several developed tissues. In joint tissue, FRP decreases the production of matrix metalloproteinases and prostaglandins from synovial fibroblasts, modifying joint inflammation and destruction in arthritic disease [4,5]. This effect appears to be associated with down-regulation of FOS/Fos expression [4,5]. In blood cells, graft-infiltrating CD8⁺ T cells produce FRP, which contributes to allograft tolerance [15]. In cardiovascular tissue, FRP inhibits smooth muscle cell proliferation and migration, and protects cardiac myocytes from hypoxia/reoxygenation-induced apoptosis [16,17]. In some cancer tissues, FRP plays roles in cell-cycle inhibition and apoptosis, and has a negative regulatory effect on growth [1,18,19]. However, the basic physiological function and the signal transduction system of FRP remain to be clarified. To address these issues, we attempted to identify molecules that interact with FRP using a two-hybrid cloning method and a Biacore system.

The *Drosophila disconnected* (*disco*) gene product, disco, is thought to be a transcription factor and is required for proper neuronal connections in both the larval and adult visual system in *Drosophila* [20–22]. Using the yeast two-hybrid system, Mukhopadhyay *et al.* [23] discovered a novel *Drosophila* protein that interacted with disco *in vitro* and in yeast. They named this molecule disco-interacting protein 2 (DIP2), and cloned a murine homolog (Dip2a), the amino acid sequence of which is approximately 60% identical to that of its *Drosophila* counterpart. The DIP2 homolog genes are evolutionarily conserved, and have been identified in organisms as diverse as *Caenorhabditis elegans* and humans. In mouse embryos, Dip2a was expressed in restricted regions of the brain (e.g. the neocortex, striatum and thalamus), and its persistence in these regions suggests that Dip2a may provide positional cues for axon pathfinding and patterning [23]. The evolutionarily conserved expression pattern suggested that the Dip2a/DIP2A gene plays an important role in the development of both vertebrate and invertebrate nervous systems.

Both FRP and DIP2A appear to regulate cell growth and migration, and to play major roles in the arrangement of cells in organs with complicated structures, such as the brain. Recently, Ouchi *et al.* [24] reported that they had identified DIP2A as a receptor for FRP by the combination of affinity chromatography and mass spectrometry. Here, we demonstrate the association of FRP with DIP2A and their signal

transduction, resulting in down-regulation of FOS, and present the analyses of their molecular interactions with other two-hybrid clones and known organizer proteins of the TGF- β superfamily.

Results

Cloned cDNAs encoding FRP-interacting protein

Six cDNAs of various lengths were obtained in this screen. Two showed false two-hybrid interactions (Fig. 1, clones B16 and B78). The remaining clones were A7 (3.8 kb), A45 (1.3 kb), A94 (2 kb) and C63 (2.8 kb): A7 was the cDNA 3' end of DIP2A; A45 was the cDNA 3' end of CD14, a monocyte differentiation antigen; A94 was the cDNA 3' end of glypican 1, a core protein of heparan sulfate proteoglycan; C63 was a partial cDNA fragment of titin (connectin), an abundant string-like protein in striated muscle (Fig. 2). Glypican 1 and CD14 are glycosyl phosphatidylinositol-anchored membrane proteins, and have no cytoplasmic region [25,26]. Titin is a cytoskeletal protein that does not appear to be directly involved in the cellular signal transduction system. Bioinformatics analysis of DIP2A using PredictProtein suggested that DIP2A could be a membrane protein with a cytoplasmic N-terminus, three transmembrane regions and an extracellular C-terminus (Fig. 3A). WoLF PSORT also estimated that DIP2A could exist in the plasma membrane. HomoloGene indicated that DIP2A has three domains, the DNA methyltransferase 1 associated protein (DMAP)-binding (amino acids 9–123), CaiC (amino acids 320–919) and adenosine monophosphate (AMP)-binding (amino acids 1017–1486) domains (Fig. 3A). This three-domain structure is roughly com-

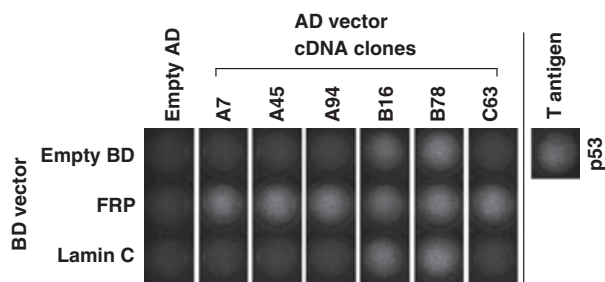


Fig. 1. Verification of the two-hybrid interaction between FRP and cDNA clones. Two-hybrid interaction was detected by the fluorescence generated by yeast cell proliferation. Positive control cells contained p53 and T-antigen cDNAs in BD and AD vectors, respectively. Negative control cells contained empty BD and AD vectors. Possible non-specific binding of cDNA products to FRP was determined based on their interaction with lamin C.

1. A7 (3.8 kb): DIP2A cDNA (3195- in AB273729)



2. A45 (1.3 kb): CD14 cDNA (45-1367 in BC010507)



3. A94 (2.0 kb): glypican 1 cDNA (1736- in X54232)



4. C63 (2.8 kb): titin cDNA (10955-13760 in X90568)

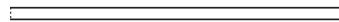


Fig. 2. Cloned cDNAs encoding FRP-interacting proteins. The open rectangular boxes show coding regions.

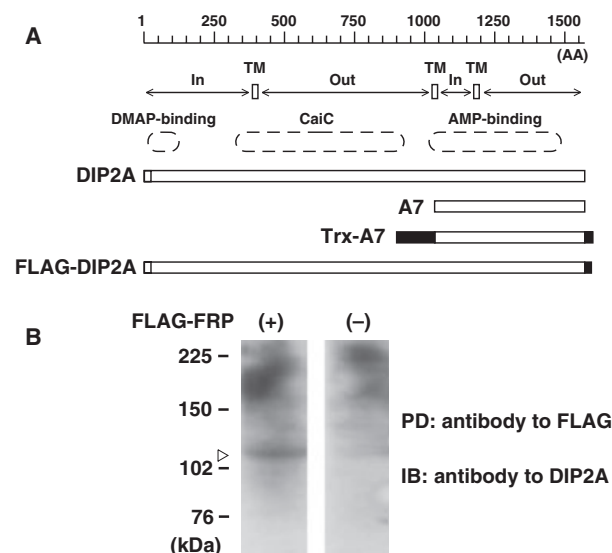


Fig. 3. Structure of DIP2A and its detection. (A) DIP2A and its recombinant proteins are shown. Closed rectangles show the fusion protein or tag sequences. Open short rectangles in the N-terminus show signal sequences. Trx-A7 is a fusion of A7 with a thioredoxin domain at the N-terminus and 6-His at the C-terminus. FLAG-DIP2A is DIP2A with a FLAG epitope at the C-terminus. (B) Samples with or without FLAG-tagged FRP were pulled down (PD) by antibody to FLAG and subjected to immunoblotting (IB).

patible with the results of our bioinformatics analysis. As DIP2A was the candidate receptor protein for FRP, a full-length DIP2A cDNA was constructed as described in Experimental procedures. Combined sequence data for this cloned full-length cDNA and A7 revealed a 6981 bp sequence for DIP2A cDNA with a polyA tail. This cDNA sequence, AB273729 (DDBJ), was almost identical to that of NM_015151.3 (RefSeq) except for four nucleotide substitutions: C \rightarrow T (position 1449), C \rightarrow T (position 1992), G \rightarrow A (position 2208) and G \rightarrow A (position 2428), resulting in a single amino acid substitution of P \rightarrow L (position 1541) (nucleotide and amino acid numbers in

AB273729). The PCR product-derived sequence data were verified against those of independently cloned PCR fragments. A pull-down assay using a magnetic column showed that DIP2A in loaded SF-1 cell lysate was associated with FLAG-tagged FRP trapped by magnetic bead-conjugated protein G via antibody to FLAG. DIP2A was detected in the eluate as a protein of approximately 110 kDa (Fig. 3B).

FOS down-regulation signaling of FRP through DIP2A

To estimate FRP signaling through DIP2A, we established a stable cell line, TetOn-SF-1/FLAG-DIP2A,

that expresses FLAG epitope-tagged DIP2A under regulation by doxycycline (Dox) (Fig. 4A). Like the parental synovial fibroblast cell line SF-1 [4], TetOn-SF-1/FLAG-DIP2A secreted FRP in an autocrine manner. Dox induced the expression of FLAG-tagged DIP2A and augmented FOS suppression by FRP in a dose-dependent manner (Fig. 4C, FRP 0 ng·mL⁻¹), and this effect was enhanced by addition of exogenous FRP (Fig. 4C, FRP 100 ng·mL⁻¹) ($P < 0.005$ for Dox and $P < 0.05$ for FRP). Using a fixed concentration of exogenous FRP, Dox-induced FLAG-tagged DIP2A was found to suppress FOS expression (Fig. 4E). This suppression was blocked and prevented by the Fab fragment of IgG to FLAG in a dose-

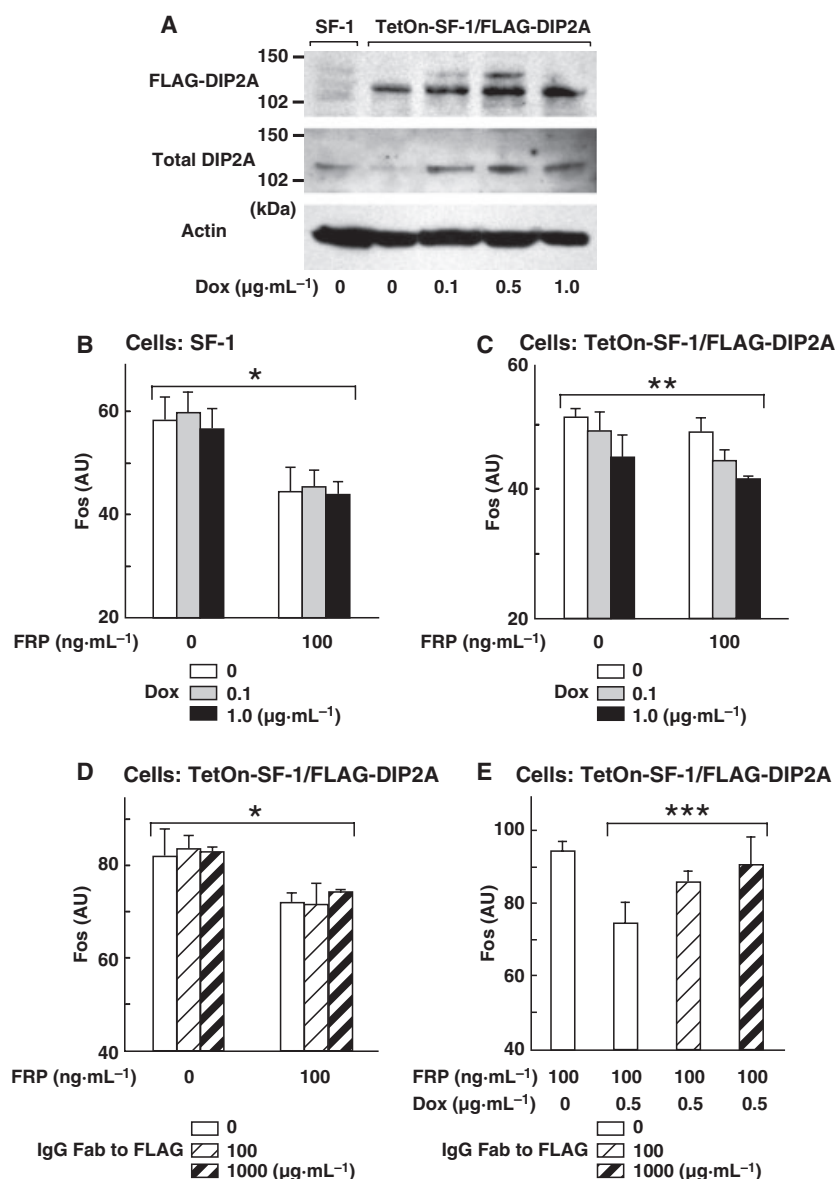


Fig. 4. DIP2A over-expression and blocking assays. (A) Dox-induced FLAG-tagged DIP2A and endogenous DIP2A were detected in TetOn-SF-1/FLAG-DIP2A. (B,C) Dox was added to SF-1 (B) or TetOn-SF-1/FLAG-DIP2A (C) cells with or without exogenous FRP. (D,E) The Fab fragment of IgG to FLAG was added to TetOn-SF-1/FLAG-DIP2A cells with or without exogenous FRP (D). In addition to exogenous FRP, Dox-induced FLAG-tagged DIP2A was blocked by IgG Fab to FLAG (E). *Difference not significant for Dox or IgG Fab to FLAG, $P < 0.001$ for FRP by two-factor ANOVA. ** $P < 0.005$ for Dox and $P < 0.05$ for FRP by two-factor ANOVA. *** $P < 0.05$ by one-factor ANOVA. The data represent at least three independent experiments that showed consistent results.

dependent manner ($P < 0.05$) (Fig. 4E). We confirmed that Dox did not affect FOS expression in the parental SF-1 line, and that IgG Fab to FLAG did not interfere with this assay system (Fig. 4B,D). Moreover, we established another stable cell line, 3T3/miR-Dip2a, that expresses microRNA (miR) against Dip2a to reduce its production. 3T3/miR-Dip2a showed 69% reduced production of Dip2a compared to 3T3/miR-LacZ (Fig. 5A). 3T3/miR-Dip2a also secreted FRP in an autocrine manner (Fig. 5D). Fos gene (*Fos*) and matrix metalloproteinase 3 gene (*Mmp3*) were significantly up-regulated under conditions of Dip2a down-regulation ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 5B,C). Exogenous FRP did not suppress *Fos* expression in 3T3/miR-Dip2a but did suppress *Fos* expression in 3T3/miR-LacZ (Fig. 5B).

Interactions among FRP, DIP2A, CD14 and TGF- β superfamily proteins

We used a Biacore system to confirm FRP interaction with the two-hybrid clones, and also to analyze FRP binding activity to proteins of the TGF- β superfamily, including activin and BMP-2, as reported by Zhou *et al.* [13]. Biacore produces sensorgrams that indicate the real-time interaction of analytes in free solution and ligands immobilized on sensor chips. The vertical axis shows the surface plasmon resonance, which reflects the change in mass of analytes bound to ligands. Therefore, the number of resonance units increases when analytes bind to ligands, and the number of resonance units decreases when analytes dissociate from ligands. The background surface plasmon resonance is eliminated by subtracting the surface plasmon resonance without ligands. Our Biacore studies indicated that FRP bound to DIP2A (Fig. 6A) and CD14 (Fig. 6B), and also to follistatin (Fig. 6C), activin A (Fig. 8A),

transforming growth factor β (TGF- β 1) (Fig. 8B), BMP-2 (Fig. 8C), BMP-4 (Fig. 8D), activin receptor type IIB (ActR-IIB) (Fig. 8E), transforming growth factor β type II receptor (TGF- β RII) (Fig. 8F) and bone morphogenetic protein receptor type IA (BMPRIa) (Fig. 8G). Neither glypican 1 nor titin were tested due to lack of protein samples. The K_D values are summarized in Table 1. These were larger than the K_D values of follistatin for its binding partners as determined by Biacore: activin A ($0.465\text{--}4.32 \times 10^{-10}$ M), BMP-4 (2.9×10^{-9} M), BMP-6 (5.1×10^{-9} M) and BMP-7 (3.7×10^{-8} M) [27,28]. The K_D values for binding of FRP to TGF- β RII, FRP to BMP-2, DIP2A to TGF- β 1 and DIP2A to BMP-4 could not be determined due to poor sensorgram fitting. FRP was characterized by extremely high association and dissociation rates, and comparatively low affinity binding for partner molecules (Fig. 6A–C), except for TGF- β 1, BMP-2/4 and BMPRIa (Fig. 8B–D,G). Activin A, CD14, FLRG and BMP-2 did not bind to DIP2A (Fig. 7A). However, follistatin bound weakly and quite slowly to DIP2A (Fig. 6D), and FRP binding to DIP2A was blocked by CD14, follistatin, activin A and BMP-2 (Fig. 7B–E).

Interestingly, TGF- β 1 and BMP-4 bound to DIP2A with relatively high affinity (Fig. 9A,B). However, bound TGF- β 1 or BMP-4 appeared to be substituted by FRP, based on the characteristic rectangular sensorgram (Fig. 9C,D). FRP blocked activin A binding to ActR-IIB (Fig. 8H) and BMP-2 binding to BMPRIa (Fig. 8J), but only slightly weakened the binding of TGF- β 1 to TGF- β RII (Fig. 8I). In addition, FRP did not weaken BMP-4 binding to BMPRIa: in fact, FRP formed a ternary complex with BMP-4 and BMPRIa (Fig. 8K). These data are helpful when considering the various effects of FRP on the signaling of TGF- β and BMP subtypes.

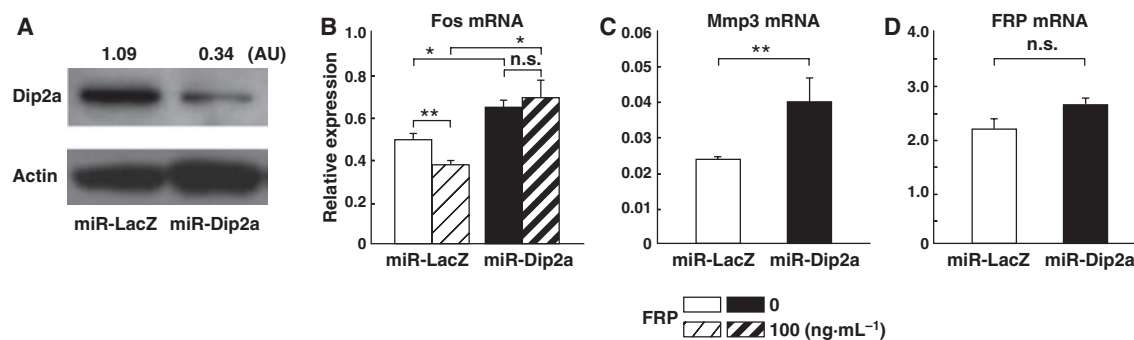


Fig. 5. Dip2A RNAi analysis using 3T3/miR-Dip2A. (A) Dip2a reduction in 3T3/miR-Dip2A cells was verified by Western blotting. The numbers show the expression levels of Dip2a relative to actin. (B–D) Gene expression in 3T3/miR-Dip2A was evaluated by quantitative PCR. 3T3/miR-LacZ is the control. Exogenous FRP was added (B). Gene expression values were normalized against *Hprt*, and calibrated against those in wild-type NIH-3T3 cells. * $P < 0.01$ and ** $P < 0.05$ by Student's unpaired *t* test. n.s., not significant.

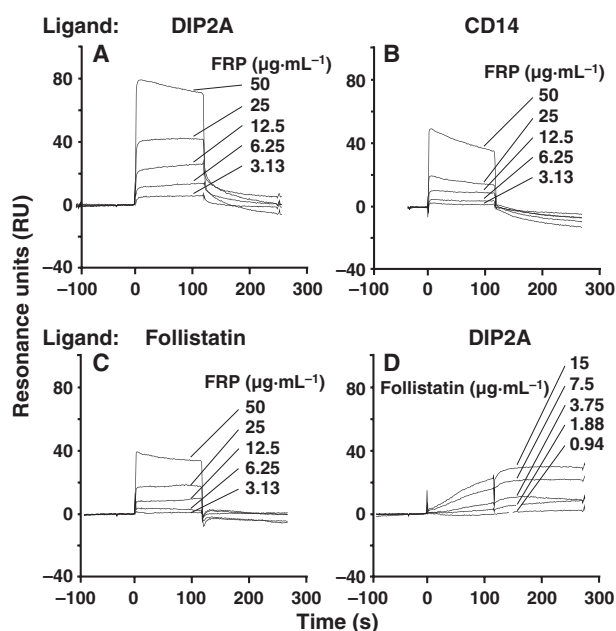


Fig. 6. Binding activities among FRP, DIP2A, CD14 and follistatin. Sensorgrams show interactions of FRP with DIP2A (A), CD14 (B) and follistatin (C), and that of follistatin with DIP2A (D). Ligands were immobilized 940 RU of DIP2A, 1770 RU of CD14, and 1980 RU of follistatin. The injected analytes were various concentrations of FRP or follistatin.

Table 1. Dissociation equilibrium constants determined using Biacore. ND, not determined.

Ligand	Analyte	K_D value (M)
Activin A	FRP	1.43×10^{-6}
ActR-IIB	FRP	3.14×10^{-4}
BMP-2	FRP	ND
BMP-4	FRP	1.91×10^{-7}
BMPR1A	FRP	3.51×10^{-7}
CD14	FRP	1.72×10^{-4}
DIP2A	FRP	4.20×10^{-6}
Follistatin	FRP	1.88×10^{-4}
DIP2A	Activin A	No binding
DIP2A	BMP-2	No binding
DIP2A	BMP-4	ND
DIP2A	CD14	No binding
DIP2A	FLRG	No binding
DIP2A	Follistatin	ND
DIP2A	TGF- β 1	ND
TGF- β 1	FRP	1.28×10^{-8}
TGF- β R11	FRP	ND

Discussion

We cloned four cDNAs encoding proteins that interact with FRP, i.e. DIP2A, CD14, glypican 1 and titin, using the yeast two-hybrid system. Then, Biacore

studies indicated that, in addition to interacting with DIP2A and CD14, FRP also interacted with proteins of the TGF- β superfamily, including activin, TGF- β , BMP-2/4, their receptors and follistatin. DIP2A had high affinity among these receptor-type proteins, together with BMPR1A. However, FRP binding to glypican 1 and titin was verified only by yeast two-hybrid reaction due to lack of protein sample availability. Cripto, another TGF- β superfamily co-receptor, has been reported to bind to glypican 1 [29]. Although we did not test Cripto in these experiments, it may be a candidate binding partner for FRP.

Previously, we demonstrated that FRP down-regulates FOS/Fos expression [4,5]. Here, we showed that DIP2A actually takes part in the FOS down-regulation signal of FRP. This FOS down-regulation of FRP was minimal, and may not reflect FRP signaling directly. With regard to other effects of FRP, Oshima *et al.* [17] reported that FRP activated phosphorylation of V-akt murine thymoma viral oncogene homolog 1 (Akt1) in neonatal rat ventricular myocytes. Moreover, Ouchi *et al.* [24] demonstrated DIP2A-mediated Akt1 phosphorylation by FRP. Although we did not evaluate phosphorylated Akt1 in these experiments, we are currently assessing other possible FRP signaling markers, including phosphorylated Akt1, that may be more sensitive than FOS.

Our bioinformatics analysis, extracellular antibody blocking of FLAG-tagged DIP2A, and extracellular soluble molecule binding to DIP2A confirmed that DIP2A is a cell-surface protein. It is unclear whether DIP2A has any co-receptor(s), and whether any other receptor molecules interact with FRP. As TGF- β and BMP-4 also bind to DIP2A, we cannot exclude the possibility that TGF- β or BMP-4 may transmit a signal(s). Moreover, FRP bound DIP2A in a substituting manner for TGF- β and BMP-4, and it appears that they may elicit different signals against DIP2A. The purpose of Biacore experiments was to quantitatively analyze the interaction of FRP with DIP2A and CD14, and to ascertain whether FRP could bind to any TGF- β superfamily proteins. In future experiments, we intend to investigate the effects of FRP and DIP2A on activin, TGF- β and BMP-2/4 signaling systems.

DIP2 was originally cloned as a protein that interacts with disco in *Drosophila* [23]. Disco regulates proper neuronal connections in the visual system of *Drosophila*, probably in conjunction with DIP2. Disco is thought to be a transcription factor due to its zinc finger motif [21]. Definite homologs of disco have not been identified in vertebrates. In mouse embryos, as in *Drosophila*, Dip2a is expressed in the central nervous

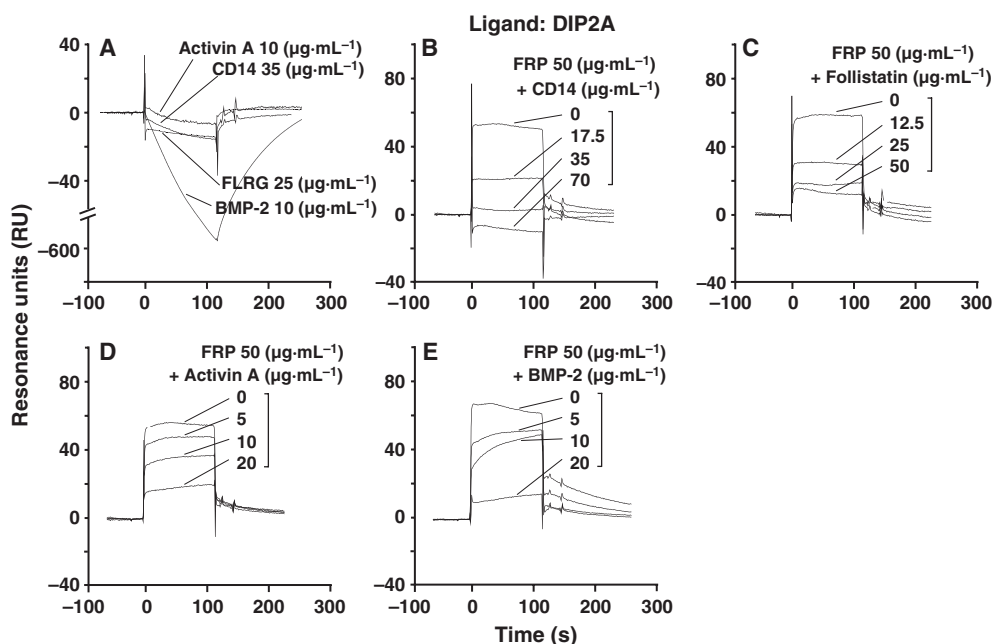


Fig. 7. There was no binding activity of DIP2A for activin A, CD14, FLRG or BMP-2, and DIP2A binding activity for FRP was blocked by CD14, follistatin, activin A and BMP-2. (A) The injected analytes were activin A, CD14, FLRG and BMP-2. (B–E) The injected analytes were FRP, with various concentrations of CD14, follistatin, activin A and BMP-2, respectively. The ligand was immobilized 940 RU of DIP2A.

system during development with a specific spatio-temporal pattern [23]. FRP also shows regionally and temporally characteristic expression in monkey brain [6–8]. Therefore, it is not surprising that FRP and DIP2A interact with each other and exert physiological functions. DIP2B, another DIP2 family protein, has been reported to be associated with at least one human neurocognitive disorder [30].

The molecular anatomical function of DIP2A remains to be clarified. Bioinformatics analysis using PredictProtein and HomoloGene suggested that DIP2A is a type I receptor molecule with three domains, i.e. DMAP-binding, CaiC and AMP-binding domains from the N- to the C-terminus (Fig. 3A). FRP associated with A7, the 513 amino acid C-terminal sequence of DIP2A, *in vitro* and in yeast. As the AMP-binding domain is also located in A7, bound FRP may have an effect at this site. AMP-binding domains are found in the family of enzymes that includes luciferase, long-chain fatty acid CoA ligase, acetyl CoA synthetase, and various other related synthetases. The CaiC domain, which also binds AMP, is found in acyl CoA synthetases and AMP-acid ligases that are involved in lipid metabolism, secondary metabolite biosynthesis, transport and catabolism; CaiC is a bacterial inner membrane enzyme with crotonobetaine-CoA ligase and carnitine-CoA ligase functions [31]. These two

domains may function cooperatively through AMP binding. The DMAP-binding domain is found in vertebrate DNA methyltransferases, such as the product of the mouse kreisler mutation gene (*kr*), which binds to DNA methyltransferase 1 associated protein 1 (DMAP1) [32,33]. Thus, it is possible that DIP2A may be transiently localized in the cytoplasmic or nuclear region.

Our Biacore data showed that FRP significantly interfered with the ligand–receptor interaction for activin A and BMP-2, but not for TGF- β 1 or BMP-4 (Fig. 8H–K). Interestingly, in zebrafish development experiments, Esterberg *et al.* [34] found that FRP antagonized BMP-4 signaling. This is similar to the observation that follistatin forms a tertiary complex with BMP-4 and its receptor to block BMP-4 signaling [35]. Follistatin and type II receptors have overlapping binding sites on a subset of TGF- β ligands [36], and therefore are not thought to interact with each other. Interestingly, our data suggest that FRP can bind directly to ActR-IIIB and TGF- β RII. Further experiments are required to confirm this differential binding of follistatin and FRP to type II receptors.

In the immune system, FRP suppresses matrix metalloproteinases and prostaglandins, and acts as a protective effector under the control of TGF- β [4,5]. This effect does not contradict the constructive role of

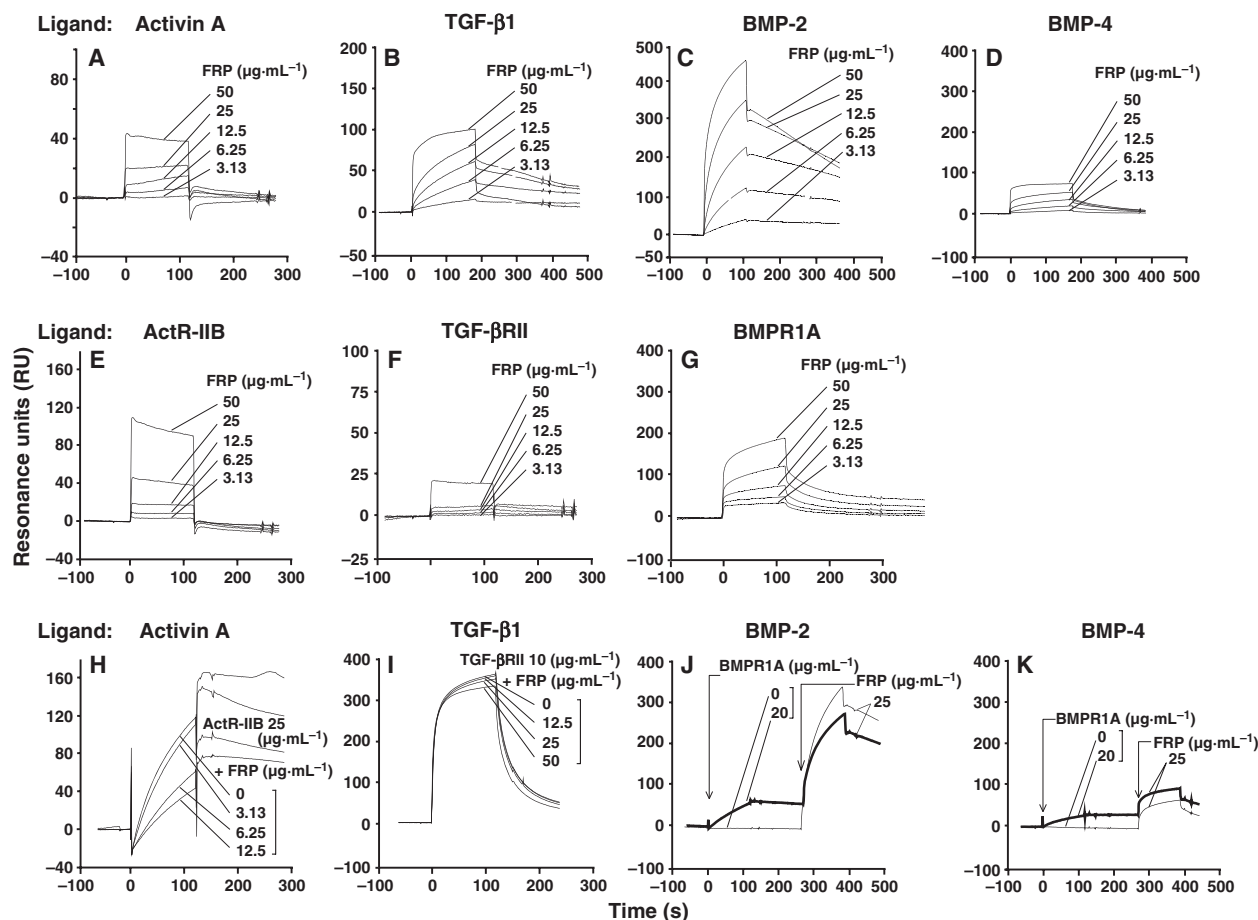


Fig. 8. Interactions of FRP with TGF- β superfamily proteins and their receptors. (A)–(G) Sensorgrams showing interactions of FRP with activin A (A), TGF- β 1 (B), BMP-2 (C), BMP-4 (D), ActR-IIB (E), TGF- β RII (F) and BMPRI1A (G). The injected analytes were various concentrations of FRP. (H)–(K) Sensorgrams showing the effects of FRP on ligand–receptor interactions of activin A with ActR-IIB, TGF- β 1 with TGF- β RII, BMP-2 with BMPRI1A and BMP4 with BMPRI1A. (H,I) The injected analytes were mixed samples of a fixed concentration of ActR-IIB (H) or TGF- β RII (I) with various concentrations of FRP. (J,K) Analytes were sequentially injected, with the first sample comprising buffer control (thin lines) or BMPRI1A (bold lines), and the second sample comprising a fixed concentration of FRP. Ligands were immobilized 1960 RU of activin A, 1060 RU of TGF- β 1, 3500 RU of BMP-2, 1520 RU of BMP-4, 2970 RU of ActR-IIB, 190 RU of TGF- β RII and 1970 RU of BMPRI1A.

the interaction between FRP and DIP2A described above, and is supported by the observation that FRP induced allograft tolerance in rat heart transplantation [15]. However, it has been reported that FRP promotes inflammation by up-regulating cytokines, such as interleukin-6, both *in vitro* and *in vivo* using an adenoviral over-expression system [37]. We are currently investigating the immunological effects of FRP in various systems.

SPARC, another member of the follistatin family, elicits changes in cell shape, inhibits cell-cycle progression, and influences the synthesis of extracellular matrix. The absence of SPARC in mice gives rise to aberrations in the structure and composition of the extracellular matrix, resulting in generation of cata-

tracts, development of severe osteopenia, and accelerated closure of dermal wounds [38,39]. These observations suggest that FRP and SPARC are functionally similar. However, interaction of SPARC with TGF- β superfamily proteins has not been reported.

In conclusion, we identified DIP2A as a candidate receptor molecule for FRP. In addition, FRP also binds TGF- β superfamily proteins. Its multi-specific binding nature, especially with organizer proteins, suggests that FRP modulates their activities and thereby contributes to proper formation of tissue structures. Functional analysis of FRP and DIP2A, and their associated protein interactions, will provide further insight into the links between cell differentiation, tissue morphogenesis and homeostasis.

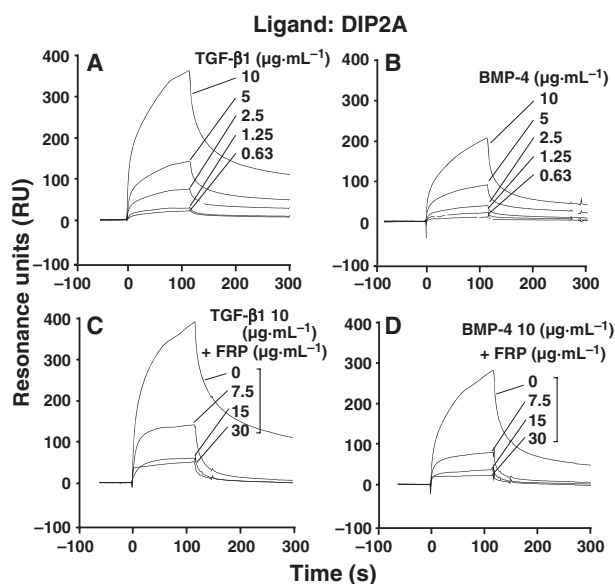


Fig. 9. Binding activity of DIP2A for TGF- β superfamily proteins and its disturbance by FRP. (A,B) The injected analytes were various concentrations of TGF- β 1 or BMP-4. (C,D) The injected analytes were mixed samples of a fixed concentration of TGF- β 1 or BMP-4 and various concentrations of FRP. The ligand was immobilized 940 RU of DIP2A.

Experimental procedures

Cloning of the molecules that interact with FRP

Cloning was performed using a commercial yeast-Gal4 two-hybrid system kit (Matchmaker Two-Hybrid System 3; Clontech, Mountain View, CA, USA). The bait protein was human full-length FRP. As FRP is expressed abundantly in the heart [3], the prey protein was from a human heart cDNA library (Matchmaker pre-transformed human heart library; Clontech). Mated yeast clones were first screened on agar plates containing minimal synthetic dropout medium lacking adenine, histidine, leucine and tryptophan (QDO medium) supplemented with 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (X- α -Gal; Clontech). The growing blue clones were selected and secondarily screened by replication on QDO agar plates containing X- α -Gal and 75–100 mM 3-amino-1,2,4-triazole (Sigma-Aldrich, St Louis, MO, USA). Plasmids in the screened yeast clones were isolated using a commercial kit (Yeastmaker yeast plasmid isolation kit; Clontech).

Verification of the two-hybrid interaction

Isolated plasmid clones were re-transfected into the yeast, and two-hybrid interaction was detected using oxygen biosensor plates (Matchmaker biosensor kit; Clontech). The fluorescence of the reactants was measured using a Fluor-Imager SI (GE Healthcare, Piscataway, NJ, USA).

Sequence and structure analysis of cloned cDNAs and their translation products

Sequence analysis of cloned cDNAs was performed using the FASTA program available from the DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp>). The structure of unknown proteins was predicted using several programs available online: PredictProtein (Columbia University; <http://www.predictprotein.org/>) and WoLF PSORT (University of Tokyo, Japan; <http://psort.ims.u-tokyo.ac.jp>). The domain structure was analyzed using the National Center for Biotechnology Information (NCBI) HomoloGene system (<http://www.ncbi.nlm.nih.gov/HomoloGene/>).

Cloning of full-length human DIP2A cDNA

Our original cDNA fragment of DIP2A, A7, lacked the 5' region that spans residues 1–3194 in AB273729 and includes the open reading frame (Fig. 2). Because of cloning difficulties, the full-length DIP2A cDNA was reconstructed from two cloned cDNA fragments by PCR from Human Heart Marathon-Ready cDNA (Clontech). The shorter N-terminal fragment (SN, residues 1–1541 of AB273729) with a 5' end *Mlu*I site was amplified using primers 5'-TTACGCGTGCCATGGCTGACCGCGGGTGCCCG-3' and 5'-GTCCCTGTGTCCTGGGCCAGAGGGTGCC-3'. The longer C-terminal fragment (LC, residues 1112–4737 of AB273729) with a 3' end *Xba*I site was amplified using primers 5'-CTCTACCGGTAACTTTGGAGTCGG-3' and 5'-AATCTAGACTATCACATGTTGTAGGCGACA-TAG-3'. These *Mlu*I- and *Dra*I-digested SN and *Dra*I- and *Xba*I-digested LC fragments were ligated with *Mlu*I- and *Xba*I-digested plasmid pALTER-MAX (Promega, Madison, WI, USA) to produce a full-length cDNA. Mutated nucleotides due to PCR were repaired using a QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

Pull-down assay of the FRP–DIP2A interaction

Binding of FRP to DIP2A was analyzed using a Multi-MACS protein A/G kit (Miltenyi Biotec, Auburn, CA, USA), which is a column-based pull-down assay system that utilizes magnetic bead-conjugated protein G. Aliquots (50 μ L) of M2 antibody to FLAG (Eastman Kodak, New Haven, CT, USA), 100 μ L SF-1 cell lysate, 500 μ L of FLAG-tagged FRP-expressing cell culture or control supernatant [4], and 100 μ L of magnetic bead-conjugated protein G were loaded onto the column, and eluted after washing according to the protocol described by the manufacturer. The eluate was assayed by Western blotting using antibody to DIP2A (sc-67555; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and peroxidase-conjugated rabbit antibody to goat IgG (H + L) (61-1620; Invitrogen, Carlsbad, CA, USA).

Preparation of thioredoxin–A7 fusion protein (Trx–A7)

To produce recombinant A7 protein, we used a prokaryotic thioredoxin fusion protein expression system (pBAD102 directional TOPO expression kit; Invitrogen). Expression of Trx–A7 was induced in *Escherichia coli* using 0.002% arabinose for 5 h, and purified sequentially using a TALON resin column (Clontech) and a 6-His antibody column. The 6-His antibody column was prepared by coupling rabbit affinity-purified antibody to 6-His (Bethyl, Montgomery, TX, USA) using N-hydroxysuccinimide (NHS)-activated Sepharose 4B (GE Healthcare).

Establishment of a doxycycline-regulated FLAG-tagged DIP2A-expressing cell line

The cDNA of full-length DIP2A fused to a C-terminal FLAG epitope tag (FLAG-DIP2A) was cloned by a linker primer method using high-fidelity DNA polymerase (*PfuUltra*; Stratagene) [3]. The 5' primer was 5'-TTACGCGTGCCATGGCTGACCGCGGGTGC-3', and the 3' primers were 5'-AATCTAGACTACTTATCGTCGTCATCCTTG-3' and 5'-AATCTAGACTACTTATCGTCGTCATCCTTGTAAATCCATGTTGTAGGCGACATAGATGGGGTCCAG-3' at concentrations of 0.75, 0.75 and 0.03 pmol·μL⁻¹, respectively, and the annealing temperature was 68 °C. FLAG-DIP2A cDNA was inserted between the *MluI* and *XbaI* sites of the Dox-regulated expression vector pTRE-Tight (Clontech) to produce pTRET-FLAG-DIP2A. The regulator vector pTet-On (Clontech) and pTRET-FLAG-DIP2A with a linear hygromycin selection marker (Clontech) were sequentially transfected into synovial fibroblast cell line SF-1 [4] under selection with neomycin and hygromycin, to create the cell line TetOn-SF-1/FLAG-DIP2A.

In vitro assay of FRP signaling through DIP2A using the Tet-On system

FOS expression by TetOn-SF-1/FLAG-DIP2A cells was evaluated using a TransAM ELISA-based method (Active Motif, Carlsbad, CA, USA). TetOn-SF-1/FLAG-DIP2A cells (2.5×10^5 per well) were inoculated into the wells of six-well plates filled with 2 mL of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and incubated overnight. When the cells adhered to the wells, the medium was exchanged for fresh medium containing 0.1% fetal bovine serum and various concentrations of Dox, recombinant FRP and the Fab fragment of IgG to FLAG. Recombinant FRP was prepared from COS-7 cells (CRL-1651, American Type Culture Collection, Manassas, VA, USA) as reported previously [4]. Fab was produced from M2 antibody to FLAG (Eastman Kodak) using an ImmunoPure Fab preparation kit (Pierce, Rockford, IL, USA). After a further incubation for 48 h, the

cells were harvested and their FOS expression was evaluated.

RNA interference (RNAi) analysis of Dip2a

A plasmid vector expressing microRNA (miR) against Dip2a, pcDNA6.2-GW/miR-Dip2a, was prepared using a BLOCK-iT PolII miR RNAi expression vector kit and primers (NM_001081419_1672_top/bottom; Invitrogen). The Dip2a knockdown and control cell lines, 3T3/miR-Dip2a and 3T3/miR-LacZ, were established by transfecting pcDNA-GW/miR-Dip2a and pcDNA-GW/miR-LacZ into NIH-3T3 cells (CRL-1658, American Type Culture Collection), and selecting them using Blasticidin ($10 \mu\text{g}\cdot\text{mL}^{-1}$) (Invitrogen). The expression levels of target genes are expressed in terms of their $2^{-\Delta\Delta C_t}$ values determined by quantitative RT-PCR using SYBR Green I (Applied Biosystems, Austin, TX, USA). In each sample, the ΔC_t value was normalized to that of the endogenous control gene hypoxanthine guanine phosphoribosyl transferase (*Hprt*) (Mm01324427; Applied Biosystems). For each gene, the $\Delta\Delta C_t$ value is the ΔC_t value calibrated to that for wild-type NIH-3T3 cells. The primer pairs were as follows: 5'-ATGGGCTCTCCTGTCAACAC-3' and 5'-CTGTCAACCGTGGGGATAAAG-3' for *Fos*, and 5'-TGGAGATGCTCACTTTGACG-3' and 5'-GCCTTGGCTGAGTGGTAGAG-3' for *Mmp3*.

Binding kinetics analysis of FRP and its interacting proteins

Protein–protein interactions were estimated by surface plasmon resonance using a Biacore 2000 system and BIAEVALUATION software version 4.1 (GE Healthcare). Ligand proteins were immobilized on a carboxymethyl dextran flow cell (CM5; GE Healthcare). The buffer for sample dilution and running buffer was HBS-P buffer (GE Healthcare) containing 2 mM CaCl₂. The ligand and analyte samples were Trx–A7 for DIP2A, FRP (FSTL1; Genway Biotech, San Diego, CA, USA), CD14 (R&D Systems, Minneapolis, MN, USA), activin A (R&D Systems), follistatin (follistatin 300; R&D Systems), FLRG (R&D Systems), ActR-IIB (activin RIIB/Fc chimera; R&D Systems), TGF-β1 (Peprotech, Rocky Hill, NJ, USA), soluble TGF-βRII (TGF-β sRII; R&D Systems), BMP-2/4 (R&D Systems) and BMPRI1A (soluble BMPRI1A; Acris Antibodies GmbH, Hiddenhausen, Germany). K_D values were determined by a global fitting method using BIA-evaluation version 4.1 (GE Healthcare). We excluded fitting results with χ^2 values > 20 resonance units (RU) [40].

Statistics

ANOVA and Student's unpaired *t* test were performed using STATVIEW 5.0 (SAS Institute Inc., Cary, NC, USA). In all analyses, *P* values < 0.05 were considered significant.

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