RESEARCH ARTICLE

The splicing factor PQBP1 regulates mesodermal and neural development through FGF signaling

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ABSTRACT

Alternative splicing of pre-mRNAs is an important means of regulating developmental processes, yet the molecular mechanisms governing alternative splicing in embryonic contexts are just beginning to emerge. Polyglutamine-binding protein 1 (PQBP1) is an RNA-splicing factor that, when mutated, in humans causes Renpenning syndrome, an X-linked intellectual disability disease characterized by severe cognitive impairment, but also by physical defects that suggest PQBP1 has broader functions in embryonic development. Here, we reveal essential roles for PQBP1 and a binding partner, WBP11, in early development of Xenopus embryos. Both genes are expressed in the nascent mesoderm and neurectoderm, and morpholino knockdown of either causes defects in differentiation and morphogenesis of the mesoderm and neural plate. At the molecular level, knockdown of PQBP1 in Xenopus animal cap explants inhibits target gene induction by FGF but not by BMP, Nodal or Wnt ligands, and knockdown of either PQBP1 or WBP11 in embryos inhibits expression of fgf4 and FGF4-responsive cdx4 genes. Furthermore, PQBP1 knockdown changes the alternative splicing of FGF receptor-2 (FGFR2) transcripts, altering the incorporation of cassette exons that generate receptor variants (FGFR2 IIIb or IIIc) with different ligand specificities. Our findings may inform studies into the mechanisms underlying Renpenning syndrome.

KEY WORDS: Alternative splicing, FGF, FGF receptor, Mesoderm, Neural, PQBP1, Renpenning syndrome, WBP11, Xenopus

INTRODUCTION

Polyglutamine binding protein-1 (PQBP1) is a 38 kDa nuclear protein abundantly expressed in the adult mammalian central nervous system (Komuro et al., 1999a; Waragai et al., 1999), and mutations in the human PQBP1 gene cause X-linked intellectual disability (XLID) diseases that include Renpenning, Sutherland–Haan, Hamel cerebropalatocardiac, Golabi–Ito–Hall and Porteous syndromes (Kalscheuer et al., 2003; Lenski et al., 2004; Stevenson et al., 2005; Cossee et al., 2006; Lubs et al., 2006; Martinez-Garay et al., 2007). These are collectively referred to as Renpenning syndrome, and affected patients display unifying clinical features, including intellectual disability, microcephaly and short stature. However, a variety of other physical manifestations can be observed, including midline and cardiac defects, small testes, lean muscle and reduced body mass (Stevenson et al., 2005; Germanauda et al., 2010). These phenotypes suggest that, in addition to brain development and cognition, PQBP1 regulates a broader scope of body patterning events, yet the normal developmental functions of PQBP1 and the pathogenic mechanisms underlying Renpenning syndrome remain largely undeciphered.

At the biochemical level, PQBP1 functions in transcription and pre-mRNA splicing. PQBP1 interacts with the C-terminus of activated RNA polymerase II (Okazawa et al., 2002) and with spliceosome components, including U5-15kD (Dim1p) (Waragai et al., 2000; Zhang et al., 2000) and the U2 snRNP component Sf3b1 (Wang et al., 2013). Most human disease-causing PQBP1 mutations truncate the C-terminal domain of PQBP1 that is required for interaction with the spliceosomal protein U5-15kD (Waragai et al., 2000; Zhang et al., 2000). Knockdown of PQBP1 in mouse embryo primary neurons reduces general splicing efficiency and promotes the use of alternative splice sites in a variety of transcripts (Wang et al., 2013). A missense mutation within the WW domain at the N-terminus of PQBP1 in Golabi–Ito–Hall (GIH) patients (Y65C) causes abnormal pre-mRNA splicing and inhibits PQBP1 binding to spliceosome components and a partner protein, WBP11 (Lubs et al., 2006; Tapia et al., 2010; Sudol et al., 2012). PQBP1 and WBP11 both associate with the BAU1 or PRP19 spliceosomal complexes (Makarova et al., 2004; Deckert et al., 2006) and colocalize to nuclear speckles enriched in splicing factors (Komuro et al., 1999b; Llorian et al., 2004, 2005; Nicolaescu et al., 2008). A central, polar amino acid-rich domain (PRD) in mammalian PQBP1 can bind to proteins with polyglutamine (polyQ) tracts, such as transcription factors Brm2 and Ataxin-1, and the cytoplasmic transport protein Huntingtin (Htt), which is enhanced in poly(Q)-expanded pathogenic mutants of these human proteins (Waragai et al., 1999; Okazawa et al., 2002; Busch et al., 2003).

Although the molecular effects of PQBP1 have been studied in cultured cells and some model animals, little is known about its physiological function in developing embryos. Partial loss of function of PQBP1 in mice and Drosophila causes neurobehavioral phenotypes with no reported developmental defects (Ito et al., 2009; Tamura et al., 2010). However, a homozygous knockout null mouse line apparently cannot be generated, probably due to lethality, suggesting an essential role for PQBP1 in embryonic development. Overexpression of PQBP1 is also deleterious, promoting neuronal cell death in mice (Okuda et al., 2003; Marubuchi et al., 2005), and impaired long-term memory and behavioral abnormalities in Drosophila (Yoshimura et al., 2006). A PQBP1 gene duplication has been found in a patient with Renpenning syndrome, in which the PQBP1 protein may be overexpressed (Flynn et al., 2011). All of these findings suggest that maintaining PQBP1 within a restricted concentration range is essential for its proper biological actions.

Here, we use the animal model Xenopus laevis to investigate the embryonic expression and functions of PQBP1 and to identify potential molecular targets. We also evaluate one of its physiological partner proteins, WBP11, for which no developmental expression or functional information has been reported. We have found that both
genes are expressed in the developing mesoderm and nervous system, and that loss of function (by morpholino knockdown) of either one results in abnormal gastrulation and neurulation. We also find that inhibiting PQBP1 or WBP11 causes similar defects in mesodermal and neural differentiation in Xenopus embryos, particularly in FGF-responsive gene expression. Mechanically, PQBP1 knockdown causes a shift in the alternative splicing of FGF receptor-2 (FGFR2) pre-RNA, and a loss of MAPK activation in response to FGF4 (eFGF). Our findings may provide clues to the cause of developmental abnormalities associated with human PQBP1 mutations in Renpenning syndrome, with the particular suggestion that PQBP1 mutations may result in aberrant FGF signaling in embryonic and postnatal Renpenning patients.

RESULTS

PQBP1 knockdown causes mesodermal and neural defects in Xenopus embryos

Two X. laevis pqbp1 genes were revealed by BLAST searches of Xenopus expressed sequence tag (EST) and genome databases using mammalian pqbp1 sequences, which are homeologs that result from an ancient interspecies hybridization event that gave rise to the allotetraploid X. laevis lineage (Hughes and Hughes, 1993). The two predicted proteins (PQBP1a and PQBP1b) share 94% sequence identity, and are highly homologous to human PQBP1 (79-80% identity) except for the poly(Q)-binding PRD region, which is less conserved among species (supplementary material Fig. S1A).

To lay the groundwork for predicting and testing potential developmental functions of PQBP1, we determined the spatiotemporal expression of these genes in developing Xenopus embryos by whole-mount in situ hybridization (WISH) and semi-quantitative RT-PCR (qPCR) (Fig. 1; supplementary material Fig. S2). During early cleavage (~64 cells), pqbp1 transcripts were present in animal pole blastomeres (Fig. 1A), and in the early gastrula (stage 10.5) pqbp1 was expressed throughout the mesoderm (Fig. 1B,C). In the late gastrula, pqbp1 expression coincided with both chordin (marking the notochord) and sox2 (marking the prospective neural plate) (Fig. 1D). At neurula stages, pqbp1 was expressed in the neural plate and the head primordium (Fig. 1A,E) in a region that overlapped with ncam expression but also encompassed the cranial neural crest (Fig. 1E) and cells in the ventrolateral neural tube (supplementary material Fig. S3). In tailbud tadpole stages, pqbp1 was expressed in head, eyes, spinal cord and branchial arches (Fig. 1A).

To determine which early developmental processes require PQBP1, we performed gene knockdown in X. laevis embryos with translation-blocking antisense morpholino oligonucleotides (MOs) that target each individual pqbp1 homeolog (MOa and MOb for pqbp1a and pqbp1b, respectively), or another MO predicted to inhibit both homeologs simultaneously (MO1; Fig. 2). The ability of MO1 to block pqbp1 mRNA translation was confirmed by western blot (Fig. 2G). We first examined the effects of PQBP1 knockdown in the mesoderm by bilaterally injecting MOs into the marginal zone of two-cell stage blastulae (Fig. 2). MO-injected embryos are referred to as morphants, and MO1 morphant phenotypes commonly included shortened anteroposterior (AP) body axes, small heads and no tail structures (Fig. 2C). These phenotypes were also accompanied by some cell dissociation at neurula stages. Interestingly, embryos injected singly with either MOa or MOb showed only mild developmental defects (Fig. 2D,E), but when these MOs were combined the resulting morphants lacked head and tail structures that were very similar to, but more severe than, the defects caused by MO1 (Fig. 2F). The congruent effects of knocking down one or both PQBP1 homeologs indicate that the individual PQBP1 morphant phenotypes are specific to the MOs and that both homeologs contribute similarly to normal X. laevis development.

We next tested the effects of PQBP1 knockdown on development of the dorsal, Spemann–Mangold organizer mesoderm and adjacent neural tissue. MO1 was injected into the dorsal marginal zone (DMZ) of four-cell embryos, and the resulting morphants showed abnormal cell movements associated with gastrulation and neural

![Fig. 1. pqbp1 and wbp11 genes are similarly expressed in mesoderm and neural tissues during Xenopus development. (A) WISH detection of pqbp1 and wbp11 transcripts in 64-cell blastula, neurula and tailbud tadpole; lateral views except anterior (ant) view of neurula. (B-E) WISH on intact animal pole ectoderm and marginal zone in early gastrula (stage 10.5), upper panel; sense probe, lower panel. (C) Expression of pqbp1 and chordin in gastrulae, stage 11.5. (D) Expression of pqbp1, wbp11, chordin and sox2 in late gastrulae, dorsal-posterior views. Expression of pqbp1 overlaps with chordin in dorsal/axial mesoderm (arrows) and with sox2 in anterior neuroectoderm. (E) Expression of pqbp1 and ncam mRNA in the neural plate of early neurula embryos. Note pqbp1 expression is in a broader region than ncam. an, anterior neur ectoderm; chd, chordin; S, pqbp1 sense probe.](image-url)
plate folding, and later stage tadpoles had reduced head and shortened AP axial structures. The severity of the phenotypes was dose-dependent and increased as the amount of MO1 was raised (Fig. 2H). In some cases, open blastopores and abnormally folded neural plates were clearly visible (Fig. 2J,K). Importantly, these morphant defects could be partly rescued by co-injection of synthetic *pqbp1* mRNA (0.4 ng or 2.0 ng) resistant to the PQBP1 MOs, indicating that the effects of MO1 are specific (Fig. 2J,K; supplementary material Fig. S5). Consistent with abnormal phenotypes caused by gain of function in other species, overexpression of PQBP1 in *Xenopus* embryos also caused severe developmental defects in gastrulation and neurulation (Fig. 2I; supplementary material Fig. S4), and those resembled the PQBP1 knockdown phenotypes (Fig. 2H).

To further characterize the developmental defects in PQBP1 morphants, we examined molecular markers of regional patterning. As a combination of MOa and MOb had the strongest and most consistent effects, this pair was applied together in these and in subsequent PQBP1 knockdown experiments, unless otherwise noted. Examining general mesoderm, injection of PQBP1 MOs into any region of the mesoderm reduced or eliminated *brachyury* expression in recipient cells (Fig. 2J,K). Importantly, these morphant defects could be partly rescued by co-injection of synthetic *pqbp1* mRNA (0.4 ng or 2.0 ng) resistant to the PQBP1 MOs, indicating that the effects of MO1 are specific (Fig. 2J,K; supplementary material Fig. S5). Consistent with abnormal phenotypes caused by gain of function in other species, overexpression of PQBP1 in *Xenopus* embryos also caused severe developmental defects in gastrulation and neurulation (Fig. 2I; supplementary material Fig. S4), and those resembled the PQBP1 knockdown phenotypes (Fig. 2H).
As seen in Fig. 2C, we also observed some cell dissociation at neurula stages, typified by sox2-positive cells detaching from the neural plate in PQBP1 morphants (Fig. 3D, magnified).

**WBP11 is essential for normal development**

The spliceosome protein WBP11 is an endogenous partner of mammalian PQBP1 (Nicolaescu et al., 2008; Tapia et al., 2010), but despite its functional importance in pre-mRNA splicing, nothing is known about its developmental expression or function. We identified two homeologs in *X. laevis* that encode predicted proteins with 95% (604/636) identity to each other and 74% identity to human WBP11 (supplementary material Fig. S1B). In developing *Xenopus* embryos, *wbp11* is expressed maternally, with maternal transcripts persisting through blastula stages. Postzygotically, *wbp11* is expressed in ectoderm and mesoderm during gastrulation, in neural plate during neurulation and in spinal cord and brain in tadpole stages (Fig. 1A; supplementary material Fig. S2). *Wbp11* expression closely coincides with that of *pqbp1*, consistent with a physical and functional partnership between these proteins in mammalian systems. We validated the ability of *Xenopus* PQBP1 and WBP11 proteins to physically associate by co-immunoprecipitation from mammalian cultured cells (supplementary material Fig. S6A), and by observing their ability to colocalize to nuclei (supplementary material Fig. S6B).

Potential embryonic functions for WBP11 have not been evaluated in any species. Therefore, we tested whether WBP11 is required for development by inhibiting expression of the two *X. laevis* homeologs with translation-blocking MOs (Fig. 4A). We confirmed the specificity of the WBP11 MOs by showing that they block endogenous WBP11 translation in *X. laevis* embryos (Fig. 4B). Embryos injected bilaterally with WBP11 MOs displayed shortened AP axes, open blastopore remnants, small or absent heads and truncated tails (Fig. 4C). These phenotypes closely resembled those of PQBP1 morphants, so we tested whether combined knockdown of PQBP1 and WBP11 might prove more deleterious than individual gene knockdowns. Embryos were targeted in the marginal zone with low doses of either the PQBP1 or WBP11 MO, which alone allowed normal gastrulation and caused only a slight perturbation of neural folding (Fig. 4D). However, simultaneous low-dose knockdown of PQBP1 and WBP11 blocked gastrulation (note open blastopores) and neural folding (Fig. 4D). These seemingly additive phenotypic effects suggest that these proteins perform similar developmental functions.

**Defective mesodermal and neural marker expression in PQBP1 and WBP11 morphants**

To better understand the PQBP1 and WBP11 morphant phenotypes, we scored regional and tissue-specific marker gene expression by qPCR on embryos injected bilaterally into the marginal zone at the two-cell stage and harvested at early gastrulation (stage 10.5). Results in Fig. 5A show that expression of several general mesodermal markers was reduced in morphants by individual or combined knockdown of PQBP1 or WBP11. To assess general mesoderm specification, we evaluated *brachyury* and found it was reduced by about 40% of control levels by PQBP1 knockdown.
Two other mesodermal markers, *fgf4* and *cdx4*, were the most significantly disrupted in PQBP1 morphants (Fig. 5A). Single knockdown of PQBP1 or WBP11 resulted in 70-90% reduction of *fgf4* and *cdx4*, whereas combined knockdown almost completely eliminated their expression. Expression of another mesodermal FGF gene, *fgf8*, was normal in all PQBP1 or WBP11 morphants. The specificity of PQBP1 morpholino was further confirmed by the ability of MO-resistant *pqbp1* mRNA to rescue *fgf4* and *cdx4* expression in PQBP1 morphants (supplementary material Fig. S7B), adding support to rescue experiments in whole-embryo morphants (Fig. 2J). Specificity is also supported by results showing that different PQBP1 morpholinos (MO1, MOa or MOb) had similar effects (supplementary material Fig. S7A).

In the neural ectoderm, single or combined knockdown of PQBP1 and WBP11 caused significant reduction (~60%) in the expression of the early neural marker, *sox2* (Fig. 5C), consistent with reduced *sox2* and ncam expression in PQBP1 morphants observed using WISH (Fig. 3C,D).

We also tested whether PQBP1 knockdown in related *X. tropicalis* embryos, and found phenotypes similar to those of *X. laevis* PQBP1 morphants, with missing head and tail structures and short body length, as well as reduced *fgf4* and *cdx4* expression in early gastrulae (supplementary material Fig. S7C). The results of morphant analyses in *X. laevis* and *X. tropicalis* are consistent with each other and provide more general evidence that PQBP1 regulates essential developmental functions.

**PQBP1 knockdown impairs responses to FGF but not other inductive signals**

Multiple signaling pathways regulate mesoderm and neural induction, tissue patterning and morphogenesis in *Xenopus* embryos, including FGF, Nodal/Vg1, BMP and Wnt. Inhibition of any of these pathways might account for the phenotypes of PQBP1 or WBP11 morphants. To delineate which signaling pathways might be impaired in PQBP1 morphants, we examined the effects of PQBP1 knockdown on the induction of marker genes by FGF, Nodal, BMP or Wnt signals in *Xenopus* animal caps. Growth factors (as mRNAs) together with morpholinos were injected into animal poles of two-cell embryos, and caps were cut and screened for induction of target genes by qPCR. We found that PQBP1 morphant animal caps responded normally to Wnt8, Nodal2 (Xnr2) and BMP4, but response to FGF4 was significantly inhibited (Fig. 6B). Specifically, induction of *cdx4* and *bra* by FGF4 was diminished in PQBP1 morphants, despite normal induction of *cdx4* by Wnt8 or *bra* by BMP4. Furthermore, induction of *fgf4* by FGF4, induced by FGF4/Brachyury-positive feedback loops (Isaacs et al., 1994; Fujii et al., 2008), was also blocked by PQBP1 knockdown.

We also tested whether PQBP1 might be required for normal operation of the FGF receptor tyrosine kinase (RTK) signaling cascade by analyzing MAPK (Erk) phosphorylation in PQBP1 morphant animal caps. Injection of 1pg *fgf4* mRNA into animal caps induced phosphorylation of MAPK, but co-injection of either PQBP1 MO1 or MOa+MOb blocked MAPK phosphorylation (Fig. 6C). In sum, the results of experiments performed in whole embryos (Fig. 5) and isolated animal caps (Fig. 6) demonstrate that responses to FGF signaling require PQBP1, whereas Nodal, BMP and Wnt pathways do not.

**PQBP1 regulates alternative splicing of an FGF receptor**

One plausible explanation for the deleterious effects of PQBP1 knockdown on FGF responses is that PQBP1 acts at the level of gene transcription or pre-mRNA splicing for FGF signaling components.
At the ligand level, alternative splicing of fgf4 and fgf8 pre-mRNA can generate protein variants with distinct biological activities (Fletcher et al., 2006; Guo and Li, 2007; Mayshar et al., 2008). In particular, two FGF8 isoforms, FGF8a and FGF8b, respectively, induce neural and mesodermal tissues in *Xenopus* embryos (Fletcher et al., 2006). However, we observed no differences in the splicing patterns of fgf4 or fgf8 transcripts in PQBP1 and/or WBP11 morphants compared with controls (data not shown).

PQBP1 or WBP11 knockdown defects could also be explained by mis-splicing of the FGF receptors. Although only four genes encode FGF receptors, alternative splicing results in numerous isoforms with different ligand specificity (Ornitz et al., 1996; Eswarakumar et al., 2005; Zhang et al., 2006). FGF receptors 1, 2 and 3 incorporate exons 8a or 8b through alternative splicing of their pre-mRNAs, resulting in receptor isoforms IIIb or IIIc, respectively. These isoforms possess distinct ligand specificity resulting from unique residues in the immunoglobulin-like loop III of the extracellular, ligand-binding domain encoded by exon 8. Among mammalian receptors, the IIIb isoforms of FGFR1-3 preferably bind FGF3, FGF7 and FGF10, whereas IIIc isoforms prefer FGF4 and FGF8 (Eswarakumar et al., 2005; Holzmann et al., 2012). FGFR4 does not undergo alternative splicing of exon8, but binds FGF4 and FGF8b (Blunt et al., 1997).

In order to determine whether expression or splicing of FGF receptors was modified, we surveyed the levels of FGFR1-3 IIIb or IIIc isoform transcripts, as well as the total expression levels of all four FGF receptors, in control (control MO-injected), PQBP1 or WBP11 morphant embryos at early gastrulation (stage 10.5). To assay IIIb and IIIc isoforms, we used primers that targeted exon8a or exon8b on the upstream side, and thus amplified only spliced transcripts that contained one of these alternative exons in fgfr1-3 (as illustrated for fgfr2 in Fig. 7C,D). In wild-type or control embryos, qPCR indicated the presence of all potential receptor isoforms, but relative isoform levels varied (Fig. 7; supplementary material Fig. S8). We observed a dramatic change in the isoform ratio of fgfr2 IIIb and IIIc transcripts, but no change in the total level.
β of but blocked by co-injection of (C) Phosphorylation of MAPK (Erk) was induced in and either control MO (50 ng), triplicate biological replicates. Animal caps were injected with reduced by PQBP1 knockdown (* P <0.05 or ** P <0.01; Student’ s t-test, n=3). (B) Marker gene induction by FGF4 was significantly decreased in PQBP1 knockdown (50 ng MO). There was no statistically significant difference between CT and PQBP1 MO-injected caps treated with each ligand (Student’s t-test, n=3). (A) Marker gene induction by Wnt8 (50 pg), BMP4 (500 pg) and Nodal2 (Xnr2; 100 pg) was not affected by PQBP1 knockdown (50 ng MO). There was no statistically significant difference between CT and PQBP1 MO-injected caps treated with each ligand (Student’s t-test, triplicate biological replicates). Animal caps were injected with fgfr4 mRNA (1 pg) and either control MO (50 ng), pqbp1 MO1 (50 ng) or MOa+MOb (25 ng each). (C) Phosphorylation of MAPK (Erk) was induced in fgfr4-injected animal caps, but blocked by co-injection of pqbp1 MO, either MO1 or MOa+MOb. The levels of β-tubulin and total MAPK protein did not change among these samples.

Fig. 6. Effects of PQBP1 knockdown on animal cap response to growth factors. (A,B) Two-cell embryos were injected into the animal pole with growth factor mRNAs and MOs, animal caps were cut at mid-blastula and harvested at the equivalent of early gastrula stage, followed by qPCR, as depicted (top). Results were analyzed and plotted as per Fig. 5. (A) Marker gene induction by Wnt8 (50 pg), BMP4 (500 pg) and Nodal2 (Xnr2; 100 pg) was not affected by PQBP1 knockdown (50 ng MO). There was no statistically significant difference between CT and PQBP1 MO-injected caps treated with each ligand (Student’s t-test, n=3). (B) Marker gene induction by FGF4 was significantly reduced by PQBP1 knockdown (* P<0.05 or **P<0.01; Student’s t-test, triplicate biological replicates). Animal caps were injected with fgfr4 mRNA (1 pg) and either control MO (50 ng), pqbp1 MO1 (50 ng) or MOa+MOb (25 ng each). (C) Phosphorylation of MAPK (Erk) was induced in fgfr4-injected animal caps, but blocked by co-injection of pqbp1 MO, either MO1 or MOa+MOb. The levels of β-tubulin and total MAPK protein did not change among these samples.

PQBP1 and one of its binding proteins, WBP11, are implicated in RNAPII-dependent transcription and pre-mRNA splicing, but neither protein has clearly delineated roles or gene targets in developing embryos. Here, we have shown that during development of Xenopus embryos, PQBP1 and WBP11 are co-expressed in nascent mesodermal and neural tissues, and loss of function of these genes causes defects in mesodermal and neural patterning accompanied by abnormal gastrulation and neurulation, as well as by reduced mesodermal gene expression. At the molecular level, we find that PQBP1 is required for MAPK activation, target gene expression and mesoderm induction by FGF4 ligand, but not for responses to other mesodermal inducers and patterning agents, including Nodal2, BMP4 or Wnt8. Furthermore, we find that PQBP1 regulates alternative splicing of FGFR receptor-2 transcripts, influencing the relative abundance of two FGFR2 isoforms (IIb and IIIc) that have different binding specificities for FGF ligands. Our results reveal important roles for PQBP1 in vertebrate embryonic development, and those may be relevant to the molecular mechanisms of human developmental or neural/cognitive defects caused by pqbp1 mutations. Although WBP11 is not implicated in human disease or birth defects, our study is the first to indicate a normal developmental role for WBP11 in any embryo, raising the possibility that wbp11 mutations could contribute to birth defects.

DISCUSSION
PQBP1 and PQBP1 and WBP11 are required for normal mesodermal and neural development

Despite the recognized importance of pqbp1 mutations in the etiology of human XLID syndromes, information about the embryonic expression and function of PQBP1 or WBP11 is limited to a description of mouse pqbp1 expression (Qi et al., 2005). Our analysis of Xenopus embryos shows that pqbp1 and wbp11 genes are maternally expressed, present throughout cleavage late blastula stages, then zygotically expressed in the mesoderm during gastrulation and the developing neural plate and nervous system of tadpoles.

The expression of pqbp1 in tailbud tadpoles appears homologous to that reported for embryonic and postnatal mice, where pqbp1 mRNA and protein are predominantly expressed in the central nervous system and neuronal stem cells (Waragai et al., 1999; Qi et al., 2005; Wang et al., 2013). The neural expression of pqbp1 in Xenopus and mouse is consistent with the fact that human pqbp1 mutations cause mental retardation and microcephaly. Moreover,
the co-expression of \textit{pqbp1} and \textit{wbp11} in the same tissues of \textit{Xenopus} embryos is consistent with the known physical and functional links between the two proteins (Komuro et al., 1999b; Llorian et al., 2004, 2005; Nicolaescu et al., 2008). Whether these proteins function together mechanistically to govern the same biochemical or embryonic processes remains to be determined. At neurula stages, \textit{PQBP1} or \textit{WBP11} morphants display incomplete neural tube closure accompanied by reduced expression of neural marker genes, underscoring their requirement for normal neural development.

The neural expression of \textit{pqbp1} in \textit{Xenopus} embryos might have been anticipated, but our finding that both \textit{pqbp1} and \textit{wbp11} are expressed and required for normal development of mesoderm is new and reveals a broader role for these genes in early developmental processes, particularly in non-neural tissues. Knockdown of either gene causes abnormal cell migration during gastrulation, notably a failure of dorsal mesodermal convergence-extension movements. These defects in gastrulation are probably linked to reduced \textit{brachyury} expression in \textit{PQBP1} morphants, as this gene is essential for mesodermal cell motility as well as differentiation. Some physical manifestations observed in Renpenning syndrome patients, including midline and cardiac defects, lean muscle and short stature, may be analogous to defects in mesoderm observed in \textit{PQBP1} morphants.

\textbf{PQBP1 is required for FGF signaling and regulates splicing of FGF receptor transcripts}

Our work further shows that the molecular and morphological defects observed in \textit{PQBP1} morphant embryos appear to be the result of aberrant FGF signaling. Direct tests of mesoderm induction in animal caps showed \textit{PQBP1} knockdown is required for signaling triggered by FGF4 but not Nodal, BMP or Wnt ligands. The loss of RTK signal transduction in particular indicates defects upstream of FGF-responsive gene transcription, and not some general defect in transcription or mRNA splicing of FGF target genes. FGF receptors, of which there are four, were foremost among candidates because their transcripts can undergo alternative splicing in various biological systems to generate receptor isoforms with different ligand-binding specificities. Our survey of \textit{fgfr} expression and splicing shows that among the four receptors, \textit{fgfr2} showed drastically altered levels of alternatively spliced transcripts encoding isoforms IIIb and IIIc in \textit{PQBP1} morphants (supplementary material Fig. S8). Normally, early gastrula stage embryos express approximately 50% more \textit{fgfr2IIIc} transcripts than \textit{fgfr2IIIb} transcripts. We found that \textit{PQBP1} knockdown reverses this ratio, lowering \textit{fgfr2IIIc} transcript levels to about half the level of \textit{fgfr2IIIb} transcripts. As the FGFR2IIIc, but not IIIb, receptor isoform binds to FGF4 and FGF8, we postulate that the lowered abundance of FGFR2IIIc reduces the ability of the embryo to respond to mesoderm-inducing FGF4/8 ligands.
Furthermore, whereas PQBP1, FGF4 and FGF8 morphants share similar mesodermal, neural and gastrulation defects (Fisher et al., 2002; Fletcher et al., 2006; Isaacs et al., 2007), PQBP1 morphant phenotypes are more severely affected, yet rather similar to those generated by a dominant negative FGF receptor (XFD) or FGFR inhibitor SU5402, which block all FGF signaling, including that stimulated by FGF4 and FGF8b (Amaya et al., 1993; Delaune et al., 2005; Fletcher and Harland, 2008). This greater similarity between PQBP1 knockdown and wholesale loss of FGF signaling is potentially explained by the reduction of the FGFRIIIic isoform in PQBP1 morphants, but we cannot exclude the possibility that PQBP1 inhibition affects expression of other essential embryonic gene targets.

FGFR2 isomeric switching via alternative splicing has been observed in multiple contexts, including normal epithelial-mesenchymal transition (Warzecha and Carstens, 2012), embryonic development (Eswanakumar et al., 2002; Rice et al., 2003; Takeuchi et al., 2005; Liu et al., 2011), human birth defects (Hajihosseini et al., 2001; Taebi et al., 2002; Ibrahimi et al., 2005), cancer and various pathologies (Katoh, 2008, 2009; Holzmann et al., 2012; Kelleher et al., 2013). Whether PQBP1 regulates fgfr2 alternative splicing in these normal and disease situations will be worth investigating. Our preliminary results also show changes in relative levels of alternative transcripts for fgfr1-3, but changes in fgfr2 transcript splicing were greater than fgfr1 and fgfr3 upon PQBP1 knockdown (supplementary material Fig. S8). The time and place of expression of all FGF receptors and their splicing variants in Xenopus embryos has not been well delineated, so further investigation is required to understand their precise roles in mesoderm and neural induction by FGFs and how pqbp1 impacts these functions.

In addition to our findings, previous studies have identified other factors regulating fgfr2 pre-mRNA splicing. Embryonic splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) govern alternative splicing of fgfr2 and other transcripts in a human epithelial cell line, and depletion of ESRP1 and ESRP2 causes splice switching from an epithelial IIIb form to a mesenchymal IIIc form (Warzecha et al., 2012). ESRP1 and ESRP2 are both involved in pre-mRNA splicing. Epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) govern alternative splicing of fgfr2 and other transcripts in a human epithelial cell line, and depletion of ESRP1 and ESRP2 causes splice switching from an epithelial IIIb form to a mesenchymal IIIc form (Warzecha et al., 2012). ESRP1 and ESRP2 are both involved in pre-mRNA splicing. Epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) govern alternative splicing of fgfr2 and other transcripts in a human epithelial cell line, and depletion of ESRP1 and ESRP2 causes splice switching from an epithelial IIIb form to a mesenchymal IIIc form (Warzecha et al., 2012). ESRP1 and ESRP2 are both involved in pre-mRNA splicing. Epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) govern alternative splicing of fgfr2 and other transcripts in a human epithelial cell line, and depletion of ESRP1 and ESRP2 causes splice switching from an epithelial IIIb form to a mesenchymal IIIc form (Warzecha et al., 2012). ESRP1 and ESRP2 are both involved in pre-mRNA splicing. Epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) govern alternative splicing of fgfr2 and other transcripts in a human epithelial cell line, and depletion of ESRP1 and ESRP2 causes splice switching from an epithelial IIIb form to a mesenchymal IIIc form (Warzecha et al., 2012). ESRP1 and ESRP2 are both involved in pre-mRNA splicing. Epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) govern alternative splicing of fgfr2 and other transcripts in a human epithelial cell line, and depletion of ESRP1 and ESRP2 causes splice switching from an epithelial IIIb form to a mesenchymal IIIc form (Warzecha et al., 2012). ESRP1 and ESRP2 are both involved in pre-mRNA splicing. Epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) govern alternative splicing of fgfr2 and other transcripts in a human epithelial cell line, and depletion of ESRP1 and ESRP2 causes splice switching from an epithelial IIIb form to a mesenchymal IIIc form (Warzecha et al., 2012). ESRP1 and ESRP2 are both involved in pre-mRNA splicing.Epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) govern alternative splicing of fgfr2 and other transcripts in a human epithelial cell line, and depletion of ESRP1 and ESRP2 causes splice switching from an epithelial IIIb form to a mesenchymal IIIc form (Warzecha et al., 2012). ESRP1 and ESRP2 are both involved in pre-mRNA splicing.
5'-GATCGTGGCGTCTACCAACAGG-3' and pqp1 reverse, 5'-GCAAAC-GCAATCTGACAGCAGT-3'. The sequence of this clone completely matched to another X. laevis EST clone (NM_001091714) except for its shorter 5' untranslated region (UTR) end. Identical sequences were identified in X. laevis genome scaffolds (Xenbase gbrowse laevis 6.0: Scaffold1707:1728996-1729209). MO-resistant Xenopus pqp1 cDNA was amplified with the primers forward 5'-CCTCAGATGGCGGTCTT- TAGGCCTCTAGCCT-3' reverse 5'-CAATCAATAGGGGGACAGATG-3', and subcloned into pCS2+ for in vitro transcription. This resulted in nine nucleotide mismatches at the pqp1/ morpholino recognition site, but normal amino acid coding was retained. C-terminal myc-tagged Xenopus PQB1 was made by PCR cloning into the myc/PKS-SK vector. Full-length X. laevis wbp1 cDNA was obtained by RT-PCR with the following primers: 5'-CCC- ATGCGATTGCGCGGCGATCCGCTTCG-3' and 5'-CGATGACATGAA- AAATGCTTACGATAATGCGC-3' (restriction enzyme sites are underlined) with design based on an EST (BC057737). All cDNA identities were verified by DNA sequencing, and subcloned into pCS2+ unless otherwise noted. Full-length X. tropicalis fgfr2IIc was a gift from Dr Richard Harland (University of California, Berkeley, USA).

**In situ hybridization**

*In situ* hybridization was performed with digoxigenin-labeled probes as previously described (Alexandrova and Thomsen, 2006). BM Purple was used as a chromogenic substrate (Roche). Sagittal or cross sections were performed during MEMPPA fixation. Template plasmids for making probes were pBS-ncam, pBS-ax-2, pGEM4Z-chordin, pXT1-brachury, pMX-sna1, pCS2-pqp1 and pCS2-wbp1.

**Quantitative RT-PCR**

Total RNA was extracted from ten animal caps or three embryos and used for cDNA synthesis as previously described by Callery et al. (2005). Quantitative RT-PCR using the LightCycler System and SYBR Green reagent (Roche Applied Science) according to Kalkan et al. (2009) using primers listed in supplementary material Tables S1 and S2. Relative amounts of PCR product were determined based on standard curves derived from 1:1, 1:10 and 1:100 dilutions of the cDNA from control embryos, with target gene expression normalized to the relative levels of ornithine decarboxylase (odc) transcripts. Most primers were designed to have annealing temperature of 60°C, and product sizes between 100-200 bp; most PCR conditions were annealing at 55°C, elongation 12 s at 72°C, denaturation at 94°C for 10 s. cDNA were from three or more biological replicate experiments were amplified in most Light Cycler reactions (see text) and statistical analyses were conducted using Student’s t-test.

**Protein analysis, immunoprecipitation and immunofluorescence**

For detection of phosphorylated MAPK, animal caps were lysed in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, together with a proteinase and phosphatase inhibitor cocktail (Roche). Solubilized proteins were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane (Bio-Rad) and stained with anti-phospho-p44/42 MAPK (Cell Signaling, 4370; 1:2000), anti-p44/42 MAP kinase (Cell Signaling, 4695P; 1:1000) and anti-jtubulin (Accurate Chemical and Scientific Corporation, BYA6068-1; 1:5000) antibodies (Suga et al., 2006). Signals were visualized by Alexa dye-conjugated goat anti-mouse and anti-rabbit antibody (Molecular Probes), and visualized using the Odyssey Infrared Imager (LI-COR). For co-immunoprecipitation, HA-pqp1 and myc-wbp1 were transfected into COS-1 cells and detected with anti-myc mouse monoclonal 9E10 and anti-HA rabbit polyclonal antibodies, detected with Alexa 488 (Molecular Probes, A-11029, 1:1000) goat anti-mouse and Alexa 594 goat anti-rabbit (Molecular Probes, A-11037, 1:1000) secondary antibodies. COS-1 cells were grown in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

Y.I. and G.H.T. conceived and designed experiments, Y.I. carried out all experiments, and Y.I. and G.H.T. wrote the manuscript.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.106658/-/DC1

**References**


