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Wbp2nl has a developmental role in establishing neural and non-neural ectodermal fates

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ABSTRACT

In many animals, maternally synthesized mRNAs are critical for primary germ layer formation. In Xenopus, several maternal mRNAs are enriched in the animal blastomere progenitors of the embryonic ectoderm. We previously identified one of these, WW-domain binding protein 2 N-terminal like (wbp2nl), that others previously characterized as a sperm protein (PAWP) that promotes meiotic resumption. Herein we demonstrate that it has an additional developmental role in regionalizing the embryonic ectoderm. Knock-down of Wbp2nl in the dorsal ectoderm reduced cranial placode and neural crest gene expression domains and expanded neural plate domains; knock-down in ventral ectoderm reduced epidermal gene expression. Conversely, increasing levels of Wbp2nl in the neural plate induced ectopic epidermal and neural crest gene expression and repressed many neural plate and cranial placode genes. The effects in the neural plate appear to be mediated, at least in part, by down-regulating chd, a BMP antagonist. Because the cellular function of Wbp2nl is not known, we mutated several predicted motifs. Expressing mutated proteins in embryos showed that a putative phosphorylation site at Thr45 and an α-helix in the PH-G domain are required to ectopically induce epidermal and neural crest genes in the neural plate. An intact YAP-binding motif also is required for ectopic epidermal gene expression as well as for down-regulating chd. This work reveals novel developmental roles for a cytoplasmic protein that promotes epidermal and neural crest formation at the expense of neural ectoderm.

1. Introduction

One of the earliest decisions in embryonic development is the formation of the three primary germ layers, and in many animals maternally synthesized mRNAs are critical for their formation. Although the embryonic ectoderm is sometimes considered a “default” germ layer because in explant culture it will develop in the absence of external signaling factors (Itoh and Sokol, 2014), it is not simply a passive fate choice in the intact embryo. In *Xenopus*, for example, there are maternal factors sequestered in the animal blastomeres that promote the embryonic ectoderm by repressing mesoderm and endoderm formation (Itoh and Sokol, 2014; Zhang and Klymkowsky, 2007). In a microarray screen for maternal mRNAs enriched in animal blastomere progenitors of the ectoderm we identified *WW-domain binding protein 2 N-terminal like (wbp2nl)*, which is maternally deposited in all four animal blastomeres, and is zygotically expressed in the animal cap ectoderm of the blastula, the embryonic ectoderm and involuting mesoderm of the gastrula, and the neural ectoderm, border zone, and dorsolateral epidermis in the neural plate stage embryo (Grant et al., 2014). This expression pattern suggests that Wbp2nl may be involved in specifying the ectoderm germ layer and/or its three derivatives: non-neural (future epidermis), border zone (future neural crest and cranial placodes) and neural (future neural plate).

Wbp2nl, also known as Peri-Acrosomal WW-domain binding Protein (PAWP), belongs to a large family of WW-domain binding proteins (WWbps) whose members are involved in a variety of cellular processes crucial for cell fate decisions, including signal transduction, protein stability, and regulation of RNA polymerase activity (Hofmann and Bucher, 1995; Sudol et al., 2001). WWbp's contain variable length proline-rich regions that bind to the WW-domains of other proteins, many of which have crucial roles in developmental signaling pathways (Salah et al., 2012; Sudol, 2012). Previous work showed that Wbp2nl is present in the peri-acrosomal region of the sperm of several vertebrates

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(bull, mouse, rat, pig, rabbit, *Xenopus*), and is required for egg activation by eliciting intracellular calcium release (Aarabi et al., 2010; Wu et al., 2007). However, the role of egg- and/or zygote-derived Wbp2nl in embryonic development has not been described.

In this study we assessed the developmental role of Wbp2nl using loss- and gain-of-function analyses. We show that Wbp2nl is involved in establishing the relative sizes of the neural plate, border zone, and epidermis territories that are derived from the embryonic ectoderm (Groves and LaBonne, 2014). The effects in the neural plate are mediated, at least in part, by down-regulating the expression of *chd*, a BMP antagonist. Bioinformatics analyses revealed numerous putative functional sites in the PH-G and WWbp domains of Wbp2nl. Mutation of some of these sites show that the putative phosphorylation of Thr45 as well as preservation of an α-helical structure in the PH-G domain are required for the protein to ectopically induce *K81, foxd3* or *zic2* in the neural plate; a YAP-binding motif in the WWbp-domain is required for ectopic *K81* expression as well as efficient *chd* repression. This first description of an embryonic role for Wbp2nl shows that it is required for the division of the ectodermal germ layer into its functional domains.

2. Materials and methods

2.1. Obtaining embryos and microinjections

Wild type, outbred *Xenopus laevis* embryos were obtained by gonadotropin-induced natural mating of adult frogs, as previously described (Moody, 2000). Embryos were picked at the 2-cell stage, when the first cleavage furrow bisects the lightly pigmented region of the animal hemisphere, to facilitate identification of the cardinal axes (Klein, 1987; Miyata et al., 1987). This ensures accurate identification of the dorsal and ventral animal blastomeres with predominantly neural versus predominantly epidermal developmental fates (Moody and Kline, 1990). When selected embryos reached 8-cells, the dorsal-animal (D1) or the ventral-animal (V1) blastomere was microinjected with mRNA or antisense Morpholino oligonucleotides (MOs) as described elsewhere (Moody, 2000).

2.2. Blastomere explants

Both ventral animal blastomeres were injected with *wbp2nl* mRNA, and upon completion of the next cell cycle, both midline daughters (16-cell V1.1 blastomeres, Moody, 1987) were dissected free and cultured as explants, as previously described (Grant et al., 2013; Gaur et al., 2016). When sibling controls reached neural plate stages, explants were fixed and processed for in situ hybridization as described below.

2.3. Construction of Wbp2nl constructs

To make a morpholino-sensitive mRNA, *Xenopus laevis wbp2nl* was obtained (Open BioSystems; BC082812.1) and the ORF plus 66 base pairs of its 5′ UTR region generated by PCR using standard procedures and cloned into the ClaI/XhoI sites of the pCS2$^+$ vector (*pCS2$^+$-wbp2nl*). To make a morpholino-resistant mRNA, the ORF was generated by PCR and cloned into the EcoRI/XhoI sites of a pCS2$^+$ vector containing a 5′ Myc-tag (*pCS2$^+$–5′MT-wbp2nl*). Mutations were introduced into the *pCS2$^+$-wbp2nl* plasmid with the QuikChange Lighting Site-Directed Mutagenesis kit (Agilent). *wbp2nl-T45A* was constructed by a one-nucleotide base change (ACA to GCA) resulting in T45A conversion, replacing a predicted serine/threonine kinase phosphorylation site. *wbp2nl-Y55F* was constructed by a one-nucleotide base change (TAC to TTC) resulting in Y55F conversion, replacing a predicted site of tyrosine kinase phosphorylation. *wbp2nl-Y91G* was constructed by changing two nucleotides (TAC to GGC) resulting in Y91G conversion. This mutation replaced a predicted site of tyrosine phosphorylation. *wbp2nl-F127P* was constructed by changing two nucleotides (TTC to CCC) resulting in F127P conversion predicted to disrupt the α-helix C-terminal to the PH-G domain. *wbp2nl-Y282F* was constructed by a one-nucleotide base change (TAC to TTC) resulting in Y292F conversion in the YAP binding motif (PPPY to PPPF), eliminating a predicted phosphorylation site. In addition, an HA tag was added to the 3′ end of the *wbp2nl* open reading frame in *pCS2$^+$-wbp2nl* using the same mutagenesis kit. All constructs were fully sequenced in both directions.

2.4. In vitro synthesis of mRNAs and antisense RNA probes

mRNAs were synthesized in vitro (mMessage mMachine kit; Ambion). They were mixed with nuclear-localized *β-galactosidase* (*nβ-gal*) mRNA as a lineage tracer (100 pg/nl) at the indicated concentrations: wild type *wbp2nl* (200 pg/nl; 400 pg/nl), *wbp2nl-T45A* (400 pg/nl), *wbp2nl-Y55F* (400 pg/nl), *wbp2nl-Y91G* (400 pg/nl), *wbp2nl-F127P* (400 pg/nl), and *wbp2nl-Y282F* (400 pg/nl). Antisense RNA probes for in situ hybridization (ISH) were synthesized in vitro (MEGAscript kit; Ambion) as previously described (Sullivan et al., 2001; Yan et al., 2009).

2.5. Antisense oligonucleotide morpholino design and validation

To knock-down endogenous levels of Wbp2nl protein in the embryo, two translation-blocking MOs that target both homeologues were purchased (Gene-Tools, LLC) (Supplemental Fig. 2). An equimolar mixture of *wbp2nl* MOs (9.0 ng per blastomere) was microinjected into one dorsal and one ventral blastomere on one side of the 8-cell embryo. Both MOs were lissamine labeled so that cells in the embryo in which knock-down was achieved could be identified. To verify the ability of the MOs to block *wbp2nl* translation, *Xenopus* oocytes were injected with 9 ng of the MO cocktail and with either 2 ng of *wbp2nl-3′HA* mRNA (pCS2$^+$-*wbp2nl* construct contains 66 bp of *wbp2nl* 5′UTR = MO sensitive) or 2 ng of *5′MT-wbp2nl* mRNA (rescue mRNA). The latter is MO-resistant because there is no *wbp2nl* 5′ UTR present and 6 copies of the Myc-tag epitope sequence precede the *wbp2nl* ORF (Supplemental Fig. 2). The oocytes were cultured overnight at 18 °C, lysates prepared and Western blotting performed with an HA-tag or Myc-tag antibody as previously described (Neilson et al., 2012) (Supplemental Fig. 3A, B). In addition, the reversal of the MO knock-down phenotype in whole embryos was demonstrated by injecting 400 pg of rescue mRNA (*5′MT-wbp2nl*) immediately after embryos were injected with 2.25 ng of the MO cocktail (Supplemental Fig. 3A, B).

2.6. Whole embryo in situ hybridization

Embryos were cultured to gastrula (st. 10.5–11.5), neural ectoderm (st. 12–14) or neural plate (st. 16–18) stages (Nieuwkoop and Faber, 1994), fixed in 4% paraformaldehyde (in 0.1 M MOPS, 2 mM EGTA Magnesium, 1 mM $MgSO_4$, pH 7.4), stained for β-Gal histochemistry if injected with mRNAs, and processed for in situ hybridization (ISH) as previously described (Yan et al., 2009). Embryos were first scored for presence of lineage marker (lissamine-labeled MOs or nβ-Gal for mRNA) to demonstrate a successful injection. Then, position, intensity and size of the expression domain were compared on the injected, lineage-labeled side to the control, uninjected side of the same embryo as a control for inter-embryo variation. Samples were derived from at least three different clutches of eggs from three different sets of outbred, wild type parents. Samples were scored independently by two authors (AM, PG, or SAM). Frequencies of an observed phenotype in two different experimental groups were compared by Chi-squared statistical analysis. Sizes of gene expression domains were compared to control sides of the same embryo by the paired *t*-test.