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Two variants of zebrafish p100 are expressed during embryogenesis and regulated by Nodal signaling

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Abstract Human p100 protein was first identified as a transcriptional coactivator of Epstein–Barr virus nuclear antigen 2, and has been shown to be a coactivator of other cellular transactivators. Its roles in development of vertebrate embryos, however, have not been reported. We have identified a zebrafish ortholog of the human p100 coactivator. The zebrafish *p100* transcript is processed to two alternative variants, long and short forms, referred to as *p100L* and *p100S*, respectively. Both GFP-p100L and GFP-p100S fusion proteins are located in the cytoplasm of transfected culture cells and microinjected embryonic cells. Analysis of transcripts with Northern blots revealed the presence of *p100L* and lower amounts of *p100S* mRNAs from the one-cell stage throughout the life cycle. Whole-mount in situ hybridization shows that *p100L* and *p100S* share the same spatiotemporal expression pattern. Their zygotic expression is initially restricted to axial mesoderm precursors during gastrulation, and then spreads over other tissues during segmentation, and finally is constrained to some internal organs at day 5. We also find that Nodal signaling is essential for the zygotic expression of *p100*. These studies pave the way to understanding in depth the role of p100 during vertebrate embryogenesis.

Key words: Zebrafish; Nodal signaling; Epstein–Barr virus nuclear antigen-2 coactivator P100; Prechordal plate; Notochord

1. Introduction

The human p100 protein was first identified as a coactivator of Epstein–Barr virus nuclear antigen (EBNA) 2 [1]. EBNA-2 plays a key role in activating other viral genes when the virus infects and immortalizes B-lymphocytes [2]. Because p100 binds TFIIE, an essential component of RNA polymerase II initiation complexes, p100 probably acts as a bridge between EBNA-2 and the basal transcription machinery [1]. The p100 protein has been found to interact with and enhance transcriptional activity of c-Myb [3,4], a hematopoietic transcriptional factor, and STAT6, a central mediator of interleukin-4-induced gene responses [5]. As a coactivator, p100 protein is exclusively localized in the nuclei in several types of human cells [1]. Interestingly, Keenan et al. [6] have identified the p100 protein as a constituent of endoplasmic reticulum and cytosolic lipid droplets from milk-secreting cells of mouse and cow [6]. The p100 protein is also enriched both in nuclei and in endoplasmic reticulum/organelle fractions of mammary epithelial cells in response to prolactin in culture and in mammary tissue during lactation [7]. These studies suggest that p100 may play a role in a variety of important cellular events. Moreover, a p100-like protein has also been identified in plants [8], suggesting evolutionary conservation of p100.

We have identified a zebrafish ortholog of the human *p100* gene. In zebrafish, the *p100* gene has two alternatively spliced transcripts, and green fluorescent protein (GFP)-p100 chimeric proteins translated from both splice variants are localized in the cytoplasm of cultured and embryonic cells. Maternal transcripts of *p100* are distributed in all embryonic cells before gastrulation. Then expression becomes largely restricted to axial mesoderm during gastrulation and early segmentation, which is regulated by Nodal signaling.

2. Materials and methods

2.1. Embryos

Zebrafish (*Danio rerio*) of the AB strain were maintained and bred as described by Westerfield [9]. Homozygous oep^{m134} mutant embryos were produced by mating heterozygous mutant fish. Embryos from a cross of oep^{m134} were injected with 100 pg wild-type *oep* mRNA and adult females homozygous for the mutant allele were identified subsequently, as described by Gritsman et al. [10]. Maternal and zygotic *oep* (*MZoep*) embryos were produced by mating homozygous oep^{m134} females to heterozygous *oep* males. All embryos were raised at 28.5°C in Holtfreter water (0.05 g/l KCl,0.1 g/l CaCl, 0.2 g/l $NaHCO_3$, 3.5 g/l NaCl).

2.2. Isolation of p100 cDNAs

The shorter transcript form of the zebrafish *p100* gene, *p100S*, was initially identified during a systematic search of a cDNA library, which was constructed in plasmid vector pcDNA3.0, for organ- or tissue-specific genes during early embryogenesis (see [11] for details). An expressed sequence tag (EST) clone (fm53a04) that contains the longer transcript form of *p100*, *p100L*, was purchased from Invitrogen. The sequences of *p100S* and *p100L* were deposited in the GenBank database with accession numbers AF422806 and AY272050, respectively.

2.3. Plasmid constructs

For determining subcellular localization of p100, the coding sequence of *p100S* and *p100L* was amplified by polymerase chain reaction (PCR) and placed immediately downstream of the GFP coding sequence of vector peGFP-C2, which generated plasmids peGFP-p100S and peGFP-p100L, respectively. A *p100L*-specific sequence of approximately 1.8 kb was amplified by PCR with a pair of specific primers (5′-GGGCGATCAAGAATGGCAAAGGAC-3′ and 5′-CAAAACATAGACATCTGACTGAGC-3′) and cloned into vector pT-Easy to generate plasmid pP100L-1.8 kb.

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2.4. Reverse transcription (RT)-PCR and Northern blot

Total RNA was isolated from embryos at different stages using Trizol (Gibco BRL). A pair of specific primers (5′-GGGCGATCAAGAATGGCAAAGGAC-3′ and 5′-TCACAGATGGTGGTATAGTTGAC-3′) was used to amplify by RT-PCR a *p100S*-specific region of 263 bp. First-strand cDNAs synthesized from total RNA were used as templates. PCR reactions were performed for 30 cycles (94°C, 30 s; 58°C, 30 s; 72°C, 1 min) and a final extension at 72°C for 10 min. As an internal control, a 475 bp sequence of β-actin cDNA was amplified by RT-PCR using two specific primers (5′-ATGGATGATGAAATTGCCGCAC-3′ and 5′-ACCATCACCAGAGTCCATCACG-3′). The amplified products were analyzed on agarose gels.

For Northern blots, total RNA was run on 1.2% formaldehyde-containing gels with 20 μg RNA per lane and then transferred onto a nylon membrane. The insert in pP100S, the original plasmid isolated from the library, was radiolabeled by random priming in the presence of [α-^{32}P]dCTP and hybridized to RNA using the ExpressHyb™ hybridization kit (Clontech). A final post-hybridization wash was carried out in 0.2×SSC, 0.1% SDS at 60°C.

2.5. RNA synthesis

Capped mRNAs for *squint* (*sqt*), *lefty1* (previously named *antivin*) and *oep* were synthesized in vitro using the T7 Cap-Scribe kit (Roche) according to the manufacturer's recommendation. RNA was purified using RNAeasy® Mini Kit (Qiagen) and diluted in nuclease-free 0.1 M KCl to an appropriate concentration prior to microinjection.

2.6. Transfection and microinjection

Plasmids peGFP-p100S, peGFP-p100L and peGFP-C2 (control) were first used to transfect HeLa cells using the calcium phosphate transfection system (Life Technologies). DNA from these plasmids was also microinjected into one-cell embryos at a dose of about 100 pg. The embryonic cells were dispersed from the injected embryos at 80% epiboly stage, and these cells as well as transfected HeLa cells were observed for GFP by confocal microscopy. RNA was microinjected into the yolk of one-cell stage wild-type embryos. The microinjection dose was referred to the amount of DNA or RNA per embryo.

2.7. Whole-mount in situ hybridization

To detect the expression pattern of *p100*, antisense RNA probes were generated by in vitro transcription in the presence of digoxigenin-UTP using different templates. The plasmid pP100S linearized with *Bam*HI was used as a template and the probe derived was used, unless otherwise stated, to detect both *p100L* and *p100S* transcripts. To make *p100S*-specific antisense probe, the template used was a 600 bp fragment recovered from a *Pst*I digest of pP100S. This fragment contains a 480 bp *p100S*-specific sequence, a 100 bp sequence common in *p100S* and *p100L*, and the SP6 promoter. For detecting *p100L* expression only, plasmid pP100L-1.8 kb was linearized with *Sac*II prior to in vitro transcription. The embryos at different stages were fixed in 4% paraformaldehyde. The in situ hybridization essentially followed the protocol described by Westerfield [9].

3. Results

3.1. Zebrafish p100 *produces two alternative transcripts*

The *p100S* cDNA was initially identified by whole-mount in situ hybridization as a tissue-specific gene in zebrafish. This cDNA has a full length of 2191 bp and contains an open reading frame (ORF) between positions 107 and 1756 that encodes a putative peptide of 549 residues. BLAST searches revealed that the putative p100S protein shares an identity of 87.4% and 86.3% with EBNA-2 coactivator p100 of human [1] and mouse (GenBank accession number BAA84944), respectively, in the N-terminal region of 542 residues. To confirm whether p100S is a zebrafish ortholog of the human p100, we determined its genetic position in the genome. As shown in Fig. 1B, *p100S* maps to linkage group 4 of the zebrafish genome, falling into a stretch of conserved synteny with human chromosome 7 (Hsa7). This is consistent with the interpretation that p100S is an ortholog of the human p100 gene.

Because human p100 consists of 885 residues, we hypothesized that p100S is a truncated form of full-length p100 of zebrafish. Northern blots revealed a major transcript (*p100L*) of about 3.7 kb together with a minor 2.2 kb transcript (*p100S*) (Fig. 1C). We identified a zebrafish EST (fm53a04) from the GenBank database, the 5′ sequence of which is identical to that of *p100S* and the 3′ sequence of which encodes an amino acid sequence sharing high homology with the C-terminal domain of human p100. Sequencing this EST revealed that its most 5′ 1710 bp sequence of the 3654 bp full-length sequence is identical to *p100S*, implying that it is an alternative transcript of the *p100* locus. We therefore name it *p100L*. A 2667 bp ORF identified in *p100L* encodes a putative peptide of 888 residues. The putative p100L protein is identical in amino acid sequence to p100S in the N-terminal region of 535 residues, and shares an overall identity of 85% with human p100.

3.2. GFP-tagged chimeric proteins of both p100L and p100S are localized in the cytoplasm

Human p100 contains four and a half repeats of sequence homologous to bacterial staphylococcal nuclease (SNase) and a tudor domain [12,13]. Similarly, zebrafish p100L has all of these domains (Fig. 1A). In contrast, p100S contains only the first three SNase repeats and an incomplete fourth repeat, and lacks the tudor domain.

As a transcription coactivator, human p100 functions in the nucleus [1,3–5]. Because the two isoforms of zebrafish p100 are very different in length, we wondered whether either of them acts in the nucleus. To test this possibility, p100L and p100S were individually fused to GFP, and the resulting fusion constructs were used for transfection into cultured HeLa cells and for microinjection into zebrafish embryos. As shown in Fig. 1E, both GFP-p100L and GFP-p100S fusion proteins became localized to the cytoplasm, rather than in the nuclei, of the transfected human HeLa cells. To avoid misinterpreting the results from the in vitro assay, we further analyzed the subcellular localization of zebrafish p100 proteins in embryonic zebrafish cells by microinjecting DNA of the fusion constructs into one-cell stage embryos and observing GFP fluorescence during gastrulation. The results showed that both GFP-p100L and GFP-p100S were exclusively present in the cytoplasm of embryonic cells. This cytoplasmic localization is unexpected, but needs to be further tested for the endogenous proteins.

3.3. Expression of p100 *is dynamic during embryogenesis*

Northern blots showed that both p100 mRNA variants are present in embryos at all tested stages despite their dramatic quantitative difference (Fig. 1C). Because the band representing *p100S* is very faint, we performed RT-PCR to confirm the expression of *p100S*. As shown in Fig. 1D, *p100S* transcript was detected at all stages tested, supporting the conclusion that *p100S*, like *p100L*, is expressed throughout the life cycle.

To detect spatial expression of *p100* in zebrafish embryos, we performed whole-mount in situ hybridization. Because *p100S* and *p100L* share the same 5′ sequence of 1710 bp, any full-length antisense probes derived from them can produce the same in situ hybridization pattern. Therefore we tried

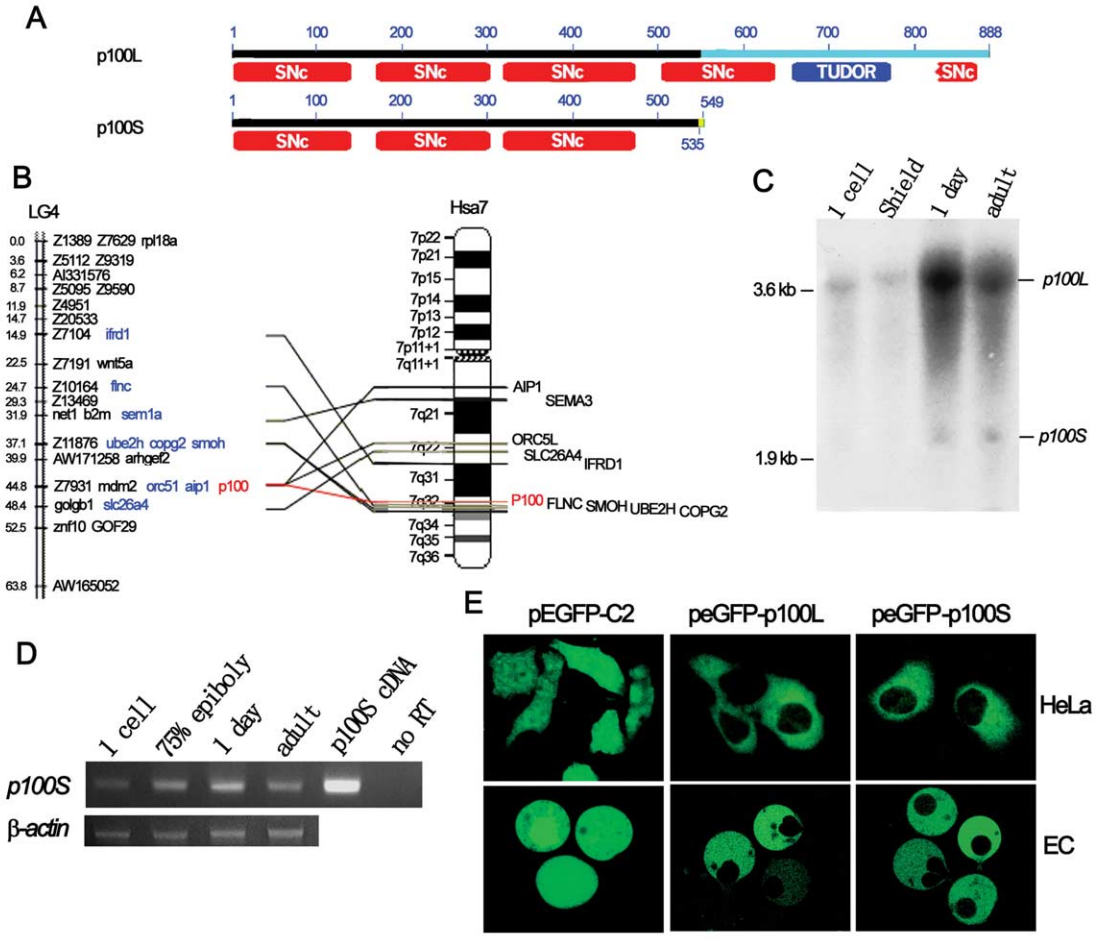

Fig. 1. Characterization of zebrafish p100. A: Sequence structures of putative p100L and p100S proteins. The first 535 residues are identical in both isoforms. Numbers above the lines indicate positions of residues. SNase repeats are shown in red boxes and the tudor domain in blue. Sequences unique to p100L and p100S are indicated by different colors (light blue or yellow). B: Chromosomal localization of zebrafish *p100* on linkage group 4 showing conserved synteny with human p100 on Hsa7. C: Northern blot showing the presence of a larger, major band (*p100L*) and a smaller, minor band (*p100S*). Molecular markers are indicated on the left. D: Amplification of a *p100S*-specific sequence by RT-PCR shows that it is present in all stages examined. The positive control used *p100S* plasmid DNA as the PCR template. The negative control (labeled as no RT) used total RNA, rather than the first-strand cDNA, of one-cell embryos as the PCR template. Lower panel shows amplification of zebrafish β-actin. E: Subcellular localization of p100L and p100S. Constructs peGFP-C2, peGFP-100L and peGFP-100S were used to transfect cultured human HeLa cells (top panel) or were injected into fertilized zebrafish eggs that were dispersed at the 80% epiboly stage (lower panel). The cells were observed for GFP by confocal microscopy. Both fusion proteins GFP-100L and GFP-100S are localized in the cytoplasm surrounding a black hole (nucleus), whereas GFP alone (peGFP-C2) is present both in the nuclei and in the cytoplasm.

to identify specific expression patterns using different probes derived from transcript-specific regions.

We first used a 1.8 kb *p100L*-specific probe to investigate the spatial and temporal expression pattern of *p100L*. Consistent with the Northern blot result, transcripts of *p100L* were first detected in the single-cell embryo, indicating their maternal origin. During the following blastula period, *p100L* transcripts were evenly distributed in all blastomeres (Fig. 2A). At the onset of gastrulation, stronger staining occurred in the embryonic shield, while in other regions staining was weaker (Fig. 2B). This difference in the distribution of *p100L* transcripts may reflect its localized zygotic expression. As embryos elongated along the anteroposterior axis by convergence, extension and involution of cells [14], the *p100L* staining domain in the shield migrated toward the animal pole and formed a longitudinal band in the dorsal midline. Accordingly, during late gastrulation, the *p100L* expression domain developed into a continuous band in the dorsal midline extending from the tailbud to the animal pole (Fig. 2C,E). This band comprises the prechordal plate anteriorly and the notochord posteriorly (Fig. 2F,G). Zebrafish *goosecoid* (*gsc*) is expressed during gastrulation in the anterior of the prechordal plate from which head mesoderm and pharyngeal endoderm derives [15]. Double staining with *p100L* and *gsc* shows that the anteriormost domain of *p100L* precedes the *gsc* expression domain (Fig. 2D). The anteriormost domain of *p100L* gives rise to the hatching gland, as also evidenced by its subsequent movement. During early segmentation, the anteriormost hatching gland domain of *p100L* grew wider, while in the forming notochord the staining intensity increased in the anterior to posterior direction (Fig. 2H–J). Thereafter, the anterior domain migrated away from the forming head and by the end of segmentation formed a ring-like band on the surface of the yolk antero-lateral to the head region (Fig. 2K,L). At this stage, the expression level was so low that it was almost undetectable in the anterior notochord but higher in the poste-

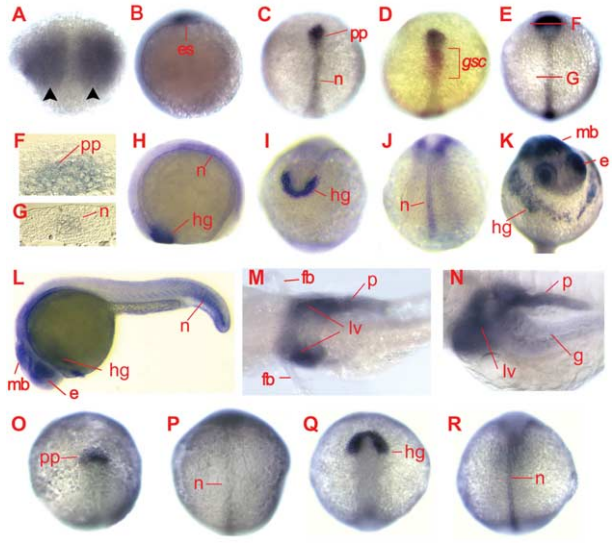

Fig. 2. Spatiotemporal expression of *p100* detected by in situ hybridization. A–N: Expression pattern of *p100L*. O–R: Expression pattern of *p100S*. A: Animal-pole view at two-cell stage showing transcript in both cells (labeled with black arrowheads) but not in the yolk cell. B: Animal-pole view at 50% epiboly showing staining in the embryonic shield. C: Dorsal view at 80% epiboly. D: Dorsal view showing that the expression domain of *p100L* (blue) extends more rostrally than that of *gsc* (red) at 80% epiboly. The gsc domain is bracketed. E: Dorsal view at the bud stage. F,G: Cross-sections of a bud-stage embryo (as in E) at the level of anterior prechordal plate (F) and the middle position (G) along the anteroposterior axis. H–J: Lateral (H), animal-pole (I) and dorsal (J) views at five-somite stage. K: Head region of a 24-h embryo. L: Lateral view of a 24-h embryo. M: Dorsal view at day 5 showing expression in internal organs. The pancreas is located on the right. N: Lateral view at a higher magnification at day 5. O,P: Animal-pole (O) and dorsal views (P) at the bud stage. Q,R: Animal-pole (Q) and dorsal views (R) at 10-somite stage. Because the *p100S*-specific probe is short, it requires a much longer staining period, which results in higher background. e, eyes; es, embryonic shield; fb, fin buds; g, gut; hg, hatching gland; lv, liver; mb, midbrain; n, notochord; p, pancreas; pp, prechordal plate.

rior notochord (Fig. 2K,L). We note that *p100L* also began to be expressed in paraxial mesoderm soon after the onset of segmentation and in many more types of tissues at later stages. For example, the transcripts were detected in the brain, eyes and somites at 24 h (Fig. 2K,L). On day 5, *p100L* was expressed in the liver, the pancreas and the intestine (Fig. 2M,N).

To look into the expression pattern of *p100S*, we used a 600 bp *p100S*-specific probe, 480 bp of which is derived from the 3′-most sequence of p100S. The short length of this probe required a much longer staining period and as a result caused higher background. Nonetheless, this probe detected *p100S* expression during gastrulation in the anterior prechordal plate and the notochord precursors (Fig. 2O–R). On day 5, very weak expression was also detected in the liver, the pancreas and the intestine (data not shown). This result suggests that the spatiotemporal expression pattern of *p100S* is identical to that of *p100L*.

3.4. *Nodal signaling is required for* p100 *expression*

Nodal proteins, members of the transforming growth factor-β superfamily, have been found to be essential for the development of mesoderm and endoderm in vertebrates [16]. Two zebrafish Nodal-related genes, i.e. *cyclops* (*cyc*) and *sqt*, have been isolated. Double mutant embryos for *cyc* and *sqt* lack a visible embryonic shield at the onset of gastrulation and later fail to form mesendodermal tissues [17]. The expression pattern of *p100* suggested the hypothesis that *p100* expression is under the control of Nodal signals. To test this idea, we first injected one- to four-cell stage wild-type embryos with different doses of *sqt* mRNA and examined *p100L* expression with the *p100L*-specific antisense probe. When the injection dose exceeded 5 pg, the expression of of *p100L* was significantly enhanced well before gastrulation (Fig. 3B). Lower doses, however, also stimulated *p100L* expression as seen at later stages. For example, 39% of embryos ($n=45$) injected at a dose of 0.5 pg had expanded expression domains of *p100L* in the prechordal plate and the notochord at the bud stage

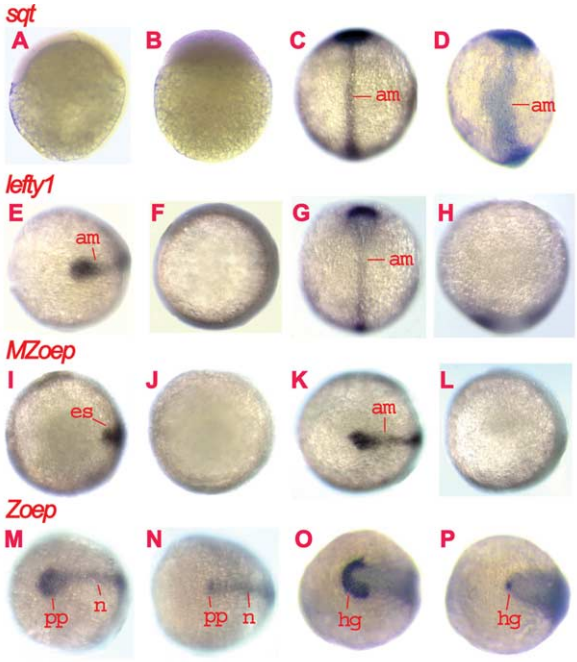

Fig. 3. *p100* expression is regulated by Nodal signals. Embryos were hybridized with antisense probe for full-length *p100S*, which detects both *p100L* and *p100S* transcripts. A,B: Lateral views of uninjected (A) and *sqt*-injected (B) embryos at about 40% epiboly. Animal pole is on the top. The staining in the injected embryo is visible while it is undetectable in the uninjected embryo. Note that the epiboly is delayed in injected embryos. C,D: Dorsal views of an uninjected (C) and *sqt*-injected embryos (D) at the bud stage. Animal pole is on the top. Note widening of the axial staining. E,F: Animal-pole views of uninjected (E) and *lefty1*-injected (F) embryos at 70% epiboly. Dorsal is to the right. G,H: Dorsal views of uninjected (G) and *lefty1*-injected (H) embryos at the bud stage. The animal pole is on the top. Note that the axial staining is missing in the injected embryos (F,H). I,J: Animal-pole views of wild-type (I) and *MZoep* mutant (J) embryos at shield stage, showing that there is no staining in the presumptive embryonic shield of the *MZoep* embryo. K,L: Animal-pole views of wild-type (K) and *MZoep* mutant (L) embryos at 80% epiboly. Dorsal is to the right. The axial staining is absent in the mutant embryo. M,N: Animal-pole views of heterozygous (M) and homozygous (N) embryos (*Zoep*) for oep^{m134} locus at 90% epiboly. Note that in the *Zoep* mutant embryo staining in the prechordal plate is reduced but the notochord staining remains unchanged. O,P: Animal-pole views of heterozygous (O) and homozygous (P) *Zoep* embryos at five-somite stage. Note that the *Zoep* mutant embryo has very weak staining in the hatching gland. am, axial mesoderm; es, embryonic shield; hg, hatching gland; n, notochord; pp, prechordal plate.

(Fig. 3D). With increasing doses of injected *sqt* mRNA, more embryos failed to undergo gastrulation and had a high level of *p100L* expression in all cells (data not shown). The proportions of embryos that failed to gastrulate were 0%, 28.8% and 55.3% at doses of 0.5 pg, 1 pg and 5 pg, respectively. We also found that injection of *sqt* mRNA similarly induced transcript that hybridized to the *p100*-specific probe (data not shown). These results suggest that Nodal signal is able to induce the expression of both *p100L* and *p100S*.

Like mouse Lefty, zebrafish Lefty1, previously called Antivin, can act as an antagonist of Nodal. Ectopic expression of *lefty1* resulted in phenotypes identical to those in *cyc*;*sqt* double mutants and such effects can be overridden by overexpression of *cyc* or *sqt* [18,19]. We speculated that overexpression of *lefty1* should inhibit zygotic expression of *p100*. Embryos injected with 100 pg of *lefty1* mRNA did not accumulate detectable amounts of p100 transcript in the embryonic shield at the onset of gastrulation in in situ hybridization experiments using the full-length *p100S* antisense probe. During gastrulation, the injected embryos did not show expression of *p100* in midline mesoderm tissues (Fig. 3E–H). This result supports the idea that zygotic expression of *p100* is under the control of the Nodal signaling pathway.

EGF-CFC proteins, acting as coreceptors, are required for Nodal signaling [20]. Loss of maternal and zygotic function of the zebrafish EGF-CFC factor Oep (in *MZoep* mutant) phenocopies the *sqt*;*cyc* double mutation [10]. In *MZoep* mutant embryos, *p100* expression was absent in the embryonic shield (Fig. 3J) and later in the midline mesoderm tissues (Fig. 3L). Maternal *p100* transcripts, however, were detected in these embryos at the one-cell stage (data not shown). These findings imply that the Nodal signal activates the zygotic transcription of the *p100* gene but is not required for its maternal expression.

To determine the role of zygotic Oep in regulating *p100* expression, we decided to examine *p100* expression in zygotic *oep* mutant (*Zoep*) embryos, which show loss or reduction of the prechordal plate and the notochord [21]. During early gastrulation, embryos produced by a pair of oep^{m134} heterozygous fish showed no obvious difference in the expression pattern of *p100* compared to wild-type embryos. By the completion of gastrulation, 23 out of 94 (24.5%) embryos, the expected proportion for homozygous mutants (25.0%), showed weaker staining in the hatching gland primordium, while the expression in their notochord was not affected (Fig. 3N). At the five-somite stage, mutant embryos had a few positive cells in the area that the hatching gland normally occupies (Fig. 3P). These results indicate that zygotically produced Oep is required for the maintenance of *p100* expression in the prechordal plate, but not in the notochord.

4. Discussion

We have identified a zebrafish ortholog of mammalian p100 protein, a coactivator of EBNA-2 [1]. Expression of the zebrafish p100 gene produces two transcript isoforms, p100L and p100S, with predominance of p100L. Like human p100, zebrafish p100L contains four and a half repeats of SNase homolog and a tudor domain. In contrast, p100S contains only the first three SNase repeats and a partial fourth repeat. Human p100 has been found to act in the nucleus as a bridge between transactivators and the basal transcription machinery [1,3–5]. We find, however, that GFP-marked chimeric proteins of both p100L and p100S are localized in the cytoplasm of cultured cells or embryonic cells. Although many studies have demonstrated that GFP-tagged proteins retain their normal biological activities (for examples see [22–24]), the faithful localization of endogenous p100L and p100S proteins requires confirmation with a p100-specific antibody and a role for these proteins in the nucleus cannot be excluded at this point. Nevertheless, cytoplasmic localization of p100 or p100-like proteins in other species has also been observed [6–8].

The zygotic expression of zebrafish p100 occurs during early gastrulation in the axial mesoderm and later on in many more tissues, suggesting a possibility that p100 proteins play roles in the formation and differentiation of these tissues. Because its zygotic expression is positively regulated by Nodal signaling, it will be interesting to investigate whether and how p100 proteins are involved in developmental processes regulated by Nodal signaling. The high reproductivity and external development of embryos have made zebrafish a vertebrate species most appropriate for large-scale chemical mutagenesis [25]. Recent advances in targeted mutagenesis in zebrafish allow rapid production of mutants deficient for specific loci [26]. Generation of mutants with loss-of-function of p100 will help to disclose functions of p100 in the development of vertebrate embryos.

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