ILF-3 is a regulator of the neural plate border marker Zic1 in chick embryos

K.J. Fishwick¹, E. Kim¹, and M. E. Bronner¹,*
¹Division of Biology, California Institute of Technology, Pasadena, California

Abstract

Background—The neural crest is a multipotent cell type unique to the vertebrate lineage and capable of differentiating into a large number of varied cell types, including ganglia of the peripheral nervous system, cartilage and glia. An early step in neural crest specification occurs at the neural plate border, a region defined by the overlap of transcription factors of the Zic, Msx and Pax families.

Results—Here we identify a novel chick gene with close homology to double stranded RNA binding protein Interleukin enhancer binding factor 3 (ILF-3) in other species. Our results show that chick Ilf-3 is required for proper expression of the transcription factor, Zic-1, at the neural plate.

Conclusion—In summary, we have identified a novel chick gene and show it has a role in the correct specification of Zic-1 at the neural plate border.

Keywords
neural crest; embryo; ILF-3; NF-90; neural plate border

1. Introduction

The induction and specification of the neural crest is a multi-step process requiring the sequential activation of batteries of transcription factors within gene regulatory networks (Sauka-Spengler and Bronner-Fraser, 2008; Betancur et al., 2010). The first step is the induction of neural crest precursors in a region of the embryo surrounding the lateral neural plate, known as the neural plate border (reviewed by (Huang and Saint-Jeannet, 2004; Barembaum and Bronner-Fraser, 2005). This population of cells is heterogeneous and multipotent such that cells residing in this domain may adopt epidermal or neural fates as well as becoming neural crest (Ezin et al., 2009), although it has been shown that cells have the capacity to form neural crest as early as HH3 (Basch et al., 2006). As neurulation proceeds, the neural plate rises up to form neural folds which eventually appose to form the neural tube. Neural crest precursors become localized at the dorsal tips of the fold/tube and express markers such as Snail-2 (Nieto et al., 1994), Sox-10 (McKeown et al., 2005) and FoxD-3 (Dottori et al., 2001).

Although many genes necessary for this process are known, others have yet to be uncovered. Using the results of a screen to identify transcripts upregulated during the process of neural crest induction (Gammill and Bronner-Fraser, 2002; Adams et al., 2008), we have identified and present here the full-length sequence for a gene similar to ILF-3 in other species but
previously unknown in chick. We further investigate the role of this gene, which we term *Ilf-3* during early chick embryonic development.

2. Results

2.1 Cloning of chick *Ilf-3*

A cDNA clone with closest homology to the product of chimpanzee *Ilf-3* gene (AY414841) was identified in a screen for genes up-regulated during neural crest development (Gammill and Bronner-Fraser, 2002; Adams et al., 2008). This produced no matches in searches of chick genome and nucleotide databases UCSC genome browser and NCBI BLAST (Altschul et al., 1997; Kent et al., 2002); beyond a 74% maximum identity (29% coverage) with Spermatid Perinuclear RNA Binding Protein (*STRBP*). (It should also be noted that, when performed now, these searches also show homology with a short/partial *Ilf-3* sequence predicted by automated computational analysis which was not present in the database when the searches were made). Analysis of the alignment between the unknown cDNA and *STRBP* shows that there are three regions of alignment and the largest two overlap with known motifs including a DSRM (dsRNA binding motif) and a DZF domain of currently unknown function (Marchler-Bauer et al.). The lack of similarity outside of these motifs indicated that this cDNA was not *STRBP*. Searches of nucleotide databases of other species showed 79% identity with 94% coverage to human ILF-3 and 77% identity with 94% coverage to mouse *Ilf-3* indicating that this gene was a previously undiscovered chick homolog of *Ilf-3* rather than *STRBP*.

The full length mRNA sequence was determined using 5′ and 3′ rapid amplification of cDNA ends (RACE) from a library of HH4-HH10 whole chick embryos. The total cDNA spans 2973 nucleotides with a predicted coding sequence of 2682 nucleotides. Figure 1a shows the full length *Ilf-3* mRNA isolated with coding sequence underlined. This produces a polypeptide of 893 amino acids. The full length chick *Ilf-3* protein alignment to mouse and human variants of ILF-3 is shown in Figure 1b.

2.2 Distribution pattern of *Ilf-3* in the early chick embryo

As a first step in uncovering the function of this novel chicken gene, we performed a detailed characterisation of the expression of *Ilf-3* mRNA throughout early embryonic development. Onset of expression was first noted at gastrulation stages, HH2-3 (Fig 2a, b) using staging based on (Hamburger and Hamilton, 1951) with very weak expression building to strong expression across the neural plate by HH4 & 5 (Fig 2c, d). As development proceeds, *Ilf-3* is broadly expressed throughout the embryo although expression is strongest within the neural fold and tube at HH6-8 (Fig 2e–g). By HH9 & 10, *Ilf-3* becomes restricted to the neural tube (‘nt’) and neural plate (‘np’) and lateral plate mesoderm (‘lpm’) (Fig 2h, i). In transverse cross-section at HH9 *Ilf-3* is observed most strongly in the ectoderm (‘ec’), dorsal neural tube (‘dNT’), endoderm (‘en’) (Fig 2hii), notochord (‘n’) (Fig 2hiii–v), lateral plate mesoderm (‘lpm’) (Fig 2hiv) and ventral segmental plate mesoderm (‘vsm’) (Fig 2hv).

2.3 ILF-3 is required during early embryonic development as a regulator of the neural plate border gene Zic-1

Neural crest development occurs over an extended time period with the progressive activation of batteries of gene expression controlling steps from initial induction and specification to final migration and differentiation into specialized cell types. Because of the strong early expression of *Ilf-3* in the neural plate, we asked whether *Ilf-3* was necessary for the induction of neural crest occurring at early stages. To address this, an antisense morpholino oligonucleotide (‘morpholino’ or ‘MO’) was designed against the translation
start site of Ilf-3. When introduced into one side of the neural plate via electroporation, Ilf-3 protein levels are reduced on the morpholino-treated side relative to the non-electroporated side (Fig 3c).

Neural crest cells arise from the neural plate border region, between the ectoderm and neural plate, which expresses a battery of transcription factors known as neural plate border specific genes. These include Zic-1, Msx-1 and Pax-7. Expression of the neural plate border/neural gene Zic-1 was reduced on the Ilf-3 MO electroporated side but not in the unelectroporated half (7/13 embryos treated with Ilf-3 MO show loss, 0/7 control MO embryos show loss (Fig 3a, b, quantified as a percentage of total numbers of embryos examined in d). Interestingly, Zic-1 reduction appeared selective to the neural plate border region (red arrowhead, Fig 3a), but unchanged in the more anterior, future placode region. Although the control morpholino electroporation did not cause a reduction of Zic-1, we sought to further verify the specificity of the Ilf-3 morpholino using a 5 base pair mismatch control morpholino. 6/6 embryos similarly showed no effect on Zic-1 levels on the electroporated side (Fig 3e).

2.4 Other neural plate border/neural/ectodermal markers are unaffected by loss of ILF-3

Neural crest gene induction is specified by a group of transcription factors including Zic-1, Pax-3 and Msx-1 (Meulemans and Bronner-Fraser, 2004). We examined a comprehensive panel of these known neural plate border specific genes (Khudyakov and Bronner-Fraser, 2009) but found that only Zic-1 was altered. Expression of Msx-1 is unaffected in 5/5 Ilf-3 MO treated embryos, and Pax-3 expression unaffected in 3/4 embryos (Fig 4a, b). In addition, neural crest specifier genes that are expressed around the time of neural plate border formation, such as AP-2 and N-Myc, show no differences at this stage (Fig 4c, d; 6/7 and 5/5 embryos respectively). We also investigated bona fide neural crest genes, activated downstream of neural plate border specifier genes. Neither FoxD-3 (5/7 embryos) nor Snail-2 (4/5 embryos) were reduced following Ilf-3 MO electroporation (Fig 4e and f respectively).

In addition to the neural crest, this region contributes to neural plate or ectodermal fates. Similarly, neural plate markers Sox-2 and Sox-3 were unchanged by the loss of Ilf-3 (Fig 4g, h; 4/4 for Sox-2 and 6/6 for Sox-3). The placodal marker Eya-2 is also unaffected by Ilf-3 MO electroporation in 4/4 Ilf-3 MO treated embryos (Fig 4i). Dlx-3, which is expressed broadly in the epiblast, neural plate and neural plate border and pre-placodal region, and Dlx-5, expressed in the pre-placodal region only, were similarly unchanged in expression (Fig 4j, k; 5/5 and 3/3 embryos respectively).

Because of the striking effect of Ilf-3 loss on Zic-1, we examined the effect of Ilf-3 loss on additional Zic family genes. Zic-2 and Zic-3 show no apparent mis-regulation as a result of Ilf-3 loss (11/11 Ilf-3 MO treated embryos show no change in Zic-2 (Fig 4l); 4/4 Ilf-3 MO treated embryos show no change in Zic-3 (Fig 4m).

3. Discussion

We have identified the chicken homolog of Ilf-3 and show that it is expressed from very early developmental stages. We demonstrate that reduction of Ilf-3 results in loss of the caudal extent of the Zinc finger gene Zic-1. Zic-1 is expressed in the early neural plate and neural plate border region as well as the preplacodal domain. Cells from this region may contribute to the neural crest, central nervous system or ectodermal placodes. Studies in frog have shown Zic-1 to be responsive to BMP inhibition but not to require Wnt signalling (Sato et al., 2005; Tropepe et al., 2006; Hong and Saint-Jeannet, 2007). In light of this, it is possible that Ilf-3 exerts its effects on Zic-1 through modulation of BMP signalling.
However, other evidence indicates that this is not the case. The expression of Msx-1, known to be responsive to BMP2/4 signalling is unchanged by Ilf-3 MO injection; and inhibition of BMP signalling in the ectoderm induces expression of Sox-2, which is similarly unchanged. Intermediate levels of BMP signalling at the neural plate border have been shown to activate genes including FoxD-3 and AP-2, which are unaffected by Ilf-3 MO (Meulemans and Bronner-Fraser, 2004). Thus, it seems likely that the effect of Ilf-3 on Zic-1 is mediated either directly or by pathways other than BMP signalling.

\(\text{Ilf-3}\) has been cloned independently by multiple groups and has accumulated a list of alternate names. It was first termed \(\text{NF90}\) for ‘nuclear-factor 90kD’ and found to bind nucleotides representing NFAT (Nuclear Factor of Activated T-cells) in the IL-2 promoter of T-cells (Corthesy and Kao, 1994; Kao et al., 1994). The first record of this gene being named \(\text{ILF-3}\) (Interleukin enhancer binding factor 3) is in 1996 and the same group later mapped human \(\text{ILF-3}\) to chromosome 19. (Marcoulatos et al., 1996; Marcoulatos et al., 1998). Similar transcripts which share homology to \(\text{NF90}\) in the N terminal region were independently identified by two groups both using DNA dependent protein kinase (PKR) as bait in a yeast 2-hybrid screen (Patel et al., 1999; Saunders et al., 2001b) and termed double-strand RNA binding protein 76 (\(\text{DRBP76}\)) and nuclear factor associated with dsRNA (\(\text{NFAR}\)) respectively. It was further noticed that the \(\text{NFAR}\) gene encoded two splice variants, a shorter 90kD transcript named \(\text{NFAR-1}\) and a longer 110kD transcript named \(\text{NFAR-2}\) (Saunders et al., 2001a). Another group identified the same transcript, which they termed human translational control protein 80 (\(\text{TCP80}\)) by screening expression libraries using beta-glucosidase as bait (Xu and Grabowski, 1999; Xu et al., 2000). This gene was also isolated in a screen for M-phase phospho-proteins and termed M-phase phospho protein 4 (\(\text{MPP4}\)) (Matsumoto-Taniura et al., 1996). It was also discovered independently in \(\text{Xenopus}\), as a double stranded RNA binding protein, 4F (Bass et al., 1994) and later identified as a member of a transcription factor complex called CBF (CCAAT box transcription factor). Hence, we have named this gene ‘\(\text{Ilf-3}\)’ in line with what appears to be the most commonly used nomenclature.

\(\text{ILF-3}\) was originally found as a regulator of Interleukin-2 (IL-2) transcription (Corthesy and Kao, 1994; Shi et al., 2007a; Shi et al., 2007b); however further study of this gene has elucidated multiple roles, including regulating translation, regulating stability of mRNA, microRNA and viral replication and to bind to both protein and 3’UTRs of transcripts. As a double stranded RNA binding protein with two dsRNA binding domains, ILF-3 has been shown to bind AU-rich elements in 3’UTRs. These include IL-2 which leads to the stabilization/reduced degradation rate of IL-2 following T-cell activation (Shim et al., 2002), and the 3’UTR of VEGF to enhance stability in hypoxia (Vumbaca et al., 2008). It was also shown to stabilize MAP Kinase Phosphatase-1 (\(\text{MKP-1}\)) following upregulation in oxidative stress (Kuwano et al., 2008) and stabilize MyoD and p21\(^{\text{WAF1/CIP1}}\) (Shi et al., 2005). Loss of NFAR was found to render fibroblasts more sensitive to viral infection and mice with mutations in the NFAR gene were born with reduced size relative to wild type littersmates and died shortly after birth from respiratory failure resulting from neuromuscular respiratory defects (Shi et al., 2005). A separate group attempting to knock out NFAR gene function using mice generated from ES cells reported that they were unable to obtain viable mutants even as heterozygotes; and chimeric embryos were only found up to E14.5 (Pfeifer et al., 2008) indicating that \(\text{ILF-3}\) is critically important for development.

In \(\text{Xenopus}\), CBTF has shown to bind the GATA-2 promoter necessary for the onset of zygotic GATA-2 transcription and directly regulates \(\text{Xgata-2}\). Similar to the chick, strong expression was noted in the ectoderm in early stage embryos. Loss or over-expression of \(\text{XIlf-3}\) leads to strongly aberrant dorsal-ventral axis patterning (Brewer et al., 1995; Orford et al., 1998; Scarlett et al., 2004; Cazanove et al., 2008), in contrast to the chick embryo.
where a reduction in Ilf-3 levels affects only a single gene. This difference may be due to the fact we are unable to target Ilf-3 for knock down in chick embryos as early as in frog, or it is possible that greatly increased levels of morpholino may be able to cause similar a similar phenotype. It is also interesting that, given the effect of Ilf-3 reduction on Zic-1, and the known role of Zic-1 in regulating downstream neural crest genes including FoxD-3 and Snail-2 in Xenopus (Sato et al., 2005), downstream genes such as Snail-2 are unaffected by loss of ILF-3. In all cases, we are only able to cause a reduction in the posterior expression of Zic-1 and this may be insufficient to cause a noticeable phenotype of loss of downstream genes, or in chick, Zic-1 may not be necessary for the correct specification of neural crest genes Snail-2 and FoxD-3.

In conclusion, with the exception of the study of expression of Ilf-3 and its role in the regulation of GATA-2 in Xenopus, very little is known about the role of ILF-3 in development. However the finding of clones of this dsRNA binding protein in a screen for neural crest specifier led us to examine firstly whether ILF-3 has been previously overlooked in the chicken and secondly if it has a role in development of the early embryo. Here we demonstrate both the existence of this gene in the chick embryo and a role for the protein in formation of the neural plate border.

4. Experimental Procedures

Cloning and primer design

Partial cDNA clone (2.59 kb) was isolated from a screen performed by (Gammill and Bronner-Fraser, 2002) and cloned into the NotI site of pCS107.

A RACE library was generated from HH4-HH10 whole chick embryos using GeneRacer kit (Invitrogen Life Technologies) following the manufacturers instructions. 5′ RACE was carried out using the GeneRacer kit with supplied 5′ primer and ILF3 gene specific reverse primer: TCAAGGCCGTCTCCGATTGGATCGA. PCR conditions used for 5′ RACE: 94°C 2mins; 94°C 30sec, 72°C 4 mins repeat (5 cycles); 94°C 30 sec 70°C 4 mins (5 cycles); 94°C 30sec, 65°C 30sec, 72°C 4mins (35 cycles); 72°C 10 mins. Buffer used was Expand Long Template Buffer 1 (Roche) and Polymerase used was standard Taq (Roche). RACE was carried out using the GeneRacer kit with supplied 3′ primer and ILF3 gene specific forward primer: GCACGGCGGTAAGAAGCAGCAGCAC. PCR conditions used for 3′ RACE: 94°C 2mins; 94°C 30sec, 68°C 6 mins repeat (5 cycles); 94°C 30 sec 66°C 6 mins (5 cycles); 94°C 30sec, 65°C 30sec, 68°C 6mins (35 cycles); 68°C 10 mins. Buffer used was Expand Long Template Buffer 1 (Roche) and Polymerase used was standard Taq (Roche); 5% DMSO was added to the reaction mixture.

The assembled sequence for full length chick ILF-3 has been submitted to GenBank with the Accession number JQ845950.

Alignment

ClustalW (Goujon et al.; Larkin et al., 2007) was used to align chick ILF-3 with human ILF3 (NP_036350.2) and mouse ILF-3 (NP_001036172.1).

Morpholino design

A FITC-conjugated anti-sense morpholino oligonucleotide (‘morpholino’ or ‘MO’) (Gene Tools LLC) was designed against the translation start site. To inject, 1 or 1.5mM concentration of morpholino was prepared with 2μg/μl pCIG carrier DNA in EB buffer (Qiagen). ILF-3 MO sequence: TCACGAAGATCCGCATCAGGCGCAT; Control MO
sequence:CCTCTTACCTCAGTTACAATTTATA; 5bp mismatch control MO sequence: TCACcAacATCgGCATgAcGCgCAT.

Chick culture & electroporation
Fertilized Rhode Island Red chicken eggs (McIntyre Poultry, CA) were incubated to HH3+/HH4 according to the classical staging method (Hamburger and Hamilton, 1951). Embryos were placed in EC culture (Chapman et al., 2001) for electroporation or collection for in-situ hybridization. For electroporation 5 pulses of 6V (50ms ON 100ms OFF) were passed across the embryo. The embryos were then cultured on a thin layer of albumin until they reached the required stage.

In-situ hybridization
For in-situ hybridization, embryos were fixed overnight at 4°C in 4% paraformaldehyde. Embryos were subsequently washed in phosphate-buffered saline and taken through sequential dehydration into 100% Methanol and stored overnight. In-situ hybridization was conducted according to a standard laboratory protocol using digoxygenin labelled antisense RNA probes. Antisense Ilf-3 probe was from synthesised from the partial cDNA clone ‘msa333’ (Gammill and Bronner-Fraser, 2002; Adams et al., 2008) in pCS107 by S. McKeown. Plasmid probes used AMyc (L.Keruso), Sox-10, Snail-2, Dlx-3, FoxD-3, Sox-2, Dlx-5, Pax-3, Sox-3, Zic-2 and Zic-3. EST-based probes were Zic-1 (ChEST 459n6), AP-2 (ChEST765g1), Msv-J (ChEST900p21) and Eya-2 (ChEST576g13).

Western blotting
Embryos were collected on ice and stored in lysis buffer. Western blotting and transfer were carried out using standard protocols. Primary antibodies used were Ilf-3 (2ug.ul, AbCam) and Tubulin (1/100, Sigma) and secondary antibody used was anti-Mouse conjugated-HRP (Promega). Image shown in representative of 2 repeated experiments.

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Original cDNA from library was cloned into pMES expression vector and the probe synthesized by S. McKeown. This work was funded by NIH grant HG004071 to Marianne E. Bronner.

References


Key findings

- Ilf-3, a novel gene in the chick, is expressed at early developmental stages.
- Downregulation of Ilf-3 causes a reduction in Zic-1 expression at the neural plate border.
- No other neural plate border gene examined is affected by loss of Ilf-3.
Figure 1.
(a) Chick ILF-3 mRNA with coding region underlined (b) protein sequence with aligned with ILF-3 in human and mouse.
Figure 2.
Expression of ILF-3 during early embryonic development (a) HH2; (b) HH3+; (c) HH4; (d) HH5; (e) HH6; (f) HH7; (g) HH8; (h) HH9; (g) HH10; transverse cross-section of (h) in (hi-vi). Figure labels: ‘nt’ = neural tube, ‘lpm’ = lateral plate mesoderm, ‘np’ = neural plate, ‘ec’ = ectoderm, ‘en’ = endoderm, ‘dNT’ = dorsal neural tube, ‘n’ = notochord, ‘vsm’ = ventral segmental plate mesoderm’.

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Figure 3.
(a) Western blot Ilf-3 levels in embryos electroporated with Ilf-3 MO on one side of the neural plate (left) (‘+ ILF-3 MO’) compared to non-electroporated side (right) (‘-ILF-3 MO’); (b) Zic-1 expression in embryo electroporated with Ilf-3 MO; (b) Zic-1 expression in embryo electroporated with control MO; (d) quantification of severity of phenotype in morpholino treated embryos expressed as a percentage of total numbers of embryos examined; (e) Zic-1 expression in embryo electroporated with mismatch control MO.
Figure 4.
Effect of Ilf-3 MO treatment on early neural plate, neural plate border and ectodermal markers. Embryos electroporated with Ilf-3 MO showing expression of (a) Msx-1; (b) Pax-3; (c) AP-2; (d) NMYC; (e) FoxD-3; (f) Snail-2; (g) Sox-2; (h) Sox-3; (i) Eya-2; (j) Dlx-3; (k) Dlx-5; (l) Zic-2; (m) Zic-3.