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A functional interaction between Irx and Meis patterns the anterior hindbrain and activates *krox20* expression in rhombomere 3

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ABSTRACT

Patterning of the vertebrate hindbrain involves a segmentation process leading to the formation of seven rhombomeres along the antero-posterior axis. While recent studies have shed light on the mechanisms underlying progressive subdivision of the posterior hindbrain into individual rhombomeres, the early events involved in anterior hindbrain patterning are still largely unknown. In this paper we demonstrate that two zebrafish Iroquois transcription factors, Irx7 and Irx1b, are required for the proper formation and specification of rhombomeres 1 to 4 and, in particular, for *krox20* activation in r3. We also show that Irx7 functionally interacts with Meis factors to activate the expression of anterior hindbrain markers, such as *hoxb1a*, *hoxa2* and *krox20*, ectopically in the anterior neural plate. Then, focusing on *krox20* expression, we show that the effect of Irx7 and Meis1.1 is mediated by element C, a conserved *cis*-regulatory element involved in *krox20* activation in the hindbrain. Together, our data point to an essential function of Iroquois transcription factors in *krox20* activation and, more generally, in anterior hindbrain specification.

Introduction

Antero-posterior (AP) patterning of the vertebrate brain is initiated during gastrulation in the newly formed neural plate. In the hindbrain, AP patterning involves a segmentation process that leads to the formation of seven bulges called rhombomeres (r). The rhombomeres are cellular compartments and constitute segmental units for neuronal differentiation and gene expression (Lumsden and Krumlauf, 1996; Moens and Prince, 2002; Schneider-Maunoury et al., 1998). Among the numerous genes involved in the formation and specification of rhombomeres, the zinc finger gene *Krox20/Egr2* (*krox20/egr2b* in zebrafish), expressed in r3 and r5, plays an essential role in the expansion and specification of these two rhombomeres (Giudicelli et al., 2001; Voiculescu et al., 2001), thereby subdividing the hindbrain into odd-numbered and even-numbered rhombomeres. Thus, discovering how *krox20* expression is regulated is essential to fully understand the process of hindbrain segmentation.

In zebrafish, the sequence of events leading to caudal hindbrain segmentation and *krox20* activation in r5 has been partially uncovered. During gastrulation, retinoic acid (RA) produced in the paraxial mesoderm activates *hoxb1b* expression up to the future r3/r4 boundary (Maves and Kimmel, 2005). *hoxb1b* initiates a genetic cascade leading to the subsequent subdivision of this r4–r7 domain (Choe and Sagerstrom, 2004) and, together with *hoxb1a*, is an essential determinant of r4 identity (McClintock et al., 2002). RA also activates the expression of the homeobox gene *vhnf1* posterior to the r4/r5 boundary (Hernandez et al., 2004; Maves and Kimmel, 2005; Wiellette and Sive, 2003). Vhnf1 synergises with Fgfs secreted by r4 to activate *valentino* (*val*, the zebrafish orthologue of *MafB*), a gene coding for a bZIP transcription factor, in r5–r6 and *krox20* in r5 (Sun and Hopkins, 2001; Wiellette and Sive, 2003). Thus, the caudal (r4–r7) hindbrain is subdivided into individual rhombomeres at the beginning of somitogenesis.

In contrast, the mechanisms involved in the specification of the anterior hindbrain and in its subdivision into individual rhombomeres, particularly in the activation of *krox20* expression in r3, have been poorly characterised. Recently, several studies have demonstrated a requirement for the families of Hox cofactors Pbx and Meis/Prep in this process (Choe et al., 2002; Deflorian et al., 2004; Waskiewicz et al., 2001, 2002). In zebrafish, at least five *meis/prep* genes (*meis1.1*, *meis2.1*, *meis2.2*, *meis 3.1* and *prep1.1*) and two *pbx* genes (*pbx2* and *pbx4*) are expressed in the hindbrain at the onset of segmentation (Waskiewicz et al., 2001; Zerucha and Prince, 2001). Genetic analysis has identified

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lazarus/*pbx4* (*lzr*) as globally required for *Hox* genes function along the AP axis (Popperl et al., 2000). Interestingly, embryos lacking all *Pbx* function in the hindbrain, obtained by injection of *pbx2* Mo into a *lzr* null mutant background, present a transformation of all rhombomeres into r1 identity; in particular, *krox20* expression in r3 and r5 is lost (Waskiewicz et al., 2002). Inhibition of Meis function by dominant-negative forms also results in the disruption of hindbrain segmentation, supporting the idea that *Pbx* and *Meis* genes function in a common pathway (Aamar and Frank, 2004; Choe and Sagerstrom, 2004; Choe et al., 2002; Deflorian et al., 2004; Dibner et al., 2001; Dibner et al., 2004; Salzberg et al., 1999; Vlachakis et al., 2001; Waskiewicz et al., 2001). Since Meis and Pbx act as Hox cofactors, it has been proposed that Hox genes could have a role in the specification of the whole hindbrain. This hypothesis is supported by experiments in *Xenopus* embryos, which show that the loss of function of all paralogous group 1 (PG1) *Hox* genes leads to a transformation of all rhombomeres into r1 identity, a phenotype similar to the Pbx-less embryos (McNulty et al., 2005).

Two zebrafish *Iroquois* (*Irx*) genes, *irx1b* and *irx7*, are expressed during gastrulation in the prospective midbrain and anterior hindbrain (Itoh et al., 2002; Lecaudey et al., 2001, 2004). Irx proteins belong to the same TALE superfamily of transcription factors as Pbx and Meis (Burglin, 1997); they are involved in various embryonic patterning processes (Cavodeassi et al., 2001; Gomez-Skarmeta and Modolell, 2002) and in the activation of proneural gene expression (Gomez-Skarmeta et al., 1996, 1998; Itoh et al., 2002). We have shown previously that *irx7* was involved in positioning the r4/r5 boundary (Lecaudey et al., 2004). More anteriorly, *irx7* and *irx1b* are implicated in the formation of the midbrain–hindbrain boundary (mhb) (Itoh et al., 2002). In the present report, we uncover a novel function of *irx7* and *irx1b* in the anterior hindbrain.

Experimental procedures

Zebrafish lines and maintenance

Zebrafish (*Danio rerio*) were raised and staged as previously described (Kimmel et al., 1995; Westerfield, 1995). The *vhnf1*^{hi2169} mutant (Sun and Hopkins, 2001), the *val*^{b337} mutant (Moens et al., 1996) and the Isl1-GFP transgenic (Higashijima et al., 2000) lines have been described previously.

Constructs

Constructs for the *in vitro* synthesis of mRNAs were generated by cloning cDNAs into the pCS2^{+} or pCS2^{+}MT vectors. The *meis1.1myc* and *irx7* expression constructs have been described previously (Lecaudey et al., 2004; Waskiewicz et al., 2001). To make the *eltC:gfp* transgenesis construct, the 1263 bp zebrafish element C was PCR amplified using primers 5'-gcgatgcatcattgataaatggtttctaatgattgg-3' and 5'-gcggtcgaccgctgatgagagcaaacg-3' and cloned in front of the GFP coding sequence into the Tol2 transgenesis vector (Kawakami et al., 2004) modified by addition of a *Gata2* promoter (T. Becker, personal communication).

DNA, RNA and morpholino injections, transgenesis and cell transplantations

Capped RNAs were transcribed with SP6 RNA polymerase and injected in one cell at the four cell stage at the following concentrations: *meis2.1*, *meis3.1*, *prep1.1*, *meis1.1myc* at 80 ng/μL, *irx7* at 25 ng/μL, *pbx4* and *gfp* at 60 ng/μL, *irx1b* at 30 ng/μL. *eltC:gfp* DNA (25 ng/μL) was coinjected with Tol2 transposase RNA (25 ng/μL) (Kawakami et al., 2004) at the one cell stage. To obtain Tg(eltC:gfp) transgenic lines, embryos injected with *eltC:gfp* DNA and Tol2 transposase were selected for fluorescence in the r3–r5 region at 24 hpf, then raised to adulthood and outcrossed. Morpholinos for *irx7* (Mo7), *hoxb1a* (Mohoxb1a), *hoxb1b* (Mohoxb1b) and *p53* (Mop53) were already described (Lecaudey et al., 2004; McClintock et al., 2002; Robu et al., 2007). We designed an *irx1b* morpholino (Mo1b): acatgtccaactcccgaggaactct and an *irx7* splice morpholino (Mosp7): gtcaaaatactacttacaatgtgtg. Morpholinos (Gene-Tools) were resuspended in water at 4 mM and diluted to working concentration in Danieau buffer. Mo7, Mosp7 and Mo1b: 1 mM; Mohoxb1a: 0.25 mM; Mohoxb1b: 0.5 mM. For Mop53, the concentration used equalled the total concentration of the coinjected morpholinos. For mosaic rescue experiments, Mo1b and Mosp7 were injected at the one cell stage, and *irx7* and *gfp* RNAs were injected at the 8–32 cell stage. For cell transplantations, donor embryos were injected at the one cell stage with tetramethylrhodamine dextran (M_r 70×10^3, Molecular Probes) and *irx7*+*meis1.1* RNA. Transplantations of donor cells into host embryos were carried out at sphere stage.

Whole mount in situ hybridisation and immunohistochemistry

In situ hybridisation (ISH) and immunohistochemistry (IHC) were performed as previously described (Lecaudey et al., 2004). For immunohistochemistry, the following antibodies were used: rabbit anti-phospho-Histone H3 antibody (Upstate 06-570, 1:200) and rabbit anti-Caspase 3 antibody (R&D systems AF835, 1:200). For double fluorescence ISH, *krx20* and *hoxb1a* were detected using the tyramide-FITC signal amplification method (Perkin Elmer) and Fast Red (Roche), respectively. Confocal optical sections of flat-mounted embryos were obtained with an inverted Leica DMIRBE microscope.

Anti-Irx7 antibody generation and Western blot

A rabbit polyclonal antibody was raised against two Irx7 peptides: RGGPYYTPYRPIPAD (amino acids 88–102) and SPVNLSTHDLLKQSQ (amino acids 300–314). Peptide synthesis and antibody preparation were performed by Eurogentec. The antibody was characterised by Western blot on nuclear extracts from 100% epiboly stage embryos (Fig. S4 in supplementary material), using the preimmune serum as control.

Co-immunoprecipitation assays

Proteins were produced either *in vivo* in COS7 cells or *in vitro* using an SP6 Coupled Reticulocyte Lysate System (Promega, Madison, WI). For *in vivo* production, the expression plasmids were transfected into COS-7 cells using the Lipofectamine Transfection Reagent (Invitrogen). Cell lysates were prepared as described (Dignam et al., 1983). Nuclear membranes were disrupted by the addition of 0.5% Nonidet P-40, the suspension was brought to 0.4 M NaCl and 0.2 mM EDTA. The Irx7HA-tagged protein was purified using the anti-HA Affinity Matrix (Roche). For co-immunoprecipitation (coIP) of *in vitro* translated ^{35}S-labelled proteins, 40 μL (for 2 proteins) or 60 μL (for 3 proteins) were added to 300 μL of 1X binding buffer (10 mM Tris–HCl at pH 7.5, 75 mM NaCl, 1 mM ethylenediamine-tetraacetic acid [EDTA], 1 mM dithiothreitol [DTT]), along with 1% bovine serum albumin [BSA], 1X Pefabloc and 2 μg of poly(I-C), and incubated overnight at 4 °C in the presence of 3 μL of anti-Myc antibody (Upstate Biotechnology 06-549) and 30 μL of protein A-Sepharose beads. Beads were washed 5 times with 1 mL of 1X binding buffer, and precipitated proteins were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography. For coIP of *in vivo* produced proteins, 30 μL of each protein produced in COS7 cells were added to 300 μL of Binding Buffer (10 mM Tris HCl, pH7.5; 75 mM NaCl; 1 mM EDTA; 1 mM DTT; 0.1% NP40; 0.1% BSA; 1 mM phenylmethylsulfonyl fluoride; 1 μg/mL leupeptin; 1 μg/mL pepstatin; 1 μg/mL aprotinin), 8 μg poly dIdC and incubated at 4 °C in the presence of 4 μL anti-Irx7 (for Irx7 HA), or anti-Myc (9B11, Ozyme) (for Meis1.1Myc) antibodies, and 40 μL protein A-sepharose beads. The immune complexes were collected by a brief