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Nlcam modulates midline convergence during anterior neural plate morphogenesis

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ABSTRACT

During development, different cell types must undergo distinct morphogenetic programs so that tissues develop the right dimensions in the appropriate place. In early eye morphogenesis, retinal progenitor cells (RPCs) move first towards the midline, before turning around to migrate out into the evaginating optic vesicles. Neighbouring forebrain cells, however, converge rapidly and then remain at the midline. These differential behaviours are regulated by the transcription factor Rx3. Here, we identify a downstream target of Rx3, the Ig-domain protein Nlcam, and characterise its role in regulating cell migration during the initial phase of optic vesicle morphogenesis. Through sophisticated live imaging and comprehensive cell tracking experiments in zebrafish, we show that ectopic expression of Nlcam in RPCs, as is observed in Rx3 mutants, causes enhanced convergence of these cells. Expression levels of Nlcam therefore regulate the migratory properties of RPCs. Our results provide evidence that the two phases of optic vesicle morphogenesis: slowed convergence and outward-directed migration, are under different genetic control. We propose that Nlcam forms part of the guidance machinery directing rapid midline migration of forebrain precursors, where it is normally expressed, and that its ectopic expression upon loss of Rx3 imparts these migratory characteristics upon RPCs.

Introduction

Shaping the developing embryo requires the orchestration of complex morphogenetic events, ranging from cell shape changes and folding of epithelia, to cell rearrangements and migration. Together, these determine the shape, size and position of body parts and organs. These cell movements must be very tightly coordinated in space and time, and this involves the dynamic transcriptional regulation of effector genes that control cell shape, polarity, adhesion and migration.

Cellular adhesion is central to many aspects of morphogenesis. Cell shape change, rearrangement and migration all depend critically on linking the cytoskeleton of a cell to its external substrate–either another cell or the extracellular matrix (ECM)–and this is achieved primarily by adhesion molecules. There are several major families of cell adhesion molecules (CAMs), the most prevalent being cadherins (Halbleib and Nelson, 2006; Tepass et al., 2000), integrins (Bokel and Brown, 2002; Geiger et al., 2001) and members of the immunoglobulin (Ig)-domain superfamily (Rougon and Hobert, 2003). Dynamic modulation of the expression levels and subcellular localisation of these various factors is crucial to allow morphogenetic processes to occur.

The vertebrate eye provides an attractive model for the study of morphogenesis. After specification of the eye field within the neural plate, the tissue undergoes a series of complex morphogenetic events, forming the bilateral optic cups (reviewed in (Adler and Canto-Soler, 2007; Chow and Lang, 2001). The transparent teleost embryos provide a particularly amenable paradigm for analysis of these processes, and detailed descriptions of retinal morphogenesis are available (Li et al., 2000; Schmitt and Dowling, 1994). However, the underlying cellular mechanisms have only recently begun to be investigated (Cavodeassi et al., 2005; England et al., 2006; Rembold et al., 2006b). In teleosts, where the morphogenesis has been most extensively studied, the eye field originates as a contiguous group of cells, flanked by telencephalic cells anteriorly and laterally, and diencephalic precursors to the posterior (Woo and Fraser, 1995). The initial specification of the eye field, regulated by transcription factors such as Six3 and Pax6, requires the sorting out of retinal vs. non-retinal cells—a process directed by Eph-Ephrin mediated cell–cell adhesion (Cavodeassi et al., 2005; Moore et al., 2004). Subsequent formation of the optic vesicles is a two-step process,

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beginning while cells of the neural plate are still converging to the midline. Firstly, RPCs converge more slowly than the surrounding forebrain cells, creating a wider domain from which the optic vesicles will emerge. Subsequently, individual outward-directed migration of RPCs drives the splitting of the eye field and vesicle evagination. Concomitant to these RPC movements, the forebrain takes shape: lateral telencephalic cells converge rapidly towards the midline and diencephalic cells move anteriorly, filling the gap created by the splitting retinal field (England et al., 2006; Rembold et al., 2006b).

The Rx transcription factors play central roles in eye development. In *Xenopus*, fish and mouse, disruption of Rx gene function leads to small or absent eyes (Andreazzoli et al., 1999; Kennedy et al., 2004; Loosli et al., 2003, 2001; Mathers et al., 1997). Teleosts have three Rx genes, of which Rx3 is expressed earliest in the presumptive eye field. In both zebrafish and medaka, null mutants for *rx3* have no eyes. RPCs are specified, but fail to undergo morphogenesis and remain trapped within the forebrain (Loosli et al., 2003; Stigloher et al., 2006; Winkler et al., 2000). 4D imaging analysis has revealed the cellular basis of these morphogenetic defects in the medaka *eyeless* mutant (Rembold et al., 2006b): the mutant retinal cells converge fully towards the midline, and fail to migrate outwards, instead forming an epithelialised neural keel-like structure, similar to the surrounding forebrain cells. Rx3 thus influences both steps of optic vesicle morphogenesis, convergence and evagination.

The functions of Rx1 and Rx2 are less clear; in zebrafish, their expression is dependent upon Rx3 (Loosli et al., 2003), and overexpression of either can induce retinal cell fate (Chuang and Raymond, 2001). However, morpholinos directed against either paralog (or both in combination) does not give an early morphogenetic phenotype (Rojas-Munoz et al., 2005). The degree of redundancy between these three paralogous genes, however, remains to be fully determined. In *Xenopus*, where there are two Rx paralogs, Rx1 has been shown to be important for RPC proliferation, and also directs their migration (Kenyon et al., 2001; Zaghloul and Moody, 2007). In mammals, despite the differences in how morphogenesis occurs (via an outpocketing of the neural epithelium, rather than by individual cell migration), the function of Rx in directing retinal fate and morphogenesis is conserved (Mathers et al., 1997; Medina-Martinez et al., 2009). This conservation is underscored by the fact that mutations in human Rx have been associated with anopthalmia (Voronina et al., 2004).

To date, few targets of Rx genes have been postulated. Manipulation of Rx levels affects the expression patterns of various transcription factors, neurogenic genes and cell cycle regulators (Andreazzoli et al., 1999, 2003; Kennedy et al., 2004; Loosli et al., 2003, 2001; Winkler et al., 2000; Zhang et al., 2000). However, it remains unclear whether any of these factors are direct transcriptional targets of Rx. Moreover, since none of these factors are morphogenetic effectors, they cannot account for the morphological defects of Rx mutants.

In the case of Rx3, the downstream targets, either direct or indirect, should include factors that influence cell migration. Among these are likely to be adhesion molecules, whose differential expression in wild-type (wt) and *rx3*$^{-}$ embryos may help to explain the altered behaviour of mutant cells. Here, we identify one such factor: the Ig-domain CAM Nlcam (Mann et al., 2006), which shows elevated expression in the eye field of zebrafish *rx3*/*chk* mutants. Ectopic over-expression of Nlcam causes a small-eye phenotype, mimicking the *chk* mutant. Through live imaging and cell tracking experiments, we demonstrate that Nlcam modulates the migration of RPCs during the initial phase of midline convergence. Consistent with this, loss of *nlcam* function leads to delayed convergence of lateral forebrain cells. Our results provide the first link between Rx3 and the downstream cellular machinery responsible for controlling the differential migratory behaviour of forebrain and eye cells.

Materials and methods

Fish husbandry

Zebrafish stocks were maintained at 26 °C, and embryos raised at 28 °C. WIK/AB was used as a wt line. Rx3::GFP transgenic fish were obtained by injecting pBS-ISceI OlRx3::GFP (Rembold et al., 2006b) into wt embryos. Progeny with strong, eye-specific GFP expression were selected and the line maintained. The *chk*^{s399} line (Loosli et al., 2003) was used.

Cloning

The *nlcam* cDNA (coding region plus partial 5′ and 3′ UTRs) was amplified by RT-PCR from wt cDNA, using the following primers:

Nlcam-F: AATTTACTGACGTACGCAAC
Nlcam-R: ACAGCTTCTGACTCCATTTT

The resulting 1.9 kilobase pairs (kb) product was cloned into pCS2 for mRNA synthesis. The nlcam-GFP fusion was generated by PCR-cloning the *nlcam* coding region (minus the stop codon) into pEGFP-N1 (Clontech). The fusion was subcloned into pCS2 for mRNA synthesis. Rx3::nlcam was generated by replacing GFP in pBS-ISceI Rx3::GFP by the 1.9 -kb nlcam cDNA. Partial clones of *ncam2*, *ncam3* and *mcam* for *in situ* hybridization probes were amplified from wt cDNA using the following primers:

Ncam2-F: TTCGGCAGGGTGAGGTGGCTGAAGTGGTCT
Ncam2-R: ATTGATTGGCGTGTTCTTGCTTGTATTCTC
Ncam3-F: CGCCATTATCGTGTGTGATGTCATAAGCTC
Ncam3-R: TGATTAGCTGTGGCCTCTTCTTCAGTCCTC
Mcam-F: TGGCGAGAGGGATTTAATAAGCGATGA
Mcam-R: CCTTTGACATTGTTTGAAAGAAAATGCGAG

The products were Topo-cloned into pCRII-TOPO (Invitrogen).

In situ hybridisation

In addition to the amplified cDNAs, the following clones were used to synthesise probes: *alcam* (BC050482); *e-cadherin* (AI629129); *bcam* (CN831659); *integrinβ1b* (BQ075715); *integrinβ4* (BI840449); *integrinβ5* (BC124678); *emx3* (CO959873)—all obtained from ImaGenes (www.imagenes-bio.de). The *n-cadherin* cDNA was given by JD Jontes (Jontes et al., 2004), and subcloned into pBS SK+. The *ncam* cDNA was a gift from D. Grunwald.

Whole mount *in situ* hybridisations were carried out on the progeny of *chk*/+ intercrosses, using Digoxigenin-labelled probes, as described previously (Loosli et al., 1998). Embryos were sectioned by embedding in gelatin/albumen, and 25-μm vibrotome sections were cut.

Rx3 binding site predictions

The mouse RAX consensus binding site previously identified (Berger et al., 2008) was retrieved from the UniPROBE database (Newburger and Bulyk, 2008). Zebrafish *nlcam* and corresponding orthologous genes were retrieved from the EnsEMBL v50 database (Flicek et al., 2008): ENSDARG00000058538 (zebrafish), ENSTRUG00000006355 (fugu), ENSGACG00000010444 (stickleback) and ENSORLG00000005387 (medaka). Occurrences of the RAX position weight matrix were screened in the 20 -kb upstream and intronic sequences of *nlcam* and its orthologues using the POSSUM software (threshold 7) [http://zlab.bu.edu/~mfrith/possum/]. The upstream and intronic RAX binding sites were predicted at the following respective positions from the gene transcription start