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Nemo regulates cell dynamics and represses the expression of *miple*, a midkine/pleiotrophin cytokine, during ommatidial rotation

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ABSTRACT

Ommatidial rotation is one of the most important events for correct patterning of the *Drosophila* eye. Although several signaling pathways are involved in this process, few genes have been shown to specifically affect it. One of them is *nemo* (*nmo*), which encodes a MAP-like protein kinase that regulates the rate of rotation throughout the entire process, and serves as a link between core planar cell polarity (PCP) factors and the E-cadherin–β-catenin complex. To determine more precisely the role of *nmo* in ommatidial rotation, live-imaging analyses in *nmo* mutant and wild-type early pupal eye discs were performed. We demonstrate that ommatidial rotation is not a continuous process, and that rotating and non-rotating interommatidial cells are very dynamic. Our in vivo analyses also show that *nmo* regulates the speed of rotation and is required in cone cells for correct ommatidial rotation, and that these cells as well as interommatidial cells are less dynamic in *nmo* mutants. Furthermore, microarray analyses of *nmo* and wild-type larval eye discs led us to identify new genes and signaling pathways related to *nmo* function during this process. One of them, *miple*, encodes the *Drosophila* ortholog of the midkine/pleiotrophin secreted cytokines that are involved in cell migration processes. *miple* is highly up-regulated in *nmo* mutant discs. Indeed, phenotypic analyses reveal that *miple* overexpression leads to ommatidial rotation defects. Genetic interaction assays suggest that *miple* is signaling through Ptp99A, the *Drosophila* ortholog of the vertebrate midkine/pleiotrophin PTPζ receptor. Accordingly, we propose that one of the roles of Nmo during ommatial rotation is to repress *miple* expression, which may in turn affect the dynamics in E-cadherin–β-catenin complexes.

Introduction

The *Drosophila* adult eye is composed of around 800 units, or ommatidia, which are precisely oriented in mirror symmetric fashion relative to a dorsal–ventral midline, the equator. This pattern is generated during larval development in the eye imaginal disc, when ommatidial preclusters rotate 90° towards the equator adopting opposite chiral forms depending upon whether they lie dorsally or ventrally (Jenny, 2010). These patterning events closely follow a moving front of differentiation, the morphogenetic furrow (MF), which moves from posterior to anterior across the eye imaginal disc (Tomlinson and Ready, 1987). The Frizzled planar cell polarity (Fz-PCP) pathway controls the proper differentiation of R3 and R4 photoreceptors and, subsequently, the direction of ommatidial rotation (Seifert and Mlodzik, 2007). The direction of rotation depends on correct R3/R4 cell fate specification since misrotation is a common phenotype observed in loss- and gain-of-function mutants of PCP genes (Mlodzik, 1999). During this process ommatidial precursors rotate as a group, but independent of their undifferentiated, stationary neighbors, the interommatidial cells (IOCs) (Fiehler and Wolff, 2007). The exact cellular mechanisms that drive this behavior have not yet been established. In parallel to Fz-PCP signaling, which may regulate ommatidial rotation through effects on cytoskeletal elements via the Rho-Kinase Drok (Winter et al., 2001), this process is also regulated by the Epidermal growth factor receptor (Egfr) pathway (Brown and Freeman, 2003; Gaengel and Mlodzik, 2003; Strutt and Strutt, 2003). Egfr pathway members signal through both the Mitogen activated protein kinase (MAPK)/Pointed (Pnt) transcriptional cascade and Canoe (Cno) (Brown and Freeman 2003, Gaengel and Mlodzik, 2003), and also interact genetically with *E*-cadherin (*E*-cad) and *N*-cadherin (*N*-cad) during this process (Brown and Freeman, 2003; Gaengel and Mlodzik, 2003; Mirkovic and Mlodzik, 2006). Moreover, genes functionally related with cytoskeleton reorganization and cell adhesion act as downstream effectors of Egfr

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signaling, thus linking ommatidial rotation with cell adhesion and cytoskeleton rearrangements (Gaengel and Mlodzik, 2003; Mirkovic and Mlodzik, 2006). In addition, the cell adhesion molecules Echinoid (Ed) and Friend of Echinoid (Fred) are required at multiple steps during the ommatidial rotation process (Fetting et al., 2009), and Ed seems to be required to decrease Flamingo (one of the PCP core proteins) levels on non-rotating IOCs to permit correct rotation of ommatidial clusters (Ho et al., 2010). Other genes that have been shown to be required during ommatidial rotation are *nemo* (*nmo*), *scabrous* (*sca*) and *zipper* (*zip*) (Choi and Benzer, 1994; Chou and Chien, 2002; Escudero et al., 2007; Fiehler and Wolff, 2007, 2008; Mirkovic et al., 2011).

The *Drosophila nmo* gene encodes the founding member of the Nemo-like kinase (Nlk) subfamily of MAPKs (Brott et al., 1998). Nlk family members have regulatory roles in multiple developmental processes in vertebrates and invertebrates. Indeed, vertebrate NLK has been shown to participate in several signaling pathways, being activated by Transforming Growth Factor-β (TGF-β), Wnt, and IL-6 signaling (Brott et al., 1998; Ishitani et al., 1999; Kanei-Ishii et al., 2004; Kojima et al., 2005; Meneghini et al., 1999; Ohkawara et al., 2004), and to function downstream of nerve growth factor (NGF) (Ishitani et al., 2009). In addition, NLK phosphorylates and regulates the activity of several transcription factors in the nucleus such as T-cell factor (TCF)/Lymphoid enhancer factor (LEF), Signal transducer and activator of transcription 3 (STAT3), c-Myb, Smad4, the intracellular domain of Notch1 (Notch1-ICD) or Nuclear Factor-κβ (NF-κβ) through phosphorylation of its co-factor CREB binding protein (CBP) (Ishitani et al., 2010; Ishitani et al., 2003; Ishitani et al., 1999; Kanei-Ishii et al., 2004; Kojima et al., 2005; Meneghini et al., 1999; Ohkawara et al., 2004; Shi et al., 2010; Yasuda et al., 2004). In *Drosophila*, *nmo* is involved in diverse processes such as eye specification, synaptic growth, apoptosis, wing development, pair-rule patterning and circadian rhythms (Braid et al., 2010; Braid and Verheyen, 2008; Chiu et al., 2011; Merino et al., 2009; Mirkovic et al., 2002; Morillo et al., 2012; Verheyen et al., 2001; Yu et al., 2011). Moreover, it seems that *nmo* mediates crosstalk between multiple signaling pathways since it antagonizes *Drosophila* Wg signaling (Zeng and Verheyen, 2004) and attenuates BMP signaling by phosphorylating Mad during wing development (Zeng et al., 2007). Nmo was originally identified as an ommatidial rotation-specific factor (Choi and Benzer, 1994), which was subsequently shown to be essential for regulating the rate of ommatidial rotation throughout the entire process (Fiehler and Wolff, 2008; Mirkovic et al., 2011). Genetic interaction assays suggested that *nmo* could be functionally related to the JNK cascade during ommatidial rotation (Fiehler and Wolff, 2008; Mihaly et al., 2001). Furthermore, it has been recently demonstrated that *nmo* genetically interacts with several core PCP components (*prickle*, *strabismus*), members of signaling pathways (*Notch*, *spitz*, *Egfr*) and genes encoding cell adhesion proteins such as E-cad (*shotgun*) and β-catenin (*armadillo*) (Mirkovic et al., 2011). Indeed, it has been suggested that Nmo serves as a molecular link between core PCP factors and the E-cad–β-catenin (β-cat) complexes promoting cell motility during ommatidial rotation (Mirkovic et al., 2011).

In order to analyze more precisely the requirement of Nmo in the ommatidial rotation process, we used several strategies. In vivo analyses of wild-type and *nmo* mutant eye imaginal discs demonstrated that this gene regulates the speed of ommatidial rotation, as suggested from studies in fixed discs (Fiehler and Wolff, 2008). We also found that cone cell dynamics during this process is disturbed in *nmo* mutants and demonstrated that Nmo is required in these cells for correct ommatidial rotation. Our in vivo analyses also showed that interommatidial cells are less dynamic in *nmo* mutants than in wild-type discs. In addition, we performed a microarray study to identify genes that were deregulated in *nmo* mutant eye imaginal discs and that could be involved in ommatidial rotation. Four of the genes identified were validated and confirmed to be functionally linked to *nmo* by genetic interaction assays with several mutant alleles. In addition, phenotypic analyses revealed that the ommatidial rotation process is compromised when expression levels of some of those genes are modified. One of them is *miple*, which encodes a secreted heparin-binding protein that belongs to the midkine (MK)/pleiotrophin (PTN) family (Englund et al., 2006). In vertebrates, both MK and PTN are secreted cytokines that are implicated in many different processes, including cell migration (Muramatsu, 2010; Papadimitriou et al., 2009). Our results showed that *miple* overexpression causes rotation defects and that it interacts genetically with *nmo* and *nmo*-related genes, suggesting that Nmo is required to repress *miple* for correct ommatidial rotation.

Materials and methods

Fly stocks and genetics

Fly lines used in this study include: nmo^{P1} (Choi and Benzer, 1994), *sev* > *nmo* (Mirkovic et al., 2011), $aos^{\Delta 7}$ (Freeman et al., 1992), UAS-*miple* (Toledano-Katchalski et al., 2007), the *mthl8* allele $P\{Mae\text{-}UAS.6.11\}mthl8^{F29.6}$ (Mukherjee et al., 2006), UAS-$Egfr^{DN}$ (Freeman, 1996), $Egfr^{CO}$ (Clifford and Schupbach, 1989). $ptp99A^{1}$, shg^{2}, arm^{4}, *cut*-GAL4, iR*miple*, iR*LRP1*, iR*Alk*, UAS-*Dcr-2*, $P\{EPgy2\}CG32373^{EY21017}$ (named in this paper as $EP^{CG32373}$) and the *unc-13-4A* overexpression line, $EP^{EY04085}$ were obtained from the Bloomington stock center. iR*mthl8*, iR*CG32373* and iR*unc-13-4A* were obtained from the Viena Drosophila RNAi Center. For UAS-*mthl8* transgenic lines full length *mthl8* cDNA LP02895 was subcloned into pUAST vector and flies were generated at BestGene Inc. (Chino Hills, USA.) by standard methods. Expression of several lines was checked by in situ hybridization with an *mthl8* probe in *en*-GAL4/UAS-*mthl8* embryos. *GMR* > *miple*, *arm*GFP, nmo^{P1} and *cut*-GAL4, nmo^{P1} lines were generated by standard recombination methods. nmo^{DB}, FRT80 (Mirkovic et al., 2011) and *ey*-FLP; ubiGFP, FRT80 flies were used to induce mitotic recombination for nmo^{DB} clones analysis. *arm*GFP was a gift of Silvia Muñoz-Descalzo (University of Cambridge, Cambridge, UK).

Live-imaging of pupal eye imaginal discs

Time-lapse imaging of pupal eye imaginal discs was performed as described (Escudero et al., 2007). Images were taken at 15 min intervals during at least 10 h in a Leica TCS SP confocal microscope. The images obtained were assembled and analyzed with ImageJ software. Measurements of IOCs apical areas were done manually with ImageJ. To quantify the number of IOCs disappearing in vivo during ommatidial rotation we followed each cell contained within the area comprised among 4 developing ommatidia from the beginning to the end of the process. IOCs that constricted their apical surface and subsequently disappeared were considered as dying cells. A total of 18 areas in *arm*GFP control and 13 in *arm*GFP, nmo^{P1} mutant discs were scored for this analysis.

Histology and immunohistochemistry

Analysis of adult retinae was performed as previously described (Tomlinson and Ready, 1987). Sections were mounted in DPX and observed through the optical microscope in dark field. At least four eyes per genotype were analyzed. For ommatidial