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# Nemo phosphorylates Eyes absent and enhances output from the Eya-Sine oculis transcriptional complex during Drosophila retinal determination

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ABSTRACT

The retinal determination gene network comprises a collection of transcription factors that respond to multiple signaling inputs to direct Drosophila eye development. Previous genetic studies have shown that *nemo* (*nmo*), a gene encoding a proline-directed serine/threonine kinase, can promote retinal specification through interactions with the retinal determination gene network, although the molecular point of cross-talk was not defined. Here, we report that the Nemo kinase positively and directly regulates Eyes absent (Eya). Genetic assays show that Nmo catalytic activity enhances Eya-mediated ectopic eye formation and potentiates induction of the Eya-Sine oculis (So) transcriptional targets *dachshund* and *lozenge*. Biochemical analyses demonstrate that Nmo forms a complex with and phosphorylates Eya at two consensus mitogen-activated protein kinase (MAPK) phosphorylation sites. These same sites appear crucial for Nmo-mediated activation of Eya function in vivo. Thus, we propose that Nmo phosphorylation of Eya potentiates its transactivation function to enhance transcription of Eya-So target genes during eye specification and development.



## Introduction

Generation of cellular diversity in a developing organism depends on coordinated cell proliferation, differentiation, migration and morphogenesis. Dynamically controlled transcriptional programs downstream of multiple signal transduction pathways produce the specific patterns of gene expression that define unique cell types and functions. The Retinal Determination (RD) gene network, a collection of conserved transcription factors named for their essential roles in eye development in Drosophila, presents a useful model to study how input from multiple signaling pathways can modify the function of a transcriptional network to regulate specific developmental decisions.

In Drosophila, the RD network is both necessary and sufficient for eye specification. Loss of RD genes in the developing eye disk results in loss or reduction in size of the adult eye, while their misexpression in non-retinal tissues can produce ectopic eyes (Bonini et al., 1993; Czerny et al., 1999; Mardon et al., 1994; Seimiya and Gehring, 2000; Shen and Mardon, 1997). The core components of the network form a cascade of transcriptional regulation where the PAX6 homolog Eyeless (Ey) activates expression of Eyes absent (Eya) and the SIX family member Sine oculis (So), which form a bipartite transcriptional complex and drive expression of Dachshund (Dac) (Chen et al., 1997; Halder et al., 1998; Pignoni et al., 1997; Shen and Mardon, 1997). However the flow of transcriptional induction is not solely linear, as downstream members can also activate expression of upstream RD genes, thereby amplifying network output; because of these positive feedback loops, overexpression of downstream genes such as Eya or Dac can activate the entire RD circuitry to a level sufficient for driving ectopic eye formation.

The core elements of the Drosophila RD network are deployed at multiple stages of eye development. During the second instar larval stage, division of the eye-antennal imaginal disk into eye or antennal compartments occurs via downregulation of Ey in the anterior antennal region (Kenyon et al., 2003). In the third instar, Ey deploys the rest of the RD network by inducing expression of Eya, So and Dac. Their expression is maintained in the wake of the posterior-to-anterior passage of the morphogenetic furrow, a physical indentation in the epithelium that marks the transition from asynchronous proliferation to G1 arrest and differentiation (Bessa et al., 2002; Curtiss and Mlodzik, 2000; Halder et al., 1998; Pappu and Mardon, 2004; Ready et al., 1976). Cells posterior to the morphogenetic furrow develop into photoreceptor cells and nucleate formation of the ommatidia that collectively comprise the compound eye (Clandinin and Zipursky, 2002; Wolff and Ready, 1991).

Although initially identified for their role in the Drosophila eye, components of the RD gene network have multiple roles throughout

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development in metazoans, as evidenced by a broad spectrum of loss-of-function phenotypes. For instance, *EYA1* knockout mice exhibit loss of multiple organs and defects in muscle development, while mutations in human *EYA1* have been associated with branchio-oto-renal (BOR) syndrome, an autosomal dominant disorder characterized by jaw and external ear malformations, hearing loss, and renal defects (Abdelhak et al., 1997; Grifone et al., 2005; Heanue et al., 1999; Xu et al., 1999). RD network components are also expressed outside of eye tissues in Drosophila, and null mutations are generally lethal (Bonini et al., 1998; Callaerts et al., 2001; Cheyette et al., 1994).

Expression and activity of RD network members are regulated by multiple signaling pathways to produce specific developmental outcomes (reviewed by Kumar, 2009; Silver and Rebay, 2005). For example, prior to neuronal differentiation in the developing eye disk, Hedgehog (Hh) and Decapentaplegic (Dpp) signaling promote *eya*, *so* and *dac* expressions at the morphogenetic furrow (Pappu et al., 2003), whereas Wingless (Wg) signaling downregulates expression of *eya*, *so* and *dac* in the antennal disk to inhibit retinal fate (Baonza and Freeman, 2002). Although mechanisms influencing RD protein function remain less well characterized, they are likely to be equally important and to include interactions with specific binding partners and post-translational modifications. For example, distinct cofactor interactions may mediate specific roles of So during eye development (Kenyon et al., 2005), while Eya is positively regulated by MAPK phosphorylation in response to EGFR/RAS signaling during retinal determination (Hsiao et al., 2001; Rebay et al., 2000), and by Abl kinase phosphorylation during photoreceptor axon targeting (Xiong et al., 2009).

Recently, we reported that *ey*, *eya*, and *dac* genetically synergize with *nemo* (*nmo*) to promote eye specification (Braid and Verheyen, 2008). Drosophila *nmo* encodes a proline-directed serine/threonine kinase that is essential during development and is the founding member of the Nemo-like kinase (NLK) branch of the MAPK superfamily (Brott et al., 1998; Choi and Benzer, 1994; Mirkovic et al., 2002; Miyata and Nishida, 1999). NLKs are highly conserved in evolution and have multiple developmental roles in a variety of organisms, including endoderm induction in *C. elegans* (Meneghini et al., 1999), antero-posterior patterning and neurogenesis in zebra fish (Ishitani et al., 2010; Thorpe and Moon, 2004), and mouse hematopoiesis (Kortenjann et al., 2001). Functionally, NLKs act as regulators of downstream transcriptional effectors for multiple signaling pathways. One of the best-characterized roles for Nmo/NLK is in regulating Wnt/Wingless signaling. NLKs block activation of Wnt/Wg target genes (Zeng and Verheyen, 2004) by phosphorylating T-cell factor (TCF) and inhibiting the DNA-binding ability of the beta-catenin/TCF complexes (Ishitani et al., 1999; Ishitani et al., 2003). Nmo antagonizes BMP signaling in Drosophila where it suppresses the transcriptional activity of Mothers against Dpp (Mad) by preventing its nuclear accumulation (Zeng et al., 2007). In addition, Nmo has been implicated in planar cell polarity, programmed cell death, embryonic patterning, synaptic growth, and wing patterning, and is likely to mediate cross-talk between multiple signaling pathways in these contexts (Braid et al., 2010; Choi and Benzer, 1994; Fiehler and Wolff, 2008; Merino et al., 2009; Mirkovic et al., 2002; Mirkovic et al., 2011; Verheyen et al., 2001).

In the context of Drosophila eye development, we have previously shown that coexpression of Nmo potentiates ectopic eye formation driven by Ey, Eya and Dac transgenes in a dose-dependent manner (Braid and Verheyen, 2008). Here, we test the hypothesis that Nmo-mediated modulation of Eya-So transcriptional activity might provide a mechanistic explanation for the cooperative genetic interaction between Nmo and the RD network. We show that Nmo catalytic function is required to promote Eya-mediated retinal determination and to enhance activity of the Eya–So transcriptional complex. Mechanistically, Nmo can form a complex with and phosphorylate Eya at two MAPK consensus sites. This phosphorylation potentiates Eya activity in ectopic eye induction assays and enhances Eya–So mediated transcription of *lozenge* and *dachshund*. Together our results suggest that the Nmo kinase forms part of a novel regulatory complex that modulates Eya's transactivation function during Drosophila retinal determination.

## Results

### *The Nemo kinase cooperates with Eya to promote eye development*

We have previously demonstrated that *eya* and *nmo* interact genetically to promote eye specification in Drosophila (Braid and Verheyen, 2008). To begin to address the underlying mechanism, we asked if the kinase function of Nmo is required for Eya activity during ectopic eye formation by comparing the effects of coexpressing wild type and kinase inactive Nmo transgenes (Fig. 1). A weak Eya transgene, whose ectopic eye induction efficiency is only ~25% (Hsiao et al., 2001), was selected to maximize the range of responsiveness to Nmo-mediated enhancement. Importantly, this background is still sensitized to *nmo* levels, as reduced *nmo* dosage decreased Eya's ectopic eye induction efficiency more than two-fold (Fig. 1). In contrast, and as previously shown with other Eya transgenes (Braid and Verheyen, 2008), coexpressing wild type Nmo increased the penetrance and the frequency of Eya-mediated ectopic eye induction (Fig. 1). Thus over 50% of adults exhibited ectopic eyes, a two-fold increase relative to Eya alone, with approximately a quarter of those animals showing ectopic eye tissue under both, rather than under just one, antennae. In this assay, we found that kinase dead Nmo failed to enhance, and slightly suppressed Eya-mediated ectopic eye formation (Fig. 1). These results extend our previously reported Eya-Nmo synergistic interaction (Braid and Verheyen, 2008), and suggest that the kinase function of Nmo is required.

### *Nemo potentiates Eya-So mediated induction of Lozenge and Dachshund expression*

Eya has two biochemical functions, one as a transcriptional coactivator in conjunction with the DNA binding protein Sine oculis (So) and a second as a protein tyrosine phosphatase; both activities are required for full function during eye specification (Rayapureddi et al.,

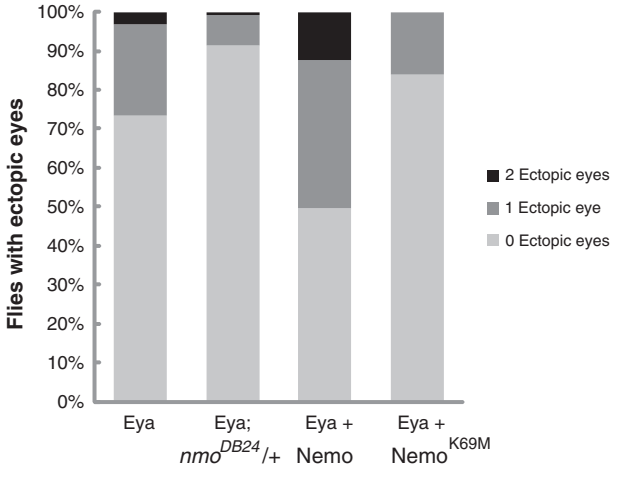


**Fig. 1.** Nemo's kinase activity is required for Eya-Nemo synergy during eye induction. Heterozygosity for *nmo* reduces the frequency of Dpp-Gal4>*UAS-eya*-mediated ectopic eye formation, while coexpression of UAS-Nmo increases both the frequency and penetrance of ectopic eyes. A Kinase-dead Nmo transgene ($Nmo^{K69M}$) fails to increase and slightly suppresses ectopic eye frequency when coexpressed with Eya. Penetrance reflects whether ectopic eye tissue was observed under one or both antennae (1 Ectopic eye, dark gray bar or 2 Ectopic eyes, black bar) and frequency refers to a binary scoring system for presence/absence of ectopic eye tissue.