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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.

### Keywords

Eye development; Transcription factor; Cell fate; NLK

### 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

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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 development in metazoans, as evidenced by a broad spectrum of loss-of-function phenotypes. For instance, *EYAI* knockout mice exhibit loss of multiple organs and defects in muscle development, while mutations in human *EYAI* 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 crosstalk 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., 2003; Silver et al., 2003; Tootle et al., 2003). Taking advantage of our recent finding that cytoplasmic Eya phosphatase function contributes to photoreceptor axon targeting in the larval brain (Xiong et al., 2009), we first asked whether Nmo might interact with Eya in this context. However, neither decreasing nor increasing *nmo* dose modified the Eya axon guidance phenotypes, nor did Nmo knockdown or overexpression have an axonal phenotype on its own (data not shown). This suggests that Nmo may not regulate Eya phosphatase function, at least in this context. Therefore, we directed our focus to the alternate hypothesis that Nmo potentiates Eya-So transcriptional activity.

To investigate this possibility, we compared the levels of ectopic induction of two known Eya-So target genes, *lozenge* and *dachshund* (Jemc and Rebay, 2007; Pappu et al., 2005; Yan et al., 2003), in the antennal and wing imaginal disks respectively, in response to overexpression of Eya versus Eya plus Nmo. Both tissues are competent to form ectopic eye tissue, but do not normally express Eya, and have been used extensively as tractable experimental systems for probing the function and regulation of the RD network (Bonini et al., 1997; Braid and Verheyen, 2008; Jang et al., 2003; Salzer and Kumar, 2010; Shen and Mardon, 1997; Tavsanli et al., 2004; Tootle et al., 2003; Weasner et al., 2009). First, we followed induction of *lozenge* expression using a previously characterized Eya-So transcriptional reporter referred to as the *lozenge* minimal enhancer element (LMEE; Mutsuddi et al., 2005; Yan et al., 2003). Consistent with our hypothesis, in transfected cultured S2 cells, addition of Nmo resulted in a 3-fold increase in LMEE-luciferase reporter activity relative to induction with Eya-So alone (Supplemental Fig. 1). Responsiveness of this *lozenge* reporter was tested in flies carrying an LMEE-*lacZ* transgene. In wild type flies, the LMEE-*lacZ* reporter is expressed in three concentric circles in the antennal disk (Fig. 2A), matching the previously reported expression pattern of Lozenge in this tissue (Flores et al., 1998). Driving Eya expression with Dpp-GAL4 resulted in ectopic reporter activity in the ventral antennal disk in 24% of disks analyzed (Fig. 2B). Coexpression of Eya and Nmo increased both the frequency (~52% of disks) and the size of the tissue patch showing ectopic reporter activity (Fig. 2C). On the other hand, coexpression of Eya and a kinase inactive form of Nmo decreased the frequency of ectopic reporter expression compared to expression of Eya alone, with only ~16% of disks showing LMEE-*lacZ* induction (Fig. 2D). Similar frequencies of induction of ectopic Lozenge were observed in disks stained with an anti-Lozenge antibody (data not shown).

In the second set of experiments, we used Dpp-Gal4 to express Eya in combination with Nmo transgenes in the wing disk and monitored induction of Dac, which has been shown to be genetically downstream of and transcriptionally regulated by Eya-So (Chen et al., 1997; Pappu et al., 2005; Shen and Mardon, 1997). In wild type wing disks Dachshund is expressed in the dorsal posterior compartment and in two anterior regions surrounding the wing pouch (Fig. 3A; Chen et al., 1997). Expression of Eya along the Dpp stripe at the antero-posterior border of the disk induced ectopic Dac in the dorsal wing disk (Fig. 3B, arrow) and in the dorsal and ventral hinge regions (Fig. 3B, arrowheads). In agreement with our observations in ectopic eye experiments (Fig. 1), this assay is sensitized to *nmo* levels, as reduced *nmo* dosage decreased Eya-mediated ectopic Dac induction (Fig. S2).

Furthermore, and consistent with the LMEE-*lacZ* induction assay in the antennal disk (Fig. 2), coexpression of Eya and Nmo, but not kinase dead Nmo, increased Dac induction relative to that seen with expression of Eya alone, particularly in the ventral wing pouch where endogenous *nmo* is not normally expressed (Figs. 3C, D, arrowheads; Chen et al., 1997). Expression of either Nmo transgene alone did not induce Dac expression (data not shown).

Transcriptional induction of *dac* presumably occurs through the normal RD network positive feedback circuitry such that ectopically expressed Eya interacts either with undetectably low levels of endogenous So in the wing or with another unknown factor to transcribe more *so*, raising So levels to a threshold sufficient for the Eya–So transcription factor to induce *dac* expression. Supporting this model, expression of Eya along the Dpp stripe at the antero-posterior border of the disk induced ectopic So expression in the wing pouch (Fig. S3), while expression of Eya in a *so*<sup>1</sup> mutant background, which deletes the eye-specific enhancer in the *so* gene (Cheyette et al., 1994; Pignoni et al., 1997), did not, resulting in a failure to induce Dac (Fig. 4A). Similar experiments using RNAi-mediated knockdown of *so* gave analogous results (Supplemental Fig. S4). Coexpression of Nmo and Eya in either the *so*<sup>1</sup> mutant or *so* RNAi background also failed to induce ectopic Dac expression (Fig. 4B, Supplemental Fig. S4), implying that the increased Dac induction seen in the Eya+Nmo background reflects increased activity of the Eya–So transcription factor.

### Eya is a novel substrate for Nemo phosphorylation

Our genetic studies consistently demonstrate that Nmo's kinase activity is necessary to promote Eya's ability to induce ectopic eyes and expression of Eya–So target genes. Since Eya activity and localization are regulated by phosphorylation (Hsiao et al., 2001; Xiong et al., 2009), we predicted that Eya may be a novel substrate for Nmo. We performed in vitro kinase assays using Nmo protein purified from HEK293T cells and recombinant full length His-tagged Eya fusion protein. Eya became phosphorylated only when incubated with the wild type Nmo protein, but not the kinase dead version; robust Nmo auto-phosphorylation was also observed, but only with the kinase active form (Fig. 5A).

We next mapped the key Eya residues phosphorylated by Nmo to further clarify their enzyme-substrate relationship. We identified the N-terminal P/S/T-rich region (PST) of Eya as the primary target of Nmo-mediated phosphorylation in kinase assays using fragments of the Eya protein (Figs. 5B, C). This region carries two consensus MAPK phosphorylation sites (defined as PXS/TP), which have been previously shown to be relevant in promoting *eya*-mediated eye specification (Hsiao et al., 2001). Since Nemo-like kinases belong to the MAPK superfamily, we reasoned that these two sites might be good candidates for Nmo phosphorylation. We mutated both phosphoacceptor serines (S402 and S407) to alanine and tested the resulting GST-Eya<sup>S-A</sup> fusion protein as a Nmo kinase substrate (Fig. 5C). Phosphorylation of GST-Eya<sup>S-A</sup> is reduced five-fold relative to GST-Eya, but not entirely abolished, suggesting that while these sites may be primary targets of Nmo kinase activity, one or more of the seven other S/TP motifs within the Eya fusion protein might also be phosphorylated.

To test whether the Nmo-Eya kinase-substrate relationship reflects a stable molecular complex, we carried out co-immunoprecipitation experiments in S2 cultured cells transiently transfected with epitope-tagged Eya and Nmo expression constructs. Wild type and kinase dead Nmo co-immunoprecipitate with Eya (Fig. 5D), suggesting Nmo and Eya may form a molecular complex that allows Nmo to directly phosphorylate Eya and potentiate its activity.



## Nemo activation of Eya during retinal specification requires two consensus MAPK sites

To follow up on the results from our kinase assays, we asked whether mutating the two Eya MAPK phosphorylation consensus sites would reduce the synergistic Eya-Nmo interaction in experiments measuring Dac induction in the wing. Consistent with our prior finding that UAS-Eya<sup>S-A</sup> transgenes have very low ectopic eye induction activity (Hsiao et al., 2001), most lines failed to induce ectopic Dac expression and so could not be used to test synergy with Nmo (data not shown). To circumvent this problem, Eya<sup>S-A</sup> transgenic lines providing unusually strong activity as judged by an ~40% ectopic eye induction efficiency and significant Dac induction (Figs. 6A–B, E) were used to test the hypothesis. In contrast to its synergistic interaction with wild type Eya transgenes, overexpression of Nmo did not cause a significant increase in Eya<sup>S-A</sup> mediated ectopic Dac, suggesting that the two consensus MAPK sites are crucial for the interaction (Figs. 6A–D, B' and C'' arrowheads, compare to Fig. 3C'''). Ectopic eye induction assays showed a similar trend, such that coexpression of Nmo did not increase Eya<sup>S-A</sup> activity (Fig. 6E).

If Nmo potentiates Eya function by phosphorylating its two MAPK consensus sites, then constitutively activating Eya via phosphomimetic (S–D) mutations in the MAPK sites should bypass the requirement for Nmo. As predicted, heterozygosity for *nmo*, which markedly suppresses the ectopic eye-inducing capacity of wild type Eya transgenes (Fig. 1 and Braid and Verheyen, 2008), did not suppress ectopic eye formation by the Eya<sup>S-D</sup> transgene (Fig. 6F). Together these observations demonstrate that the two MAPK phosphorylation consensus sites are crucial for Nmo activation of Eya during retinal specification.

## Discussion

Retinal determination genes are highly regulated effectors that receive input from a variety of signaling pathways to direct many aspects of *Drosophila* eye specification and development, including cell proliferation, differentiation and morphogenesis (reviewed by Kumar, 2009). In this study, we reveal a novel regulatory mechanism by which the proline-directed kinase Nmo phosphorylates Eya at two conserved MAPK phosphorylation consensus residues to promote activity of the Eya-So transcriptional complex during retinal specification.

While a kinase-substrate relationship often reflects a transient physical interaction, Nmo and Eya associate in a complex sufficiently stable to be detected by coimmunoprecipitation, raising the possibility that Nmo could be an intrinsic component of the Eya-So transcriptional complex assembled at target genes. Inclusion of a kinase in a transcriptional complex would provide a sensitive mechanism for rapid and dynamic modulation of transcriptional output. Mechanistically, Nmo could be recruited to DNA bound Eya-So complexes or conversely, Nmo itself could occupy specific target sites through other protein interactions and then recruit Eya-So. Consistent with such models, other MAPK superfamily members have been detected at specific target gene promoters along with their substrates (Lawrence et al., 2008; Pokholok et al., 2006). Thus chromatin immunoprecipitation studies will be a high priority for investigating possible Nmo-Eya-So co-occupancy at target genes.

Regardless of the exact biochemical mechanism, our work raises the broader question of whether interactions with Nmo augment Eya-So transcriptional activity at all target genes, or whether there are more selective, context-specific requirements for Nmo input during retinal specification and development. Overall our data support a broad, but perhaps not universal, involvement of Nmo in regulating RD network output. For example, the observation that Nmo potentiates Eya-So mediated induction of Dac expression and ectopic

eye formation would be consistent with Nmo playing a global role in regulating the overall activity and output of the RD network throughout eye development. Furthermore, the ability of Nmo to potentiate induction of *lozenge* suggests a regulatory role for Nmo not just during early eye fate determination but later during cell specification and differentiation. *lozenge* encodes a RUNX family transcription factor that contributes to prepatterning in photoreceptor precursors and cell fate establishment in cone and pigment cells (Crew et al., 1997; Daga et al., 1996). The fact that *lozenge* expression in the developing eye is regulated by multiple transcription factors in addition to Eya–So (Behan et al., 2002; Siddall et al., 2009; Yan et al., 2003) complicates genetic analysis of the Nmo input. Thus, similar to what we reported previously for Dac (Braid and Verheyen, 2008), *Lozenge* protein levels do not appear reduced in *nmo* loss of function clones (LB and EMV unpublished observation). This suggests either Nmo does not potentiate Eya–So mediated activation of *lozenge* and *dac* in this context, or that other transcriptional inputs effectively compensate for the presumed reduction in Eya–So activity.

Considering further the issue of context specificity and combinatorial transcriptional control, we found that Nmo potentiates Eya-mediated ectopic *lozenge* and *dachshund* expression in relatively small regions of the Dpp expression domain in antennal and wing disks. Keeping in mind the caveats inherent to such overexpression experiments, these observations suggest that rather than broadly activating Eya–So-mediated transcription in all cells, Nmo regulates the function of this complex in specific cellular contexts. The ability of only certain cell populations outside of the eye imaginal disk to support retinal formation has been previously described, and in agreement with our observations from misexpressing Eya alone, the wing disk pouch is not a “hot spot” of responsiveness to RD network activity (Salzer and Kumar, 2010). However, coexpression of Nmo results in high levels of ectopic Dac expression in this region, suggesting that Nmo can activate Eya–So to drive transcription of target genes in a cellular context where this complex would normally be inactive or actively repressed. One possible explanation for this observation is that regional Nmo-mediated activation of Eya–So in wing disks correlates with high levels of endogenous Nmo, which is expressed in a ring surrounding the pouch, and along the dorsoventral boundary bisecting the wing pouch (Zeng and Verheyen, 2004). Alternatively, Nmo could drive specific activation of Eya–So by acting as a transducer of other signaling mechanisms that are active in specific regions of the wing disk.

Another context in which Nmo potentiation of Eya–So activity might be relevant is in regulating cell proliferation. Loss of *eya* or *so* results in uncontrolled cell proliferation in undifferentiated cells in the early eye epithelium, followed eventually by apoptosis (Bonini et al., 1993; Pignoni et al., 1997) while ectopic expression of Eya–So also leads to significant tissue overgrowth (Bonini et al., 1997; Pignoni et al., 1997). These results suggest that proper Eya–So activity levels are important for balancing cell proliferation and tissue growth during organ development. Our analyses show that coexpressing Nmo and Eya results in tissue overgrowth in both antennal and wing disks (Figs. 2C, 3C), suggesting that Nmo could regulate Eya–So function in cell proliferation. Furthermore, our previous observation that *nmo* levels can modify growth in the eye and head suggests that Nmo also plays a role in regulating cell proliferation (Braid and Verheyen, 2008).

Although the full spectrum of Eya–So transcriptional targets relevant to regulating proliferation and tissue growth remains to be identified, we have previously proposed that Eya and So control cell proliferation at least in part through direct regulation of the expression of the cell cycle regulatory gene of *string* (Jemc and Rebay, 2007). In the third instar eye disk, *string* activity contributes to synchronization of undifferentiated cells immediately anterior to the morphogenetic furrow, which is essential for subsequent cell fate specification and ommatidial assembly (Mozer and Easwarachandran, 1999; Thomas et al.,

1994). Furthermore, transcriptional regulation of *string* in progenitor cells has been proposed as a mechanism that affects morphogenetic furrow progression (Lopes and Casares, 2010). Consistent with the possibility that Nmo potentiation of Eya–So activation of *string* could be important in this context, loss of *nmo* in eye disk clones results in a delay in morphogenetic furrow progression, (LB and EMV, unpublished data). The specific effects of Nmo, Eya and So regulation on cell cycle progression during early eye specification remain an interesting question for future studies.

Lending further complexity to these regulatory possibilities, the two MAPK consensus residues on Eya that we report as Nmo targets were previously described as regulatory sites that modulate Eya's function in eye specification in response to RTK/RAS/MAPK signaling (Hsiao et al., 2001). Similarly to Nmo, ERK-mediated phosphorylation at these sites was proposed to positively regulate Eya activity in ectopic eye induction (Hsiao et al., 2001). Thus there may be multiple MAPK family members, perhaps even extending beyond Nmo and Erk, that depending on specific context and in response to a variety of upstream signaling inputs, function either redundantly, competitively or in non-overlapping ways to phosphorylate and regulate Eya. Whether such inputs all modulate Eya–So transcriptional output, or whether MAPK-mediated regulation may also modify other Eya functions will be an interesting direction for future investigation.

## Experimental procedures

### Drosophila strains

We used the following Fly stocks: *dpp<sup>40C6</sup>-Gal4* (Staebling-Hampton et al., 1994), *ey-Gal4*, *so<sup>1</sup>*, (Bloomington Stock Center), *nmo<sup>DB24</sup>* (Zeng and Verheyen, 2004), *UAS-nmo-GFP* (Fiehler and Wolff, 2008), *UAS-nmo<sup>K69M</sup>-GFP* (Merino et al., 2009), *LMEE-lacZ* (Yan et al., 2003), *UAS-eya<sup>I</sup>*, *UAS-eya<sup>IIIa</sup>*, *UAS-eya<sup>Vb</sup>*, *UAS-Flag-eya*, and *UAS-Flag-eya<sup>1,2S-A</sup>*. *UAS-so<sup>RNAi</sup>*, were obtained from the Vienna Drosophila RNAi Center.

For ectopic eye induction assays, *dpp<sup>40C6</sup>-Gal4/TM3Sb* or *dpp<sup>40C6</sup>-Gal4, nmo<sup>DB24</sup>/TM3Sb*; *dpp<sup>40C6</sup>-Gal4/TM3Sb* flies were crossed to *UAS-eya<sup>IIIa</sup>* or *UAS-eya<sup>IIIa</sup>*, *UAS-nmo-GFP*, or *UAS-eya<sup>IIIa</sup>*, *UAS-nmo<sup>K69M</sup>-GFP*. Ectopic eyes under the antennae were scored in at least 120 progeny.

LMEE-*lacZ* reporter activity in antennal disks was evaluated by crossing *dpp<sup>40C6</sup>-Gal4*, *LMEE-lacZ/TM6BTb* to *UAS-eya<sup>Vb</sup>* or *UAS-eya<sup>Vb</sup>*, *UAS-nmo-GFP*, or *UAS-eya<sup>Vb</sup>*, *UAS-nmo<sup>K69M</sup>-GFP/TM6B*.

Induction of Dac expression was analyzed in wing disks dissected from appropriately genotyped 3rd instar progeny obtained by crossing *dpp<sup>40C6</sup>-Gal4/TM6BTb* to *UAS-eya<sup>Vb</sup>* or *UAS-eya<sup>Vb</sup>*, *UAS-nmo-GFP* or *UAS-eya<sup>Vb</sup>*, *UAS-nmo<sup>K69M</sup>-GFP/TM6B* or *UAS-Flag-eya*, *UAS-nmo-GFP*, or *UAS-Flag-eya<sup>1,2S-A</sup>*, *UAS-nmo-GFP* stocks. Requirement for So was evaluated by crossing *so<sup>1</sup>*; *dpp<sup>40C6</sup>-Gal4/TM6BTb* and *dpp<sup>40C6</sup>-Gal4*, *UAS-nmo-GFP/TM6BTb* to *UAS-eya<sup>Vb</sup>* or *UAS-eya<sup>Vb</sup>*, *UAS-nmo-GFP* or *UAS-eya<sup>I</sup>*, *UAS-so<sup>RNAi</sup>*.

### Immunostaining

Wing and eye-antennal imaginal disks were dissected from third instar larvae in S2 cell medium, fixed for 10 min in 4% paraformaldehyde with 0.1% Triton X-100, washed 3× in PBT (1× PBS, 0.1% Triton), and blocked for 30 min in PNT (1× PBS, 0.1% Triton, 1% normal goat serum). Disks were incubated with guinea pig α-Eya (1:1000), guinea pig α-So (1:1000), mouse α-Dac (1:20; Developmental Studies Hybridoma Bank), and rabbit α-βGal (1:1000) in PNT at 4 °C overnight as indicated, washed in PBT, incubated with donkey α-mouse-Cy3, donkey α-rabbit-Cy3, and donkey α-guinea pig-Cy5 (1:2000; Jackson



ImmunoResearch) for 2 h at room temperature, washed in PBT and mounted in ProLong antifade reagent (Invitrogen) for imaging on a Zeiss 510 confocal microscope. Pictures of 0.5  $\mu\text{m}$  sections were taken and stacked across the  $z$  axis using LSM Image Browser software. For quantification of Dac expression, a region of interest along the wing disk pouch was defined and analyzed using Image J software (Abramoff et al., 2004), and the integrated pixel intensity for all measured images was averaged for the analysis.

### In vitro kinase assays and immunoprecipitation

Nmo protein for in vitro kinase assays was obtained by transfecting HEK293T cells with pXJ-Flag-Nmo or pXJ-Flag-Nmo<sup>K69M</sup> vectors (Zeng et al., 2007) using the Effectene transfection reagent (Qiagen) and following manufacturer's instructions. Cells lysis and Nmo immunopurification were carried as previously described (Zeng et al., 2007). Recombinant full-length HIS-EYA, GST-EYA<sup>223–438</sup>, GST-EYA<sup>487–760</sup>, GST-EYA<sup>223–438</sup>, GST-SO, and GST were used as substrates. Kinase reactions were carried out as previously described (Zeng et al., 2007) and SDS-PAGE resolved samples were exposed on a Storm phosphorimager. Eya-Nmo coimmunoprecipitation was achieved by transfecting *Drosophila* S2 cells with pMT-Flag-EYA, pAct5-HA-Nmo, and pAct5-HA-Nmo<sup>K69M</sup> and processed as previously described (Xiong et al., 2009).

### Supplementary Material

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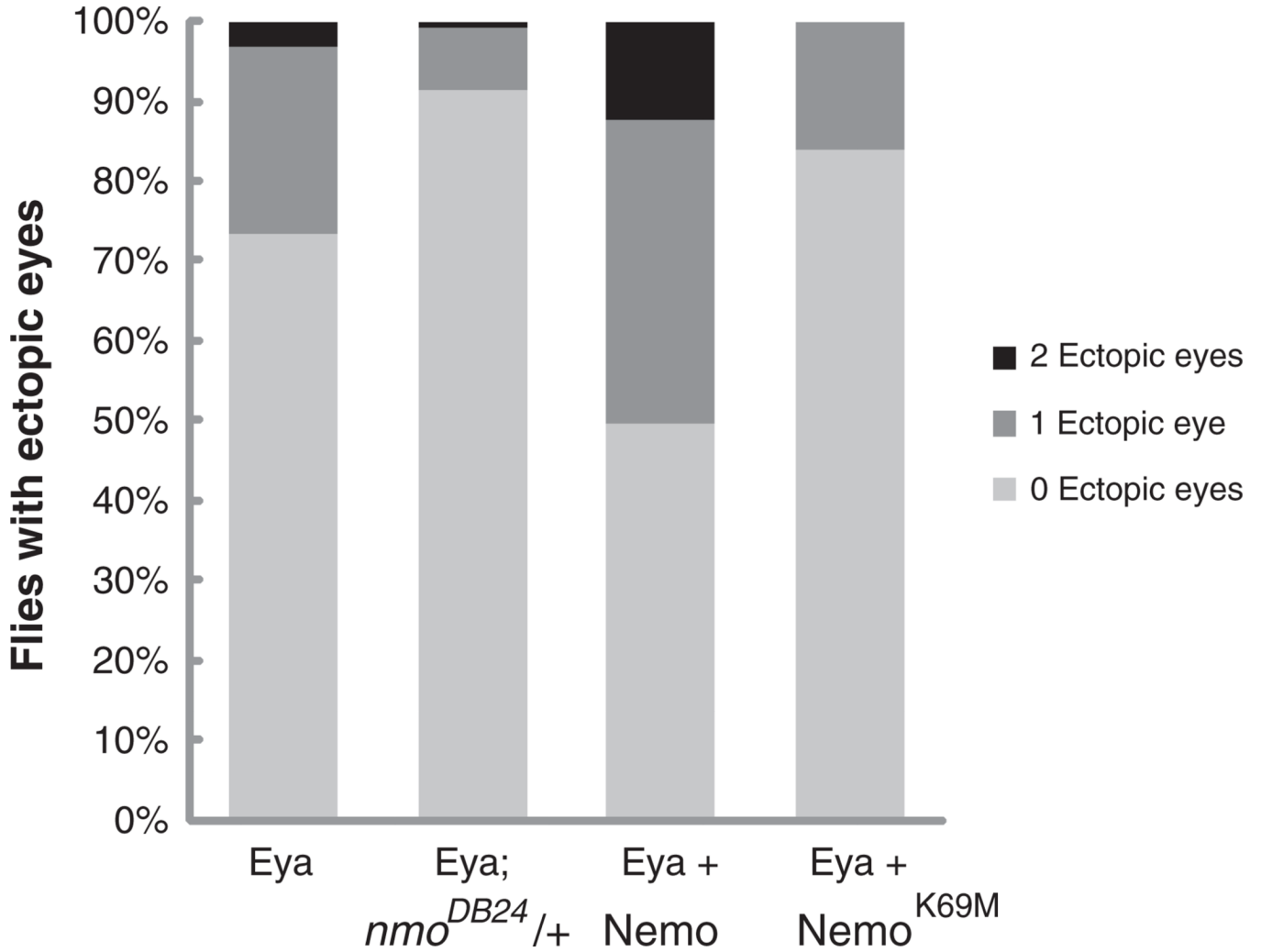
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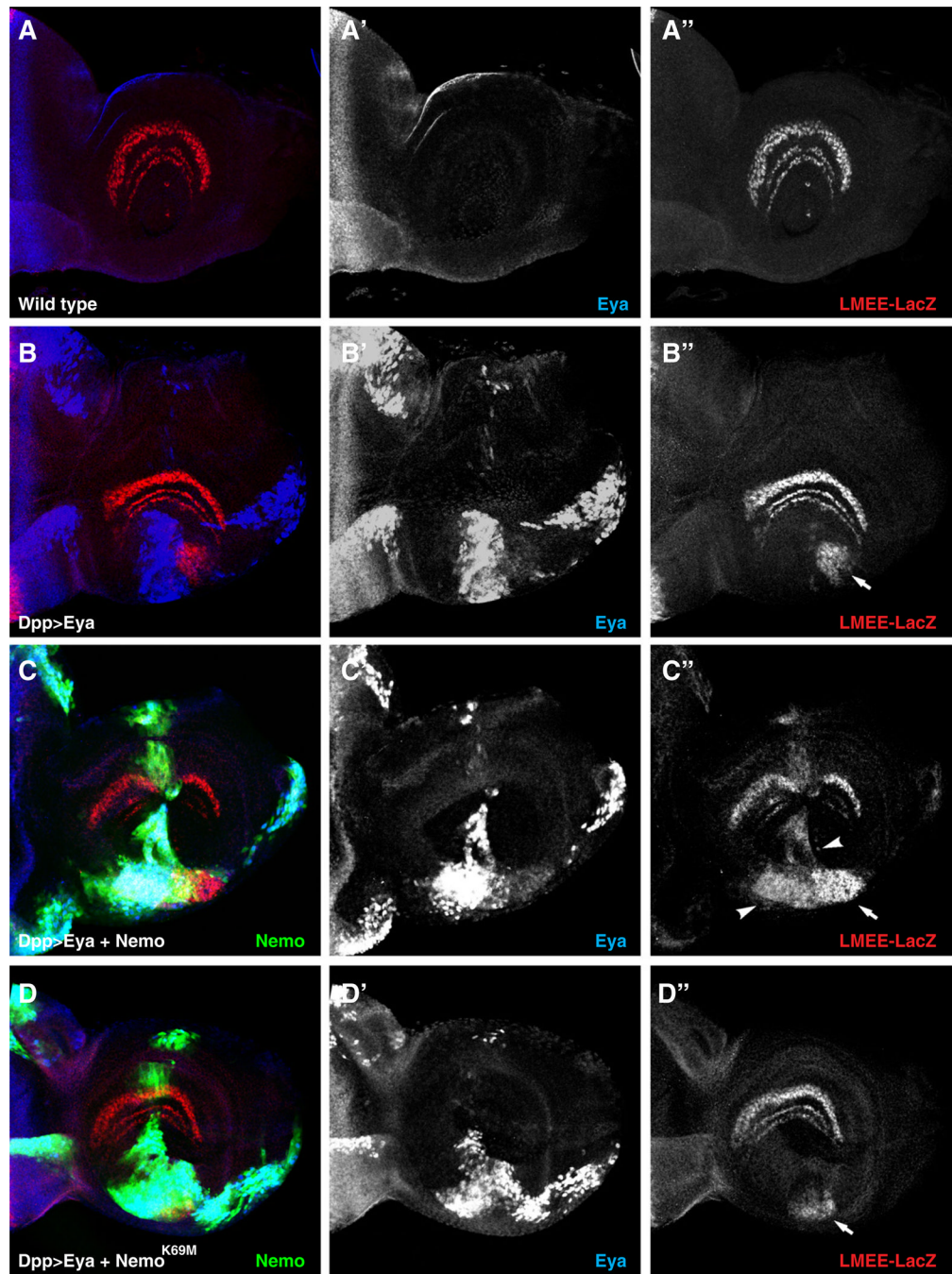
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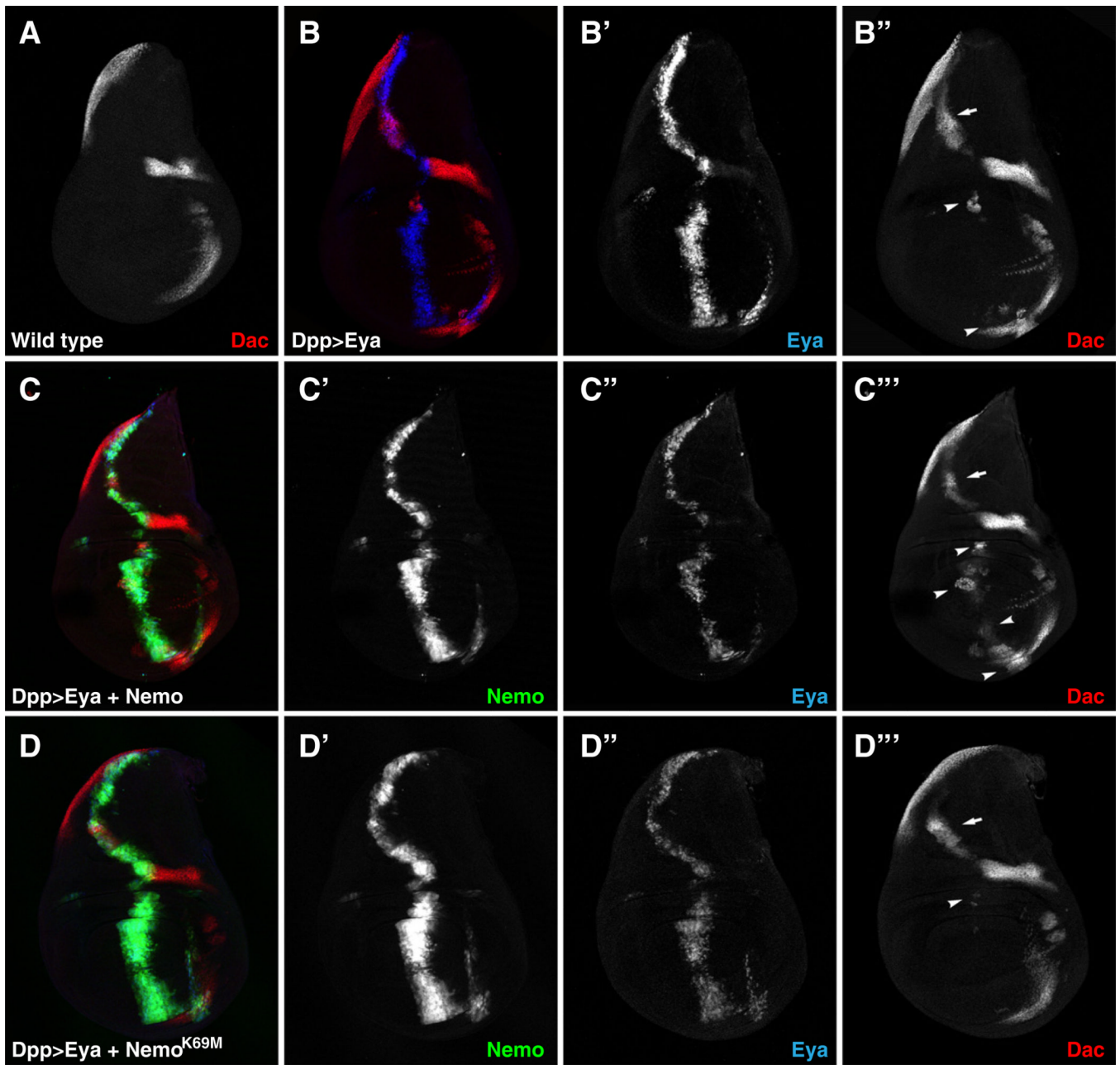




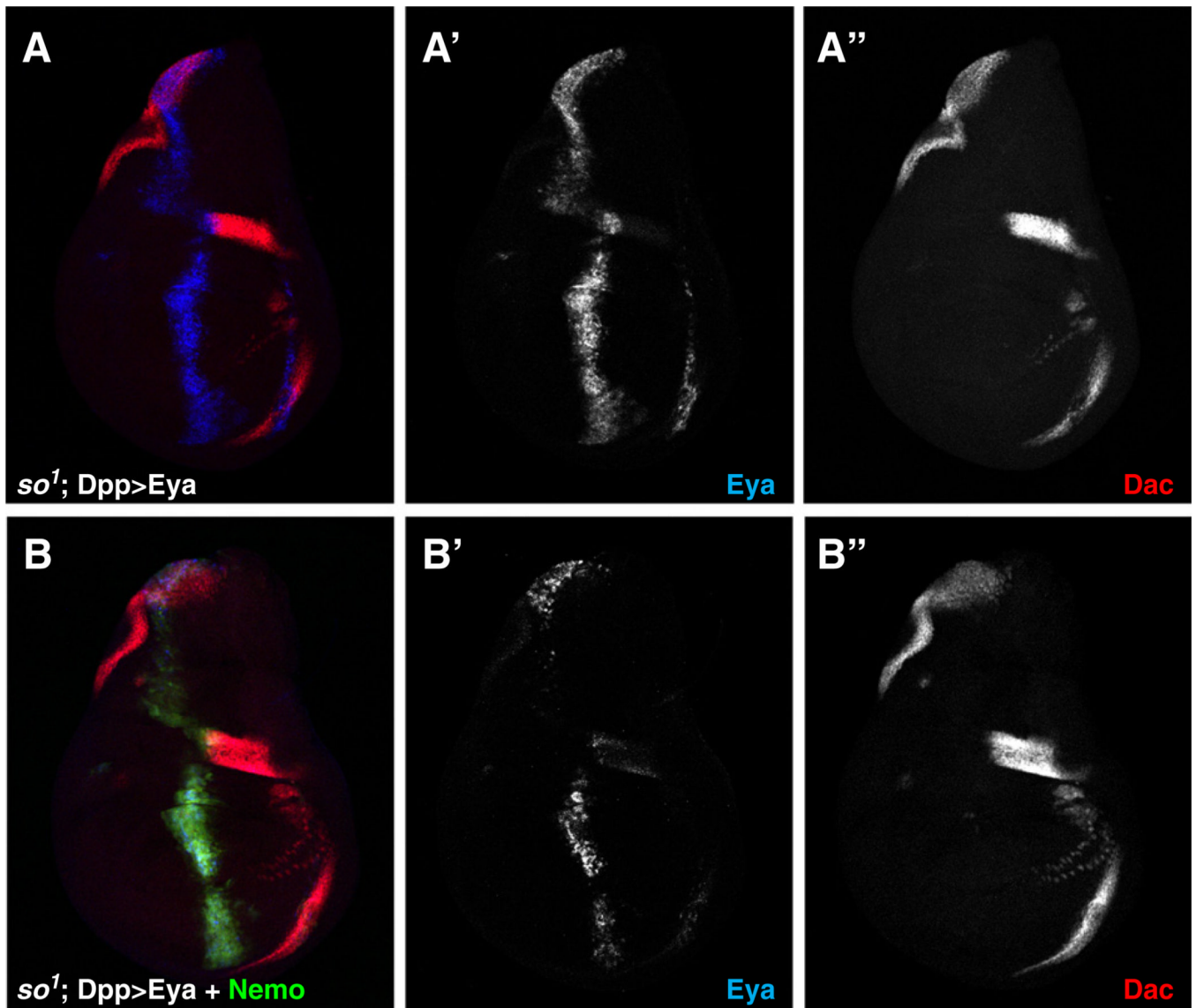
**Fig. 1.** Nemo's kinase activity is required for Eya-Nemo synergy during eye induction. Heterozygosity for *nmo* reduces the frequency of Dpp-Gal4>*UAS-eya*-mediated ectopic eye formation, while coexpression of UAS-Nmo increases both the frequency and penetrance of ectopic eyes. A Kinase-dead Nmo transgene (Nmo<sup>K69M</sup>) 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.



**Fig. 2.** Nemo increases Eya-mediated activation of a *lozenge* transcriptional reporter. (A–D) Expression of the *lozenge* LMEE-*lacZ* reporter (red), Eya (blue) and Nmo-GFP (green) in third instar antennal disks oriented dorsal up, posterior left. Dpp-GAL4 was used to drive expression of Eya and Nmo. (A) LMEE-*lacZ* reporter expression in wild type larvae is restricted to three concentric half-circles in the center of the antennal disk. (B) 24% ( $n = 25$ ) of Dpp-GAL4>UAS-Eya disks have ectopic reporter activity in the ventral antennal disk (B'', arrow). (C) Coexpression of Nmo increases both the frequency (~52%,  $n = 21$ ) and the area of LMEE-*lacZ* induction (C'', arrowheads). (D) Coexpression of kinase inactive Nmo (Nmo<sup>K69M</sup>) decreases the frequency (16%,  $n = 25$ ) and area of ectopic  $\beta$ -gal staining.

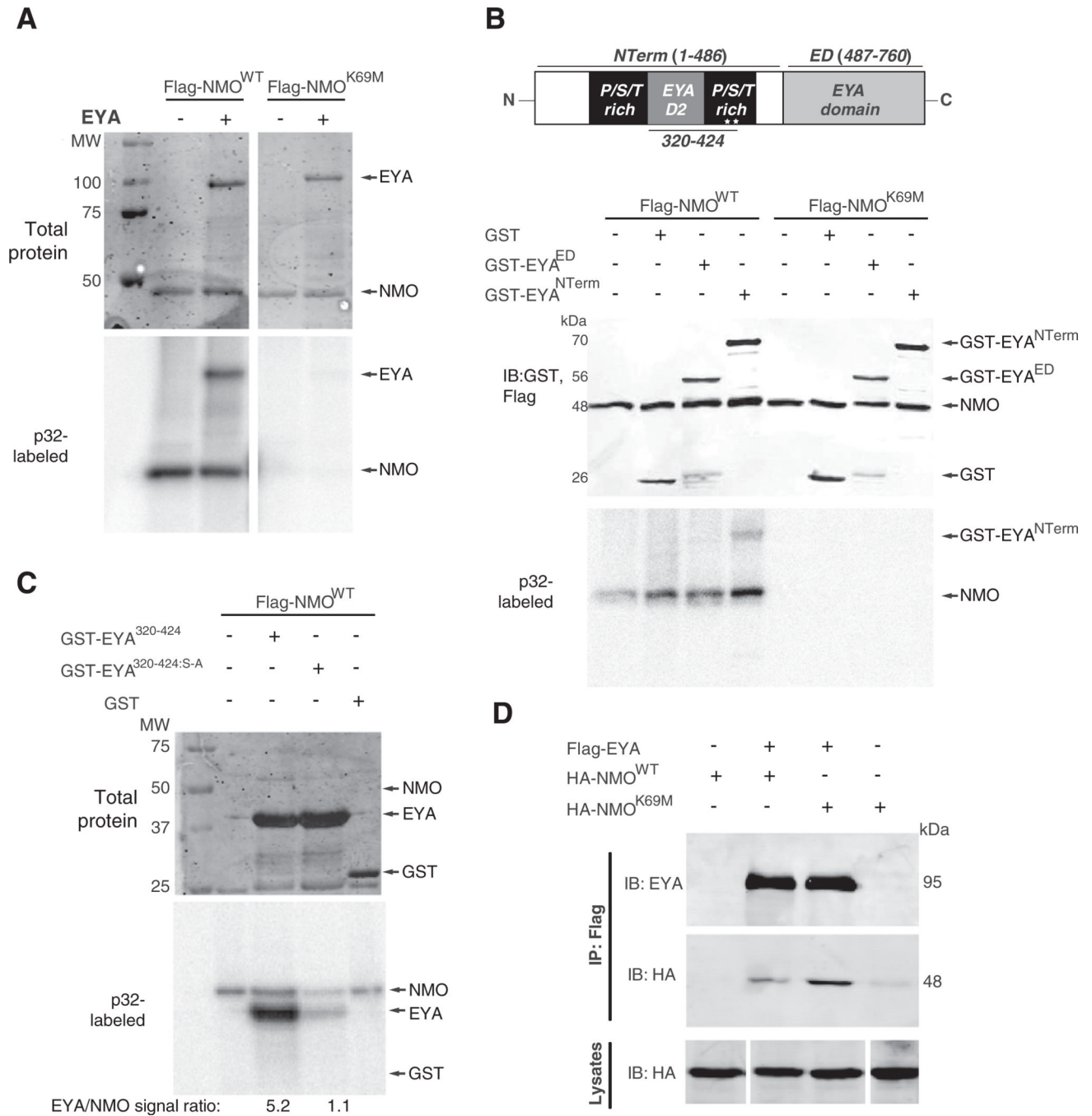


**Fig. 3.** Nemo potentiates Eya-mediated induction of Dac expression. (A–D) Dac (red), Eya (blue) and Nmo-GFP (green) expression in third-instar larval wing disks. In this and all other figures, wing disks are oriented dorsal up, anterior left. Eya and Nmo transgenes were driven using Dpp-GAL4. (A) Endogenous Dac expression. (B) Eya induces strong ectopic Dac expression in the dorsal hinge region (B'', arrow), and weaker expression in the wing disk pouch (B'', arrowhead). (C) Coexpression of Nmo increases Dac levels, particularly in the pouch and ventral region (C'', arrowheads). (D) Coexpression of kinase-inactive Nmo (Nmo<sup>K69M</sup>) decreases Eya-mediated Dac expression in the wing disk pouch (D'', arrowhead, compare to B'') but not in the hinge region (D'', arrow).



**Fig. 4.** Eya-mediated activation of Dac expression requires So. Dac (red), Eya (blue) and Nmo-GFP (green) expression in *so*<sup>1</sup> third-instar larval wing disks expressing (A) Eya alone or (B) Eya +Nemo under Dpp-Gal4 control. No ectopic Dac expression is observed.

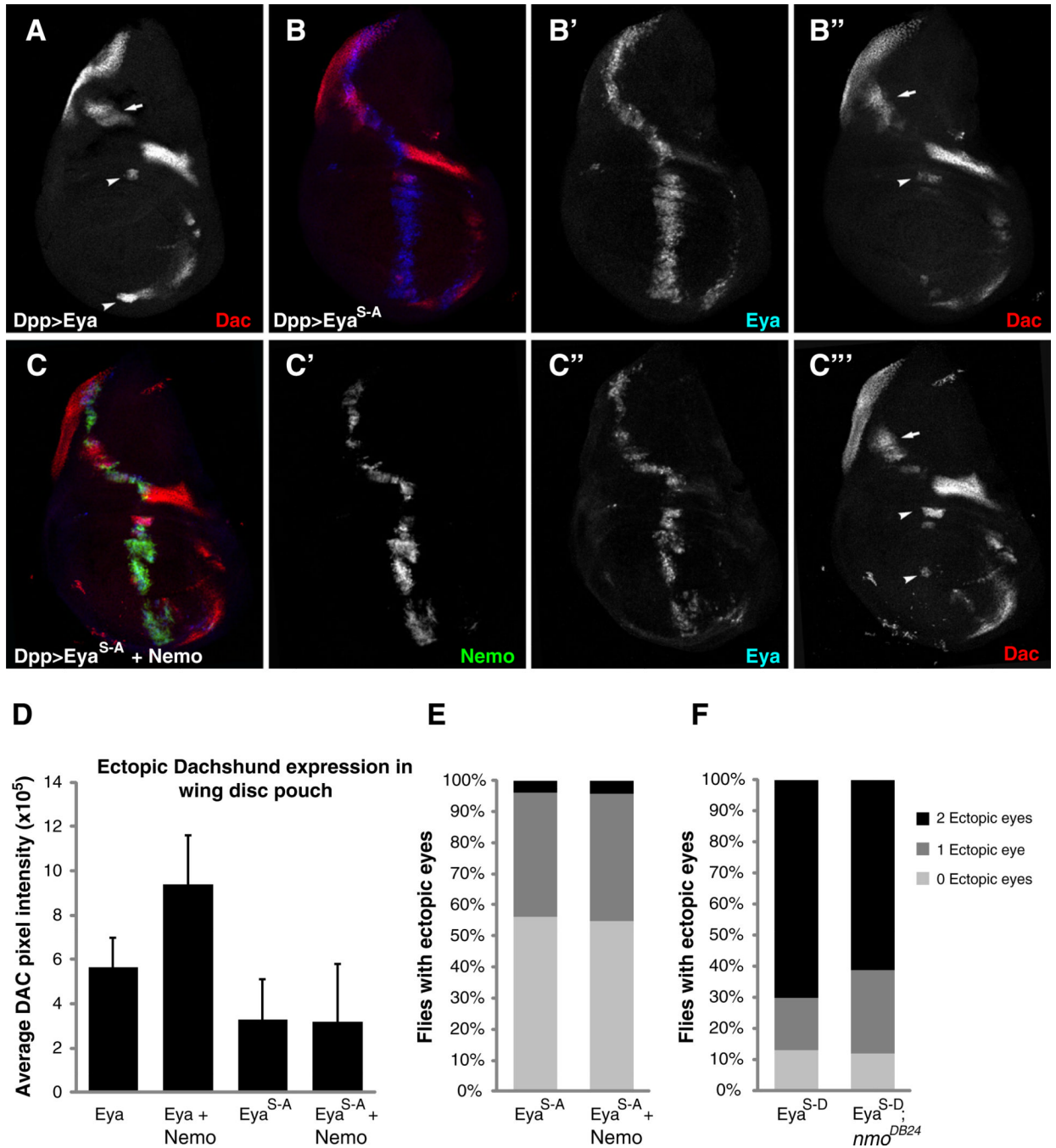




**Fig. 5.** Nemo phosphorylates Eya at consensus MAPK sites. (A–C). In vitro kinase assays using Flag-Nmo immunopurified from transfected HEK293T cells (see Methods for details). The upper panels show Coomassie staining for total protein, and the bottom panels show the phosphorimager exposure. (A) Full-length Eya protein is directly phosphorylated when incubated with wild type, but not the kinase inactive form of Nmo (K69M). Nmo autocatalytic activity is evident with the wild type, but not kinase dead form. (B) Schematic of the Eya protein, with the two MAPK phosphorylation consensus sites within the N-terminal P/S/T region of Eya represented by asterisks. In vitro kinase assays show that Nmo phosphorylates the N-terminal region of Eya, but not the ED. Immunoblot (IB) shows inputs



for Nmo, Eya and GST. (C) In vitro kinase assays using a subfragment of the P/S/T-rich region of Eya (GST-EYA<sup>320-424</sup>). Mutating the phosphoacceptor serines in the MAPK sites significantly reduces Nmo-mediated phosphorylation. (D) Immunoblots (IB) showing HA-Nmo and Flag-Eya can be co-immunoprecipitated (IP) from transfected S2 cells. The lysates (bottom panel) were run together on the same gel, but out of order with respect to the IP gels, necessitating some cutting and splicing to align the lanes.



**Fig. 6.** MAPK phosphorylation consensus sites are required for Nemo activation of Eya-So. (A–C) Dac (red), Eya (blue) and Nmo-GFP (green) expressions in third-instar larval wing discs. (A) Eya expression along the Dpp stripe causes ectopic Dac expression in the dorsal hinge region (arrow) and in the wing pouch (arrowhead). (B) Mutating the MAPK phosphorylation consensus sites (Eya<sup>S-A</sup>) does not alter Eya-mediated induction of Dac. (C) Nmo does not synergize with Eya<sup>S-A</sup>, with only a very slight increase in ectopic Dac expression observed in some disks (C''', arrowheads) as compared to the robust increase seen when Nmo is coexpressed with a wild type Eya transgene (Fig. 3C''', arrowheads). (D) Quantification of ectopic DAC along the wing disc pouch confirms the lack of significant interaction of Nmo

with Eya<sup>S-A</sup>, n = 16 disks. (E) Coexpression of Nmo does not alter the frequency of DppGal>UAS-Eya<sup>S-A</sup> mediated ectopic eye induction. (E) A phospho-mimetic form of Eya, Eya<sup>S-D</sup>, is refractory to loss of one copy of *nmo* in ectopic eye induction assays.