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Exon Junction Complex Subunits Are Required to Splice *Drosophila MAP Kinase*, a Large Heterochromatic Gene

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SUMMARY

The exon junction complex (EJC) is assembled on spliced mRNAs upstream of exon-exon junctions and can regulate their subsequent translation, localization, or degradation. We isolated mutations in *Drosophila mago nashi* (*mago*), which encodes a core EJC subunit, based on their unexpectedly specific effects on photoreceptor differentiation. Loss of Mago prevents epidermal growth factor receptor signaling, due to a large reduction in *MAPK* mRNA levels. *MAPK* expression also requires the EJC subunits Y14 and eIF4AIII and EJC-associated splicing factors. Mago depletion does not affect the transcription or stability of *MAPK* mRNA but alters its splicing pattern. *MAPK* expression from an exogenous promoter requires Mago only when the template includes introns. *MAPK* is the primary functional target of *mago* in eye development; in cultured cells, Mago knockdown disproportionately affects other large genes located in heterochromatin. These data support a nuclear role for EJC components in splicing a specific subset of introns.

INTRODUCTION

The exon junction complex (EJC) plays an important role in coupling nuclear and cytoplasmic events in gene expression; its recruitment allows nuclear pre-mRNA splicing to influence the subsequent fate of the spliced mRNAs (Tange et al., 2004). The EJC is assembled onto mRNAs during splicing, 20–24 bases upstream of each exon junction (Gehring et al., 2009a). The DEAD box RNA helicase eIF4AIII is the first subunit to associate with pre-mRNA through interactions with the intron-binding protein IBP160 (Gehring et al., 2009a; Ideue et al., 2007). eIF4AIII then recruits Magoh (known as Mago in *Drosophila*) (Boswell et al., 1991; Kataoka et al., 2001) and Y14 (Hachet and Ephrussi, 2001; Kataoka et al., 2000; Le Hir et al., 2000; Mohr et al., 2001), which stabilize eIF4AIII binding by inhibiting its ATPase activity (Andersen et al., 2006; Ballut et al., 2005; Bono et al., 2006). These three subunits constitute the pre-EJC; the fourth core subunit, MLN51 (Barentsz [Btz] in *Drosophila*) (Degot et al., 2004; van Eeden et al., 2001), is added after export of spliced mRNA to the cytoplasm (Gehring et al., 2009a; Herold et al., 2009). Many accessory proteins transiently interact with this core complex and modulate its function (Tange et al., 2004). The EJC remains bound to cytoplasmic mRNA until it is displaced by the ribosome-associated disassembly factor Pym during the first round of translation (Dostie and Dreyfuss, 2002; Gehring et al., 2009b).

The EJC has been shown to regulate posttranscriptional events that include mRNA localization, translation, and degradation. In vertebrate cells, the presence of the EJC on spliced mRNAs increases their translational yield (Nott et al., 2004; Wiegand et al., 2003), in part by recruiting S6 kinase 1 (Ma et al., 2008). The EJC is best known for its role in nonsense-mediated decay (NMD), a surveillance mechanism that degrades mRNAs containing premature termination codons (PTCs) (Chang et al., 2007). In mammals, NMD is greatly enhanced by the presence of a spliceable intron downstream of a PTC and is mediated by the EJC and accessory factors that include three up-frameshift (UPF) proteins (Cheng et al., 1994; Stalder and Mühlemann, 2008; Thermann et al., 1998). However, NMD can occur independently of splicing or the EJC in lower organisms such as *Drosophila* (Gatfield et al., 2003). In *Drosophila*, the EJC has a role in mRNA localization; all four core EJC components are required to localize *oskar* mRNA to the posterior pole of the oocyte (Hachet and Ephrussi, 2001; Mohr et al., 2001; Newmark and Boswell, 1994; Palacios et al., 2004; van Eeden et al., 2001).

We isolated mutant alleles of *mago* based on their specific defects in epidermal growth factor receptor (EGFR)-dependent processes in eye development. Phosphorylation of mitogen-activated protein kinase (MAPK) is a critical step in signal transduction downstream of the EGFR and other receptor tyrosine kinases (Katz et al., 2007). Loss of *mago* strongly reduces the total level of the mRNA encoding Rolled (Rl), the *Drosophila* extracellular signal-regulated kinase (ERK)-related MAPK. Y14 and eIF4AIII, the other two subunits of the pre-EJC, also positively regulate *MAPK* transcript levels, but Btz does not. An intronless *MAPK* cDNA is independent of *mago* and can rescue photoreceptor differentiation in *mago* mutant clones; inclusion of the introns renders it Mago dependent. Mago does not affect *MAPK* transcription or mRNA stability but

alters its splicing pattern. *MAPK* is a large gene located in heterochromatin; a genome-wide survey of Mago-regulated genes found that genes that shared these features were overrepresented. Based on these observations, we propose that the pre-EJC is essential to splice a specific set of transcripts that includes the critical signal transduction component *MAPK*.

RESULTS

mago Is Required for EGFR Signaling in Eye and Wing Development

EGFR signaling plays a critical role in *Drosophila* eye development. Differentiation of regularly spaced clusters, each containing eight photoreceptor cells, progresses from posterior to anterior across the third instar larval eye imaginal disc, led by an indentation known as the morphogenetic furrow (MF). R8, the first photoreceptor to form in each developing cluster, induces EGFR activation in surrounding cells to promote their differentiation into R1–R7 photoreceptors (Figure 1A) (Roignant and Treisman, 2009). In a genetic screen for mutations affecting photoreceptor differentiation (Janody et al., 2004), we isolated three alleles of *mago nashi* (*mago*) (Figure 1B). In large clones of *mago* mutant cells in the eye disc, R8 differentiation, visualized using the marker Senseless (Sens), initiated correctly immediately posterior to the MF; however, few other photoreceptors were recruited (Figures 1E and 1F). This phenotype resembles those reported for mutations in components of the EGFR pathway (Halfar et al., 2001; Yang and Baker, 2003).

Loss of EGFR signaling also leads to apoptosis in the eye disc (Halfar et al., 2001; Yang and Baker, 2003). *mago* mutant clones strongly accumulated activated caspases, indicative of apoptosis (Figures 1I and 1J). To test whether the lack of photoreceptor differentiation in *mago* mutant clones was simply a consequence of cell death, we blocked cell death in the eye disc by expressing the anti-apoptotic peptide p35 (Hay et al., 1994). This rescued the loss of R8 cells but did not restore their ability to recruit additional photoreceptors (Figures 1G and 1H). Like known components of the EGFR pathway, *mago* thus independently controls both photoreceptor differentiation and cell survival. A third function of EGFR signaling in the eye disc is to arrest differentiating photoreceptors in the G1 phase of the cell cycle. In the absence of EGFR signaling, re-entry of these cells into the cell cycle can be visualized by increased expression of Cyclin B, a marker of S and G2 phases (Yang and Baker, 2003). *mago* mutant clones accumulated Cyclin B in extra cells (Figures 1K and 1L), indicating a failure of G1 arrest.

To further confirm a requirement for *mago* in EGFR signaling, we examined the expression of EGFR target genes. Expression of the transcription factor Pointed P1 (PntP1) is induced by EGFR signaling as photoreceptors initiate their differentiation just posterior to the MF; in *mago* mutant clones, PntP1 expression was lost (Figures 1M and 1N). During wing development, EGFR signaling activates expression of the target gene *argos* in the wing vein primordia (Figures 1O and 1P) (Golembo et al., 1996). *argos* expression was strongly reduced in *mago* mutant cells in the wing disc (Figures 1Q and 1R). The requirement for *mago* for EGFR signaling in both eye and wing development suggests that it has a general function in this pathway. Its effect on the EGFR pathway appears quite specific because the normal pattern of R8 differentiation would be incompatible with a role for *mago* in signaling by Hedgehog, Notch, or Wingless in the developing eye (Roignant and Treisman, 2009).

Mago Acts Downstream of Raf and Upstream of MAPK Activation

To determine the point at which *mago* acts in the EGFR signaling pathway, we performed epistasis experiments in the eye disc. Spitz (Spi) is the primary ligand that induces EGFR activation in R1–R7; activated EGFR feeds into the Ras/MAPK pathway common to other receptor tyrosine kinases (Figure 2M). The GTP-bound form of Ras activates the protein kinase Raf, initiating a kinase cascade in which Raf phosphorylates. Downstream of Raf1 (Dsor1 or MEK), which in turn phosphorylates MAPK. Phosphorylated MAPK enters the nucleus and phosphorylates specific transcription factors to regulate target gene expression. We expressed constitutively active forms of these components of the pathway specifically within *mago* mutant clones. Constitutively secreted Spi (Schweitzer et al., 1995), activated EGFR (Queenan et al., 1997), activated Ras (Karim and Rubin, 1998), and activated Raf (Martín-Blanco et al., 1999) all failed to induce photoreceptor differentiation in *mago* mutant clones (Figures 2A–2F, 2I, and 2J), although each induced ectopic photoreceptors when expressed in wild-type cells (Figures 2G and 2H) (Miura et al., 2006; Roignant et al., 2006). However, an activated form of MAPK, Rolled^{SEM} (Ciapponi et al., 2001), fully rescued the lack of photoreceptors in *mago* mutant cells (Figures 2K and 2L). Similar epistasis experiments in the wing disc, using *argos-lacZ* to monitor pathway activation, likewise showed that only activated MAPK could induce *argos* expression in *mago* mutant cells (Figure S1 available online). The activity of *mago* is thus required downstream of Raf activation but upstream of MAPK activation.

Mago Is Required to Maintain MAPK Levels Sufficient for Signaling

Because *mago* encodes a subunit of the EJC, we reasoned that it might control the expression of a component of the EGFR pathway. Indeed, we found that the levels of MAPK protein were strongly reduced in *mago* mutant clones in both the eye and wing discs (Figures 3A–3D). To determine the extent of the reduction, we compared protein extracts from wild-type eye discs expressing GFP in all cells and from eye discs containing large *mago* mutant clones marked by the absence of GFP. Quantification of MAPK on western blots relative to GFP and Tubulin, to correct for the proportion of wild-type cells, showed that *mago* mutant cells expressed MAPK at only 16% of the wild-type level (Figure 3E).

We next examined whether these results could be generalized to cultured *Drosophila* S2 and S2R+ cells, in which *mago* is expressed and can be knocked down by RNA interference (RNAi) (Figures 3G and 3H). Mago depletion in these cells resulted in a 75% reduction in MAPK protein levels in comparison to Tubulin, visible both on western blots and in immunohistochemical stainings (Figures 3F–3H). This effect was specific because MEK levels were not significantly reduced (Figure 3G). As expected, the loss of MAPK protein strongly