**smoothened** and **thickveins** regulate Moleskin/Importin 7-mediated MAP kinase signaling in the developing *Drosophila* eye

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The *Drosophila* Mitogen Activated Protein Kinase (MAPK) Rolled is a key regulator of developmental signaling, relaying information from the cytoplasm into the nucleus. Cytoplasmic MEK phosphorylates MAPK (pMAPK), which then dimerizes and translocates to the nucleus where it regulates transcription factors. In cell culture, MAPK nuclear translocation directly follows phosphorylation, but in developing tissues pMAPK can be held in the cytoplasm for extended periods (hours). Here, we show that Moleskin antigen (*Drosophila* Importin 7/Msk), a MAPK transport factor, is sequestered apically at a time when lateral inhibition is required for patterning in the developing eye. We suggest that this apical restriction of Msk limits MAPK nuclear translocation and blocks Ras pathway nuclear signaling. Ectopic expression of Msk overcomes this block and disrupts patterning. Additionally, the MAPK cytoplasmic hold is genetically dependent on the presence of Decapentaplegic (Dpp) and Hedgehog receptors.

**KEY WORDS:** *Drosophila*, *moleskin*, Importin 7, Morphogenetic furrow, MAP kinase, Cell cycle, Nuclear translocation, ERK, Hedgehog, Dpp, Egfr

**INTRODUCTION**

The *Drosophila* compound eye contains about 750 facets (ommatidia), each with eight photoreceptor neurons (in a trapezoidal pattern) and 12 accessory cells (Ready et al., 1976; Frankfort and Mardon, 2002). An equator divides the eye so that the ommatidial pattern below the equator is the mirror image of that above (Ready et al., 1976). The ommatidial cells are recruited in a specific sequence by inductive and inhibitive signals via the Hedgehog, Decapentaplegic (Dpp), Notch and Epidermal growth factor receptor (Egfr) pathways (Voas and Rebay, 2004).

Patterning begins in the presumptive eye epithelium with the initiation and anterior–wards progression of the morphogenetic furrow (Ready et al., 1976; Frankfort and Mardon, 2002). The furrow is characterized by apical constriction, initial cell-type specification and patterning, as well as by cell-cycle arrest in the G1 phase (Tomlinson, 1988). Progression of the furrow requires Hedgehog signaling, which induces *dpp* expression in the furrow where the two pathways may be partially redundant (Greenwood and Struhl, 1999; Frankfort and Mardon, 2002). Additionally, G1 cell-cycle arrest in the furrow requires Dpp signaling (Penton et al., 1997; Vrailas and Moses, 2006). For simplicity, we have divided the developing eye into three stages (Fig. 1). In phase 0 (anterior to the furrow), cells are not patterned and are randomly proliferating, which requires low-level Ras pathway activity (Xu and Rubin, 1993; Halfar et al., 2001). Ectopic, high-level Ras pathway activation in phase 0 causes all cells to differentiate as photoreceptor neurons (Dominguez et al., 1998).

In phase 1 (the furrow), differentiation begins. A column of precisely spaced ommatidial founder cells (the future R8 photoreceptors) is specified every two hours (Ready et al., 1976; Basler and Hafen, 1989; Frankfort and Mardon, 2002). Founder cell specification requires the progressive restriction of the proneural transcription factor Atonal (Fig. 1) (Jarman et al., 1994; Frankfort and Mardon, 2002). Atonal is expressed in the nuclei of all cells in the furrow and is then restricted to a small cluster of cells, the ‘intermediate group’, and finally to the lone future R8 photoreceptor (Jarman et al., 1994; Dokucu et al., 1996; Frankfort and Mardon, 2002). The R8/founder cells (one per cluster) then inhibit the differentiation of their neighbors through Delta/Notch-mediated lateral inhibition (Frankfort and Mardon, 2002). pMAPK is expressed in the Atonal-positive intermediate groups, although the function of this Ras signaling is unclear (further discussed below).

In phase 2 (posterior to the furrow), the R8/founder cells reverse their inhibitory behavior and induce the recruitment of their neighbors through Egfr/Ras pathway signaling (Freeman, 1994; Tio et al., 1994; Freeman, 1996; Tio and Moses, 1997; Freeman, 2002). The first five cells recruited remain in G1 cell-cycle arrest, while the surrounding cells re-enter the cell cycle and go through a ‘second mitotic wave’, which also requires Egfr/Ras signaling (Wolff and Ready, 1991; Firth and Baker, 2003). This provides the pool of cells from which the remaining cell types will be recruited (Ready et al., 1976; Tomlinson, 1988). Later, the ommatidia become asymmetric and rotate to form the equator (Ready et al., 1976; Tomlinson, 1988). Although ommatidial chirality depends on Frizzled and Delta/Notch signaling (Mlodzik, 2002), the rotation itself is regulated, in part, by Egfr/Ras signaling (Brown and Freeman, 2003; Gaengel and Mlodzik, 2003; Strutt and Strutt, 2003).

Thus, Egfr signaling is required for proliferation and cell survival in phases 0 and 2, as well as for the reiterative induction of cell types and ommatidial rotation in phase 2. It has also been suggested that Egfr may have a crucial function in phase 1: in the initial specification of the Atonal-positive intermediate groups and/or the patterning of the R8/founder cells. Evidence for this is that Egfr-driven pMAPK is...
strongly expressed in the Atonal-positive intermediate groups (Gabay et al., 1997; Kumar et al., 1998; Spencer et al., 1998), and that the Egrf gain-of-function mutation Ellipse (EgrfEll) has reduced numbers of Atonal-positive R8/founder cells (Baker and Rubin, 1989; Baker and Rubin, 1992; Zak and Shilo, 1992).

However, we found that although a conditional-null Egrf mutation (EgrfHtl) abolishes pMAPK in the intermediate groups, it does not affect Atonal patterning (Kumar et al., 1998; Rodrigues et al., 2005). We also observed that the high-level pMAPK antigen in the intermediate groups is predominantly cytoplasmic, and proposed that there is a block to pMAPK nuclear translocation (a ‘MAPK cytoplasmic hold’) (Kumar et al., 1998; Kumar et al., 2003). Furthermore, driving MAPK into cell nuclei in phase 1 using an SV40 nuclear localization signal (NLS) reduces Atonal expression and induces precocious neural differentiation (Kumar et al., 2003). We have suggested that some nuclear activity (phosphatase or specific protease) might rapidly eliminate nuclear pMAPK, explaining why there is little detectable pMAPK in phases 0 or 2, when the pathway is known to function.

In cultured vertebrate cells, MAPK is phosphorylated by and released from MEK, it undergoes a conformational change, dimerizes and rapidly translocates to the nucleus (Cobb and Goldsmith, 2000). The dimeric form of pMAPK is thought to be too large to enter the nucleus passively, requiring Ran-mediated active transport (Cobb and Goldsmith, 2000) or a carrier-free mechanism (Whitehurst et al., 2002). However, some examples of cytoplasmic pMAPK have been reported in vertebrates (Smith et al., 2004; Ebsiuya et al., 2005). In mouse embryos, cells that receive Fgf signals have high levels of cytoplasmic pMAPK (Corson et al., 2003), and vertebrate cytoplasmic anchors for pMAPK have been suggested: Pea15 and Sef (Formstecher et al., 2001; Torii et al., 2004). However, we have been unable to identify Drosophila Pea15 or Sef homologs by BLAST analysis. Another possible mediator of MAPK cytoplasmic hold in Drosophila may be the Drosophila homolog of vertebrate importin 7, Moleskin (Msk), which has been found to bind pMAPK and is involved in its nuclear transport, with reported roles in embryonic and wing development (Lorenzen et al., 2001; Baker et al., 2002; Marenda et al., 2006).

Here, we report that Msks is required for cell proliferation and survival in phase 0, as well as for correct ommatidial rotation in phase 2. However, in phase 1, Msk protein is predominantly apical, which may serve to limit pMAPK nuclear translocation and block pMAPK signaling. Ectopic expression of Msks in phase 1 overcomes the effects of MAPK cytoplasmic hold, disrupting Atonal expression. Additionally, the Hedgehog and Dpp receptor genes smoothened (smo) and thickveins (tkv) are required for maintaining MAPK cytoplasmic hold and are genetically upstream of msk.

MATERIALS AND METHODS

Histology and microscopy

Eye discs were prepared as described by Tomlinson and Ready (Tomlinson and Ready, 1987), with modifications as described previously (Tio and Moses, 1997). For all except Msks stains, discs were mounted in Vectashield (Vector Laboratories, H-1000) and imaged by confocal microscopy. For Msks stains, peripodial membranes were removed and the antigen visualized with DAB and Ni/Co intensification, followed by dehydration and mounting in DPP (Zeiss) and bright-field microscopy. Adult eye SEM, sections and pupal eye preparations were performed as previously described (Ready et al., 1976; Cagan and Ready, 1989; Tio and Moses, 1997).

Antisera

Primary antisera: rabbit anti-Msk (1:1,000) (Lorenzen et al., 2001), mouse anti-pMAPK (1:625, Sigma) (Gabay et al., 1997), rabbit anti-Atonal (1:1,000) (Jarman et al., 1993), mouse anti-BrU (1:50, BD Biosciences), mouse anti-cyclin E (1:5, gift of B. Edgar) (Richardson et al., 1995), rabbit activated Caspase-3 (1:200, BD Biosciences) (Sriniwasan et al., 1998), rabbit anti-β-galactosidase (1:1,000, Cortex Biochem), rat anti-Evl for neurons (1:500, Developmental Studies Hybridoma Bank (DSHB)) (Robinow and White, 1988; O’Neill et al., 1994), mouse anti-Birch1 for R1 and R6 (1:10, gift of K. Saigo) (Higashijima et al., 1992), rabbit anti-SalM for R3 and R4 (1:10, gift of R. Schuh) (Kuhnlein et al., 1994; Molleraa et al., 2001), mouse anti-Cut for cone cells (1:10, DSHB) (Blohinger et al., 1990; Blohinger et al., 1993), guinea-pig anti-Senseless for R8 cells (1:1000, gift of G. Mardon) (Nolo et al., 2000; Frankfort et al., 2001), mouse anti-Pros for R7 (1:100, DSHB) (Campbell et al., 1994; Kauffmann et al., 1996), mouse anti-Cyclin A (1:10, DSHB, a gift of I. Hariharan) (Knoblich and Lehner, 1993), mouse anti-Cyclin B (1:50, DSHB, gift of I. Hariharan) (Knoblich and Lehner, 1993), mouse anti-Cyclin D (1:10, gift of K. Moberg, Emory University, Atlanta, GA) and rabbit anti-phospho-Histone H3 (1:1,000 Cell Signaling Technologies). Secondary antibodies from Jackson ImmunoReserarch were: goat anti-mouse Cy5 (1:500), goat anti-rabbit TRITC (1:250), goat anti-rat Cy5 (1:200), goat anti-rabbit HRP (1:100), and goat anti-mouse HRP (1:40). Actin was detected with Rhodamine-phalloidin (1:50, Molecular Probes).

Drosophila genotypes

Wild types: Canton-S or w1118

For ectopic expression of Msks: hs:msk and w1118 or UAS:msk, or UAS:GFP crossed to hs:GAL4 (gift of K. Moberg), or GMR:GAL4/CyO (gift of L. Zipursky, University of California, Los Angeles).

For msk null clones larval genotypes were: y w ey:FLP; P[w+mC=Ubi:GFP] P[neo FRT]80B/msk5 P[neo FRT]80B, or w hs:FLP; P[w+mC=Ubi:GFP] P[neo FRT]80B/msk5 P[neo FRT]80B, or y w ey:FLP; hs:MG UAS:lacZ/+; P[w+mC=Ubi:GFP] P[neo FRT]80B/msk5 P[neo FRT]80B, or y w ey:FLP; P[w+mC=Ubi:GFP] P[neo FRT]80B/msk5 P[neo FRT]80B, or y w ey:FLP; P[w+mC=Ubi:GFP] P[neo FRT]80B/msk5 P[neo FRT]80B, or y w ey:FLP; P[w+mC=Ubi:GFP] P[neo FRT]80B/msk5 P[neo FRT]80B, or y w ey:FLP; P[w+mC=Ubi:GFP] P[neo FRT]80B/msk5 P[neo FRT]80B, or y w ey:FLP; P[lacZ]+/H9252 P[neo FRT]80B/msk5 P[neo FRT]80B, or y w ey:FLP; P[lacZ]+/H9252 P[neo FRT]80B/msk5 P[neo FRT]80B, or y w ey:FLP; P[lacZ]+/H9252 P[neo FRT]80B/msk5 P[neo FRT]80B.

For smo, tkv and smo tkv clones: y w ey:FLP; P[w+mC=Ubi:GFP] P[neo FRT]40A/mso5 P[neo FRT]40A, or tkv8 P[neo FRT]40A, or smo8 tkv8 P[neo FRT]40A.

For smo tkv; msk clones: w y ey:FLP; P[w+mC=Ubi:GFP] P[neo FRT]40A/mso5 P[neo FRT]40A, or tkv8 P[neo FRT]40A, or smo8 tkv8 P[neo FRT]40A.

Temperature-shift regimes

For Msks expression, hs:msk or hs:GAL4:UAS:msk and controls were raised at 25°C and placed at 37°C for one hour. Eye discs were dissected immediately, after one hour, or after two hours recovery at 25°C. To induce hs:Flip clones, flies were raised at 25°C, placed at 37°C for one hour at the times indicated in the text, and then returned to 25°C until dissection in the late third instar.
DNA constructs
pP(Hsp70-CaSpeR)NK was derived from pP(Hsp70-CaSpeR) (Bell et al., 1991) by replacing the Sall, XbaI, SpeI, BamHI, Smal and PstI sites with KpnI, HpaI, EagI and NotI sites (in that order). A 3,253 bp KpnI to NotI msk-containing fragment [1049 residues of msk with a nine residue N-terminal MYC epitope tag added and two intervening residues (‘AS’)] was then inserted between the KpnI and NotI sites to yield pP(Hsp70-Msk/CaSpeR) or ‘hs:msk’, which was injected into w1118 embryos as described previously (Rubin and Spradling, 1982), at a 1:1 ratio (500 μg/ml each), with a helper plasmid driving the expression of the S129A enhanced P-transposase (Beall et al., 2002). Multiple independent lines were obtained and two viable independent insertions were retained on each major chromosome.

RESULTS
Msk is normally required for cell proliferation and survival in the developing eye in phase 0
To study the requirements for msk in eye development, we used hs:Flp to induce msk null (msk-5) clones 48, 72 and 96 hours before dissection at late third instar. When clones were induced 48 hours before dissection, we saw many, small homozygous msk null clones and homozygous wild-type twin spots (Fig. 2A), both anterior and posterior to the furrow. However, when clones were induced 72 hours before dissection there were fewer clones, and the twin spots were much larger than the msk null clones (Fig. 2B). This suggests that the msk null cells may have a growth disadvantage, or that the msk cells die, between 48 and 72 hours, indicating that Msk protein may perdure for at least 48 hours. Interestingly, at 72 hours after clone induction (Fig. 2B), msk clones survived posterior to the furrow in postmitotic territories, but there were no msk clones (only twin spots) anterior to the furrow. Additionally, clone induction 96 hours before dissection reveals even fewer but larger twin spots, and no msk clones (Fig. 2C). Thus, msk mutant cells are lost from proliferative domains. Loss of msk cells may be a result of cell competition between the msk cells and the surrounding wild-type tissue, or msk may be autonomously required for the survival of proliferating cells, much like ras, which also has a small clone phenotype (Baker and Rubin, 1992; Xu and Rubin, 1993; Halfar et al., 2001).

One possible reason that msk clones are much smaller than their wild-type counterparts might be that msk cells divide more slowly, which would suggest that msk affects the cell cycle. We, therefore, used ey:Flp to induce msk null clones, which where then examined for cell-cycle marker expression in phase 2: BrdU for S-phase (Fig. 2D-F), Cyclin E for late G1 (Fig. 2G-I), Cyclin D for G1 arrest, phospho-Histone H3 for mitosis and Cyclin A and B for G2 (data not shown). The absence of msk does not eliminate the expression of any of these markers, suggesting that there is no simple cell-cycle, stage-specific block.

Alternatively, the small clone size could be due to cell death. Larval msk clones do not stain for activated Caspase-3, a marker for apoptosis (data not shown). However, it may be that clones in phase 0 are dying by other means, such as necrosis, or that the dying cells are being cleared too rapidly to be detected. To test for this, we

Fig. 2. Cell proliferation and survival requires msk in the developing eye. (A-L) Third-instar (A-I) and 60-hour pupal (J-L) eye-imaginal discs containing clones of msk null cells, revealed by negative marking using GFP (D,G,J); outlined in (D-I). Anterior is to the right; A-C and D-L are the same scale as indicated in A and D, respectively. In E,H,K, antigens are indicated on the left; F,I and L are merged images showing GFP (green) and antigens (red); A-C shows a time course following the hsp70-driven induction of clones. Brightly labeled cells are homozygous wild-type ‘twin-spots’ (white filled arrows), gray cells are heterozygous and black cells are homozygous msk nulls (black filled arrows). Note, late clones (48 hours before dissection in A) are numerous, small, equally distributed on both sides of the furrow, and are accompanied by twin spots of roughly equal size; earlier clones (72 hours before dissection in B) are rare, smaller than their twin spots, and are present only posterior to the furrow. Note also that very early clones (96 hours before dissection in C) are absent, with only rare, large twin spots remaining. Cell-cycle markers (BrdU for S-phase (D-F) and Cyclin E for G1 (G-I) are not eliminated by msk loss of function (arrows). Note, msk null cells posterior to the furrow eventually die during pupal life, as revealed by activated Caspase 3 antigen (arrows in J-L).
overexpressed p35 to inhibit apoptosis, but this failed to rescue clone size; this was also the case for Egfr loss-of-function mutants (Yu et al., 2002). msk null cells that survive into phase 2 eventually die in the pupal stage, a time when we can detect activated Caspase 3 (Fig. 2J-L), and adult eyes have small scars in the place of msk null clones (data not shown). Taken together, these experiments suggest that msk is required for cell proliferation in the larval stage, and later for the survival of postmitotic cells.

Msk is not required for cell-type specification but is required for normal ommatidial rotation in phase 2

To determine whether loss of msk affects patterning in phase 2, we used ey:Flp to induce msk null clones and found that msk cells are able to differentiate as neurons (Elav, Fig. 3A-C), and can be specified as photoreceptor cell-types, including the R8 (Senseless, Fig. 3D-F), R3 and R4 (Spalt Major, Fig. 3G-I), and R7 (Prospero, data not shown). Additionally, they can differentiate as accessory cone cells (Cut, Fig. 3J-L).

In addition to cell-type specification, ommatidial chirality and rotation are established in phase 2. As the five-cell preclusters form, they face the same direction and have a single axis of symmetry. The preclusters then rotate 90° away from the anteroposterior axis of the eye disc with the dorsal and ventral halves rotating in opposite directions. Disruption of the direction of rotation can then be detected by the expression of BarH1, which is normally expressed in R1 and R6 (Higashijima et al., 1992; Lim and Choi, 2004). As the ommatidia clusters rotate, they lose their symmetry so that R4 is positioned posterior to R3 (Tomlinson, 1988). This repositioning of R3 and R4 can be detected with E(Spl)M-delta-0.5:lacZ, which is expressed only in the R4 (Cooper and Bray, 1999). The R3/R4 photoreceptor pair play a crucial role in the establishment of ommatidial polarity as their loss and/or misspecification disrupts the chirality of the cluster and leads to randomization of the direction and degree of rotation (Gubb, 1993; Theisen et al., 1994; Zheng et al., 1995; Fanto et al., 1998). Although chirality and rotation are linked, they are genetically separable (Rawls et al., 2002; Yang et al., 2002), and some mutations in elements of the Ras pathway affect rotation and not chirality (e.g. specific alleles of argos, spitz, pointed, nemo and Star) (Choi and Benzer, 1994; Brown and Freeman, 2003; Gaengel and Mlodzik, 2003; Strutt and Strutt, 2003).

In msk null clones, some ommatidia no longer rotate reliably (see Fig. 3M-O). As chirality and rotation can be disrupted independently of each other, we then visualized R4 fate. If the ommatidia have rotated incorrectly but chirality has remained intact, R4 would be misplaced within the cluster. In cases of miss-rotated ommatidia, we find that the R4 cell is misplaced, although in some cases two R4s are specified (Fig. 3P-R). Thus msk, like other Ras pathway elements, is required for ommatidial rotation and not chirality.

These data show that some important Egfr/Ras pathway functions are not affected in msk null clones in phase 2 (cell cycle and cell-type specification), whereas other functions are (ommatidial rotation). This suggests that in phase 2, a second pMAPK nuclear transport factor or pathway may exist, making Msk redundant at some steps. Another possibility is that enough pMAPK can translocate into the nucleus in the absence of Msk to perform some functions, but that levels are not sufficient for others. Third, some functions of pMAPK may be via cytoplasmic targets, which do not require nuclear translocation, as is known in the developing wing (Marenda et al., 2006).
Long-term ectopic expression of Msk disrupts late eye development

To study the long-term effect of the continuous ectopic expression of Msk, we used UAS:msk (Lorenzen et al., 2001) driven by GMR:Gal4 (Moses and Rubin, 1991; Hay et al., 1997) to give elevated Msk expression in phase 2 and thereafter. A limitation of this approach is that we could not observe effects on phases 0 and 1, but the advantage is that the flies survive and we can observe the effects of ectopic Msk on later stages of eye development.

Gal4 activity is cold sensitive, and we used this to control the levels of Msk expression. Flies carrying GMR:Gal4 alone or GMR:Gal4::UAS:GFP, cultured at 18°C, have full-sized and fully pigmented eyes (Fig. 4A,D; data not shown), whereas adding UAS:msk causes a roughening of the eye and a loss of some red pigment (Fig. 4B,E); this is accentuated at 25°C (Fig. 4C,F), over the effect of GMR:Gal4 alone (data not shown) (see also Kramer and Staveley, 2003). Sections of these eyes show that GMR:Gal4 control flies cultured at 18°C have the normal complement of photoreceptor and pigment cells, as seen in the adult (Fig. 4G) and in 60-hour pupae (Fig. 4J). By contrast, the addition of UAS:msk at 18°C causes some disruption, and reduced numbers of both photoreceptors and pigment cells (Fig. 4H), and this effect occurs before the 60-hour pupal stage (Fig. 4K). Elevating the temperature to 25°C kills almost all retinal cells before the adult stage (Fig. 4I), but the cells are not yet lost in the 60-hour pupa (Fig. 4L).

Msk is apically localized in the morphogenetic furrow.

We examined Msk antigen in the developing eye by removing the peripodial membrane and using DAB staining with Ni/Co intensification (see Materials and methods) (see also Lorenzen et al., 2001). Anterior to the morphogenetic furrow (phase 0), Msk antigen is evenly distributed throughout the depth of the epithelium (Fig. 5A,B). However, at the furrow (in phase 1; Fig. 5A,B) a dramatic relocalization takes place: at an apical focal plane, Msk antigen becomes concentrated in the apical tips of the cells, whereas, at a basal focal plane, the nuclei of these cells appear to be negatively stained (Fig. 5B). Apical sequestration is first seen in all cells in the furrow, and is then retained in those cells that are allocated to the ommatidial pre-clusters and lost from the cells that surround the pre-clusters (Fig. 5A). Later, the apical localization of Msk is also lost in the ommatidial clusters.

This pattern of apical Msk is very similar to the apical cell constrictions seen with cobalt or lead sulphide staining in the G1 cell cycle-arrested cells in phase 1 (Tomlinson and Ready, 1987; Wolff and Ready, 1991). We suggest that Msk is unrestricted in phases 0 and 2, facilitating pMAPK translocation into the nucleus. However, in phase 1 (in G1 arrested cells), Msk becomes sequestered apically, preventing pMAPK nuclear translocation. In late phase 1, Msk is released in cells not allocated to the ommatidial clusters (Fig. 5A).

Ectopic Msk expressed in the furrow disrupts normal Atonal and pMAPK expression

We have proposed that the elevated levels of pMAPK antigen in the furrow may be due, in part, to cytoplasmic hold, which prevents nuclear translocation and subsequent exposure to some nuclear phosphatase or protease (Kumar et al., 2003). Additionally, nuclear-directed MAPK expressed in the furrow or overexpression of ras^V12 disrupts Atonal expression (Hayashi and Saigo, 2001; Kumar et al., 2003). Thus, if we were to break cytoplasmic hold, we might expect to reduce pMAPK antigen and Atonal expression in the furrow. If apical Msk sequestration is the normal mechanism that mediates MAPK cytoplasmic hold, then high-level ectopic expression of Msk in the furrow might titrate the Msk anchoring factor(s) and allow pMAPK to enter cell nuclei, reducing pMAPK and Atonal expression.

To explore this, we used hs:GAL4 UAS:msk flies and derived hs:msk transgenic flies to express Msk by heat induction. We observe two consequences: first, pMAPK antigen is lost from the Atonal intermediate groups (Fig. 5C-F). This effect is rapid, complete (after one hour at 37°C, Fig. 4F) and reversible (pMAPK expression recovers after an hour at 25°C, data not shown). The second effect is that Atonal antigen is dramatically reduced in early phase 1 (Fig. 5G-I, compare with 5J). As with pMAPK antigen, Atonal expression recovers after an hour at 25°C (data not shown);
however, unlike the pMAPK antigen, some Atonal antigen is retained, particularly in the late, single founder cells. It could be that this is due to a failure to express sufficient ectopic Msk, or that one hour of heat induction, even at 37°C, is insufficient. It could be that this is due to a failure to express sufficient ectopic Msk, or that one hour of heat induction, even at 37°C, is insufficient. A higher induction temperature and longer times at 37°C are lethal. Therefore, to increase the levels of Msk, we combined six independent insertions together to give 12 genomic copies of hs:msk. However, the expression of multiple copies of Msk had the same affect on Atonal expression as a single copy (data not shown). As we have probably reached saturation for ectopic Msk, and this phenocopies the expression of nuclear-directed MAPK and ectopic \textit{rasv12}, the retention of Atonal in founder cells may be refractory to ectopic Msk and perhaps to pMAPK nuclear translocation.

Because ectopic expression of both \textit{rasv12} and nuclear-directed MAPK promotes precocious neural differentiation (Hayashi and Saigo, 2001; Kumar et al., 2003), and \textit{rasv12} also results in an increase in rough expression (Hayashi and Saigo, 2001), we transiently expressed Msk by heat shock and found no effect on neural differentiation or rough expression (data not shown). Thus, it may be that local levels of pMAPK nuclear translocation are sufficient for the decrease in Atonal expression, but that higher levels of pMAPK nuclear translocation, as seen in nuclear-directed MAPK, may be required for precocious neural differentiation.

If apical sequestration inactivates Msk (at least with respect to MAPK nuclear translocation), then \textit{msk} loss-of-function should not affect pMAPK or Atonal in the furrow. To test this, we stained \textit{msk} null retinal-mosaic clones for Atonal and pMAPK (Fig. 6), and observed that both are normal.

These ectopic Msk expression data in the furrow suggest that Msk is sufficient to support pMAPK nuclear translocation. Thus, we propose that Msk may be inactivated through sequestration and is normally limiting in cells in which MAPK cytoplasmic hold is required.

**MAPK cytoplasmic hold is genetically dependent on the Hedgehog and Dpp receptors Smo and Tkv.**

As MAPK cytoplasmic hold is a local and transient phenomenon limited in the developing eye to the morphogenetic furrow, we reasoned that Msk sequestration might be developmentally regulated and mediated by some signaling receptor. We initially suspected that Notch signaling might be upstream of the MAPK cytoplasmic hold in phase 1. However, it has been shown that Notch loss-of-function results in increased and persistent Atonal expression, the opposite of the result expected if MAPK cytoplasmic hold is lost (Baker et al., 1996). We repeated this experiment and obtained the same result (data not shown). Additionally, pMAPK expression is unaffected (data not shown), thus eliminating the Notch pathway as an upstream signal for MAPK cytoplasmic hold.

Next, we examined the Hedgehog and Dpp receptors Smoothened (Smo) and Thickveins (Tkv). It has been suggested that Hedgehog and Dpp signaling act together (redundantly) in the furrow, and it is observed that, in cells lacking both Smo and Tkv, Atonal expression is lost (Greenwood and Struhl, 1999). This suggests that Hedgehog and/or Dpp signaling could be required for the MAPK cytoplasmic hold.
To test this, we used ey:Flp to derive clones mutant for null alleles of smo (smo^3) and/or tkv (tkv^3). Clones lacking smo (Fig. 7A-D) have reduced Atonal expression, with the greatest loss from the intermediate groups and some remaining expression in the lone R8/founder cells (Fig. 7B,D), as seen with hs:NM and hs:rasV12^12 (Hayashi and Saigo, 2001; Kumar et al., 2003), and hs:msk (Fig. 5J). In phase 1, smo clones also lack pMAPK (Fig. 7C,D), again similar to the effect of ectopic Msk (Fig. 5F). Clones lacking tkv retain both pMAPK and Atonal expression (Fig. 7F-H), showing that tkv is not required for the MAPK cytoplasmic hold, perhaps because Dpp signaling may be downstream and partially redundant to Hedgehog signaling in the furrow (see Greenwood and Struhl, 1999). However, clones lacking both smo and tkv are devoid of both Atonal and pMAPK (Fig. 7J-L), which is again consistent with a redundant effect of Dpp and Hedgehog signaling.

These data suggest that Msk function may be genetically dependent on smo and tkv, and that one or the other of these receptors may form part of a regulated complex that sequesters Msk in the furrow. The apparent loss of MAPK cytoplasmic hold in the smo tkv clones (as seen by the loss of Atonal and pMAPK) could then be explained as a failure to anchor Msk, as loss of smo or smo and tkv results in disruption of the apical constriction of the actin cytoskeleton in the furrow (Vrailas and Moses, 2006). If this is so, msk loss-of-function should be genetically epistatic to smo and tkv for this phenotype in the furrow.

To test this, we derived triply mutant clones lacking smo, tkv and msk. Indeed, we observe a complete genetic suppression by msk of the smo tkv phenotypes: a complete restoration of pMAPK and Atonal expression and patterning (Fig. 8). In some cases we see a synthetic gain-of-function effect anterior to the furrow (phase 0):
ectopic pMAPK, but not Atonal (Fig. 8G,H). Thus, msk is genetically downstream of the Hedgehog and Dpp receptors in the furrow. We propose that the Hedgehog and Dpp pathways may act together to inhibit Msk function and hence restrict pMAPK nuclear import.

**DISCUSSION**

Early in eye development, all cells anterior to the furrow (phase 0) are primed for Ras-induced neural differentiation; ectopic activation of the pathway causes all cells to differentiate as photoreceptors, even without atonal (Dominguez et al., 1998). Normally these cells are thought to receive only low levels of Egfr-mediated Ras signaling, supporting proliferation but not differentiation (Halfar et al., 2001). Later, in the furrow (phase 1), Delta-induced, Notch-mediated lateral inhibition progressively restricts Atonal expression to single founder cells (Baker et al., 1996). Suspension of Ras signaling is required for this inhibition in order to avoid premature neuronal differentiation, and we have proposed that this inhibition is mediated by MAPK cytoplasmic hold (Kumar et al., 1998; Kumar et al., 2003). However, this block to the Ras pathway must be released in phase 2 (posterior to the furrow) to allow for developmental induction by the R8 cell (Freeman, 1997). To better understand how MAPK cytoplasmic hold is maintained in phase 1, we examined the role of the pMAPK nuclear transport factor Drosophila Importin 7/Msk, in eye development.

We suggest that in wild-type eye discs, the level of pMAPK antigen is a very misleading reporter of Egfr/Ras pathway activity, because cytoplasmic hold in phase 1 allows even a relatively low level of pathway activity to build up high levels of pMAPK antigen. We previously developed a system to reveal MAPK nuclear translocation without the use of an antibody (MG-driven reporter gene expression) (Kumar et al., 2003). However, we have since found that under all conditions tested, MG-driven reporter expression does not reveal nuclear MAPK in phase 0, where Ras pathway activation is required. We reliably see MG-driven reporter expression in phase 2, where there is thought to be high (or sustained) levels of Ras pathway activity. In phase 1, the level of pathway signaling may be insufficient for expression, and thus MG-driven reporter expression may reveal only high (or sustained) levels of nuclear MAPK. Alternatively, this could be caused by a technical limitation: the hsp70 promoter drives the expression of only low levels of MG protein (see Kumar et al., 2003). Therefore, we have turned to two less direct assays, which together, we interpret as revealing the loss of MAPK cytoplasmic hold in the furrow: (1) loss of Atonal expression (as previously demonstrated by fusing an SV40 NLS to MAPK and by the ectopic expression of RasV12) (Hayashi and Saigo, 2001; Kumar et al., 2003); and (2) loss of pMAPK antigen, which may be due to exposure to a nuclear phosphatase/protease.

We find that the MAPK nuclear transport factor Drosophila Importin 7/Msk is apically sequestered in phase 1, the time when pMAPK nuclear access is blocked. Furthermore, we find that ectopic Msk is sufficient to break the cytoplasmic hold in the furrow, as seen by loss of pMAPK antigen and suppression of the early stages of Atonal expression. However, this transient expression of Msk is unable to promote the precocious neural differentiation or the increase in rough expression, as has been seen with hs:rasV12 or nuclear-directed MAPK. Because ectopic rasV12 produces an increase in pMAPK, and the phosphorylation state of nuclear-directed MAPK is not required for nuclear translocation, it may be that the available pool of pMAPK that can be imported into the nucleus by Msk is enough to affect Atonal expression, but not to affect Elav or Rough expression. We also show genetic evidence that the MAPK cytoplasmic hold depends on the Hedgehog receptor Smo and is enhanced by the loss of the Dpp receptor Tkv. smo loss-of-function clones reduce Atonal and pMAPK expression, whereas tkv clones have much weaker effects. However, the loss of smo and tkv together completely abolishes both pMAPK and Atonal expression in the furrow. This is consistent with a previous report of the loss of Atonal expression in smo tkv clones (Greenwood and Struhl, 1999). Additionally, MAPK cytoplasmic hold in smo tkv clones is rescued by the additional loss of msk. Thus, we have shown that msk genetically antagonizes pMAPK levels in the morphogenetic furrow: msk gain-of-function reduces pMAPK and msk loss-of-function (in smo tkv clones) increases it.

Hedgehog signaling has also been reported as a positive regulator of Atonal on the anterior side of the furrow and as a negative regulator (perhaps through Rough or Bar) on the posterior side (Baker and Yu, 1997; Dominguez, 1999; Frankfort and Mardon, 2002; Lim and Choi, 2004). However, the inductive effect of Hedgehog on Atonal appears to be independent of the Hedgehog pathway transcription factor Ci (Suzuki and Saigo, 2000; Fu and Baker, 2003), which is consistent with an indirect effect through the MAPK cytoplasmic hold. We used smo tkv msk triple mutant clones to show that msk is genetically epistatic to smo and tkv in the furrow, and suggest that Msk sequestration in the furrow is required for MAPK cytoplasmic hold, and that smo and tkv are genetically upstream of this sequestration of Msk. Indeed, loss of smo and tkv results in a disruption of the actin cytoskeleton in the furrow, as well as of expression of Egfr and other signaling molecules (Baonza and Freeman, 2005; Firth and Baker, 2005; Vrailas and Moses, 2006). The loss of apical constriction may therefore disrupt Msk apical sequestration in such a way as to allow precocious Msk-mediated pMAPK nuclear import.

What is more surprising is that differentiation and ommatidial assembly, which are known to require Ras signaling (Freeman, 1997) and MAPK nuclear translocation (Kumar et al., 2003), occur normally in the absence of Msk in phase 2. It may be that cytoplasmic MAPK targets are important for ommatidial assembly or that pMAPK can translocate into the nucleus by some Ran-independent mechanism [such as that reported by Whitehurst et al. (Whitehurst et al., 2002)]. However, we favor the possibility that, in phase 2, other (possibly redundant) transport factors are expressed.

Like the Ras pathway, msk plays a role in ommatidial rotation but not chirality. It may be that in the absence of Msk, enough pMAPK can translocate into the nucleus for ommatidial assembly, but not enough for proper rotation. Additionally, in phase 0, we find that Msk is required for proliferation, which also requires Ras signaling. Therefore, Msk is required for some pMAPK nuclear translocation in phase 0 and phase 2, but is not necessary in phase 1, in order to allow for the initial specification of the Atonal-positive R8.

To conclude, we have identified the apical sequestration of Drosophila Importin 7/Msk in the morphogenetic furrow and we suggest that this may be required for the MAPK cytoplasmic hold in the developing eye. Cytoplasmic hold is required to allow initial patterning through lateral inhibition and the focusing of the proneural factor Atonal. We further suggest that this is mediated by the combined action of Hedgehog and Dpp.

We thank K. Arora, S. Bray, K. Moberg, I. Harinanan and G. Struhl for gifts of reagents, and K. Moberg for his critical reading of our manuscript. Work in the Perkins lab was supported by NIH R01 GM61707 and P01 HD39942 project 2, and in the Moses and Powers labs by NIH R01 EY12537. D.R.M. is supported by an NIH fellowship: 1 F32 GM073608.
References
Ets-related transcription factors required for Drosophila eye development are
regulated by the Ras/MAPK pathway. Cell 78, 137-147.

synchronization by decapentaplegic during Drosophila eye development. Science
275, 203-206.


Ready, D. F., Hanson, T. E. and Benzer, S. (1976). Development of the

expression induces premature entry into S phase and disrupts pattern formation

is expressed in neurons at all developmental stages. Dev. Biol. 126, 294-303.

analysis of the role of Egfr in the morphogenetic furrow of the developing

with transposable element vectors. Science 210, 348-353.

Ras-MAPK pathway mitogenic activity by restricting nuclear entry of activated
MAPK in endoderm differentiation of embryonic carcinoma and stem cells. J.

EGF receptor signaling establishes pattern across the developing Drosophila
retina. Development 125, 4777-4790.

Srinivasan, A., Roth, K. A., Sayers, R. O., Shindler, K. S., Wong, A. M., Fritz,
L. C. and Tomasselli, K. J. (1998). In situ immunodetection of activated caspase-
3 in apoptotic neurons in the developing nervous system. Cell Death Differ. 5,
1096-1106.


Drosophila larval eye development by concerted action of eyes absent, sine
oculis and hedgehog signaling independent of fused kinase and cubitus
interruptus. Development 127, 1531-1540.

Theisen, H., Purcell, J., Bennett, M., Kangasara, D., Syed, A. and Marsh, J. L.
(1994). dishevelled is required during wingless signaling to establish both cell

photoreceptor recruitment in the developing retina. Development 124, 343-351.

Tio, M., Ma, C. and Moses, K. (1994). spitz, a Drosophila homolog of
transforming growth factor-a, is required in the founding photoreceptor cells of

Development 104, 183-193.

ommatidium. Dev. Biol. 120, 366-376.

Sef is a spatial regulator for Ras/MAP kinase signaling. Dev. Cell 7, 33-44.

from the Drosophila eye. Dev. Dyn. 229, 162-175.

123, 151-165.

Whitehurst, A. W., Wilsbacher, J. L., You, Y., Luby-Phelps, K., Moore, M. S.
and Cobb, M. H. (2002). ERK2 enters the nucleus by a carrier-independent

Drosophila compound eye: the morphogenetic furrow and the second mitotic

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and

Yang, C. H., Axelrod, J. D. and Simon, M. A. (2002). Regulation of Fused by
fat-like cadherins during planar polarity signaling in the Drosophila compound

You, S. Y., Yoo, S. I., Yang, L., Zapata, C., Srinivasan, A., Hay, B. A. and Baker,
N. E. (2002). A pathway of signals regulating effector and initiator caspases in
the developing Drosophila eye. Development 129, 3269-3278.

divisions in wild-type and Ellipse eye imaginal discs. Dev. Biol. 149, 448-456.