Development 130, 845-857

doi:10.1242/dev.00312

CRM1-mediated nuclear export and regulated activity of the Receptor Tyrosine Kinase antagonist YAN require specific interactions with MAE

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Accepted 27 November 2002

SUMMARY

ETS family transcription factors serve as downstream effectors of signal transduction pathways, mediating cellular proliferation, differentiation and, when misregulated, tumorigenesis. The transcriptional repressor YAN prevents inappropriate responses to Receptor Tyrosine Kinase signaling by outcompeting POINTED for access to target gene promoters. We demonstrate that the molecular mechanism underlying downregulation of YAN involves CRM1-mediated nuclear export and define a novel role in this context for MAE, a co-factor previously implicated in facilitating MAPK phosphorylation of YAN. In addition to promoting YAN downregulation, MAE also participates in an inhibitory feedback loop that attenuates POINTED-P2 activation. Thus, we propose that MAE plays multiple independent roles in fine-tuning the levels of POINTED and YAN activity in accordance with changing RTK signaling conditions.

Key words: Receptor tyrosine kinase, Nuclear export, ETS transcription factor, *Drosophila*

INTRODUCTION

One pathway used reiteratively throughout development is the receptor tyrosine kinase (RTK) signaling network (Tan and Kim, 1999). RTKs signal through the evolutionarily conserved GTPase RAS and the mitogen-activated protein kinase (MAPK) cascade (Marshall, 1994; Zipursky and Rubin, 1994). Among the best characterized downstream targets of activated MAPK are the *Drosophila* ETS-domain transcription factors encoded by *pointed* (*pnt*) and *yan* (O'Neill et al., 1994). Use of two separate transcriptional start sites within *pnt* produces two distinct protein products, referred to as PNT-P1 and PNT-P2 (Klambt, 1993). Both function as transcriptional activators, but whereas PNT-P1 activity is not regulated by MAPK, PNT-P2 requires phosphorylation by MAPK in response to RTK/RAS signaling for activity (O'Neill et al., 1994). *yan* encodes a transcriptional repressor that competes with PNT for access to the regulatory regions of target genes (Gabay et al., 1996). In response to RTK activation, MAPK-mediated phosphorylation abrogates YAN repressor activity (O'Neill et al., 1994), allowing PNT to prevail in the competition for promoter access and turn on genes formerly repressed by YAN. Thus, the coordinate regulation of these two antagonistic transcription factors plays a key role in determining specific differentiative and proliferative responses to RTK signaling.

Both YAN and PNT-P2 appear to be evolutionarily conserved, serving as critical regulators of RTK signaling in other systems, including mammals (Hsu and Schulz, 2000). For example, the human orthologs, TEL and ETS1, respectively, are both oncoproteins (Hsu and Schulz, 2000). Like YAN, TEL functions as a transcriptional repressor (Lopez et al., 1999) and appears to be regulated by phosphorylation (Poirel et al., 1997). Translocations and deletions of the *tel* locus are the most frequent chromosomal aberrations associated with leukemia, implying an important function in proliferation control (reviewed by Rubnitz et al., 1999). The transcriptional activator ETS1 acts as a positive effector of RAS/MAPK signaling (Yang et al., 1996) and plays a significant role in mediating the invasiveness and angiogenesis of a variety of cancers (reviewed by Dittmer and Nordheim, 1998).

YAN is a general inhibitor of RTK-mediated signaling in *Drosophila*, functioning downstream of and negatively regulating multiple RTK pathways in both neuronal and non-neuronal cell types (Rebay and Rubin, 1995). Consistent with its role in mediating specific developmental transitions, YAN expression is highly regulated (Lai and Rubin, 1992; Price and Lai, 1999). In general, nuclear YAN expression is apparent in undifferentiated tissues, but disappears abruptly as the cells begin to differentiate (Lai and Rubin, 1992; Price and Lai, 1999). This pattern suggests that rapid degradation of YAN may alleviate the YAN-mediated block to differentiation. Supporting such an hypothesis, sequence analysis reveals YAN is rich in PEST sequences, a motif characteristically found in proteins with short or dynamically regulated half lives (Lai and Rubin, 1992; Rechsteiner and Rogers, 1996).

Experiments both in vivo and in cultured cells have suggested that phosphorylation of YAN by activated MAPK in response to RTK-initiated signaling may serve as the trigger for

dismantling the YAN-mediated block to differentiation. Mutating the phosphoacceptor residues of the MAPK phosphorylation consensus sites in YAN produces a constitutively 'activated' allele, YAN^{ACT}, that cannot be downregulated (Rebay and Rubin, 1995). For example, while wild-type Yan is rapidly excluded from the nucleus in RAS/MAPK-stimulated cultured cells, YAN^{ACT} remains nuclear. Further mutational analyses indicated that the first MAPK phosphorylation consensus site, Serine127, is necessary for redistribution of YAN from the nucleus to the cytoplasm in response to pathway activation in cultured cells. These data have led to the hypothesis that a primary consequence of MAPK-mediated phosphorylation might be nuclear export of YAN (Rebay and Rubin, 1995); however, the mechanism and potential in vivo relevance have not been determined.

MAPK-mediated recognition and phosphorylation of YAN at Serine127 is thought to be facilitated by a protein called Modulator of the Activity of ETS (MAE) (Baker et al., 2001). Mechanistically, MAE binds to YAN via a protein-protein interaction motif found at the N terminus of YAN and the C terminus of MAE (Baker et al., 2001), referred to as the Pointed Domain (PD) (Klambt, 1993). Interestingly, Baker et al. (Baker et al., 2001) also suggest that MAE binds to the PD of PNT-P2, and enhances the transcriptional activation of PNT-P2, leading them to propose that MAE promotes PNT-P2 phosphorylation by MAPK. Thus, they speculate that by promoting phosphorylation events that simultaneously downregulate YAN and upregulate PNT-P2, MAE facilitates downstream responses to RTK signaling.

Although it is clear that MAPK phosphorylation initiates YAN downregulation, the ensuing events, with respect to both YAN and PNT-P2, remain poorly understood. We show that nuclear export, via CRM1, is an essential step in downregulating YAN both in cell culture and in vivo. In this context, the PD of YAN plays a dual role in maintenance of nuclear localization in the absence of signaling and regulation of nuclear export upon RAS/MAPK activation. By manipulating the levels of *mae* expression in cells co-expressing specifically designed structural variants of YAN, we demonstrate that MAE plays a crucial role in mediating the nuclear export of YAN, independent of its role in promoting MAPK phosphorylation. Consistent with previous reports (Baker et al., 2001), we find that overexpression of MAE decreases transcriptional repressor activity of YAN. However, whereas the transcriptional activity of PNT-P2 was proposed to be stimulated by MAE co-expression (Baker et al., 2001), we find that overexpression of MAE inhibits the ability of PNT-P2 to activate transcription. Thus, we propose that MAE mediates downregulation of both YAN and PNT-P2. In the case of YAN, MAE facilitates MAPK-mediated phosphorylation and subsequent nuclear export, while in the case of PNT-P2, MAE could participate in a negative feedback loop that attenuates transcriptional activity.

MATERIALS AND METHODS

Molecular biology

pUAST $YAN^{N' NLS}$ was made by ligating the annealed product of the two oligonucleotides (5′ ACCCCACCTAAGAAGAAGCGCAAGG-TGGAGGACTCCCAG 3′ and 5′ GAGTCCTCCACCTTGCGC-TTCTTCTTAGGTGGGGTCTGG 3′) into the N-terminal *Bst*XI site of pUAST YAN. pUAST $YAN^{Int\ NLS}$ was made by ligating the annealed product of the two oligonucleotides (5′ GATCTACCCC-GCCAAAGAAGAAGCGCAAGGTGGAGGACG 3′ and 5′ GATC-CGTCCTCCACCTTGCGCTTCTTCTTTGGCGGGGTA 3′) into the unique internal *Bam*HI site of pUAST YAN. The underlined residues were changed from A to C, and from T to G to create pUAST $YAN^{Mut\ NLS}$. Transgenic lines were generated as previously described (Rebay et al., 1993).

$YAN^{\Delta NES1}$, $YAN^{\Delta NES1,2}$, $YAN^{\Delta NES3+PD,}$ and $YAN^{\Delta N'}$ have amino acids 1-17, 1-48, 48-117 and 1-117 deleted, respectively. Unless otherwise noted, these and all other constructs were expressed under the metallothionein promoter using the plasmid pRMHa-3.

$YAN^{Mut\ Ets}$ was made using Stratagene's QuikChange Site-Directed Mutagenesis system with oligonucleotides 5′ GGACTGGCAAA-GTTGGGAGGCATCCAGGGGAACCATCTGTCC 3′ and its reverse complement. The underlined nucleotides indicate the mutated base pairs, which result in W438G and K443G.

MYC-MAE was generated by PCR amplifying *mae* out of a cDNA library using primers 5′ CAAGTGGAATCGAGCTATACC 3′ and 5′ CTATGATAGCAGGGCCATTGCTCGG 3′. The product was N-terminally tagged with a MYC epitope, verified by sequencing, and shuttled into both pRMHa-3 and pUAST.

pUAST flag PNT-P2 was generated by adding an N-terminal FLAG epitope tag to the full length PNT-P2 coding sequence.

The EBS-luciferase reporter was created by placing six tandem copies of an ETS-binding site (O'Neill et al., 1994) upstream of the luciferase gene.

Additional subcloning details available upon request.

Immunohistochemistry

Fixation and staining of S2 cells and embryos were performed as previously described (Fehon et al., 1990; Fehon et al., 1991). S2 cells staining was performed using Anti-YAN MAb 8B12 at 1:250 or anti-MYC MAb 9E10 (a gift from R. Fehon) at 1:100, with CY3-conjugated goat anti-mouse secondary (1:10000) and DAPI (100 μg/ml at 1:5000). Staining of double-labeled embryos was performed using 8B12 (1:750), CY3 goat anti-mouse (1:1000), rat anti-ELAV MAb 7E8A10 (1:500), and CY2-conjugated goat anti-rat (1:2000). All secondary antibodies were from Jackson ImmunoResearch. Monoclonal supernatants were generated by growing hybridoma lines obtained from the Developmental Studies Hybridoma Bank in DMEM supplemented with 10% fetal bovine serum and 10-% NCTC-109 (Gibco).

Transcription assays

Drosophila S2 cells were transfected using the calcium phosphate method as previously described (Pascal and Tjian, 1991). pAc5.1-*lacZ* (Invitrogen) was used as a transfection control. Transfected cells were harvested, washed with media, and lysed by rocking at 4°C for 20 minutes in 250 μl of lysis buffer (Tropix/Applied Biosystems). Quantitation of luciferase and β-galactosidase activity was carried out using a Luciferase Assay Kit (Tropix/Applied Biosystems) or Galacto-Star Assay kit (Tropix/Applied Biosystems) in a tube luminometer (EG&G Berthold AutoLumat LB953). Each transfection was performed in quadruplicate, tested in triplicate and the data points averaged. The average luciferase/β-galactosidase signal for EBS-luciferase alone was set to 1 and the experimental averages were normalized relative to this value. Data were analyzed and graphed using Microsoft Excel.

RNAi

dsRNAs were generated using PCR primers containing T7 polymerase recognition sequences (5′ GAATTAATACGACTCAC-TAT 3′) at the 5′ ends followed by 21 nucleotides of the target sequence, and were designed to span ~500 bp of coding sequence (*crm1* 5′ T7-ATGGCGACAATGTTGACA 3′, 5′ T7-TTGTTCA-

TGCACAGGC 3′; *mae* 5′ CAAGTGGAATCGAGCTATACC 3′, 5′ CTATGATAGCAGGGCCATTGC 3′). The PCR products were extracted from 1% agarose gels and purified using Qiagen's QIAquick PCR purification kit. dsRNAs were made according to the directions of Ambion's MEGAscript in vitro transcription kit. RNAi experiments in S2 cells were performed by adding 10 μg of dsRNA to the transfection mix. Cells were analyzed at 3-7 days post transfection, as determined for maximum effect (3 days for RNAi of *crm1* and 7 days for RNAi of *mae*). RNAi was injected into embryos according to standard injection protocols (Rebay et al., 1993) at a concentration of less than 5 μM.

Histology

Adult flies were prepared for scanning electron microscopy by fixation in 1% glutaraldehyde/1% paraformaldehyde in 0.1 M sodium phosphate (pH 7.2) for 2 hours. The fixed tissue was dehydrated through an ethanol series. Samples were critical point dried, sputter coated, and pictures taken on a scanning electron microscope (JEOL 5600LV). Fixation and tangential sections of adult eyes was performed as previously described (Tomlinson et al., 1987).

Co-immunoprecipitation

Transfected cells were harvested, and lysed by rocking at 4°C for 20 minutes in 1 ml of lysis buffer [100 mM NaCl; 50 mM Tris, pH7.5; 2 mM EDTA; 2 mM EGTA; 1% NP-40 + one Complete, Mini protease inhibitor cocktail tablet (Roche)/10 ml]. Clarified lysates were subjected to immunoprecipitation (anti-MYC 1:50 for 3 hours at 4°C), followed by the addition of 20 μl of Protein-A Sepharose beads (Zymed) (1.5 hours at 4°C). Beads were washed twice with lysis buffer and twice with PBS. The immunoprecipitates were boiled in 40 μl of 2×SDS buffer, and western blotting was carried out as previously described (O'Neill et al., 1994) (anti-MYC 1:100, anti-YAN 1:500, anti-FLAG 1:50000).

RESULTS

CRM1 mediates YAN nuclear export

Although redistribution of YAN from the nucleus (Fig. 1A) to the cytoplasm (Fig. 1B) upon RAS/MAPK activation in S2 cultured cells is suggestive of nuclear export, it is formally possible this shift results from degradation of YAN in the nucleus, coupled with a failure of newly synthesized and phosphorylated YAN to enter the nucleus. To determine if the cytoplasmic accumulation of YAN in RAS^{V12} stimulated S2 cultured cells is a consequence of nuclear export, we asked whether blocking the nuclear export machinery would result in nuclear retention of YAN. YAN, which is predicted to be 78 kDa, is too large to diffuse through the nuclear pore, and thus its export must occur by facilitated transport. CRM1, a common exportin, mediates translocation of nuclear export sequence (NES) containing proteins from the nucleus (Fornerod et al., 1997). We found that in RAS^{V12}-stimulated S2 cultured cells, YAN was retained in the nucleus in the presence of Leptomycin B (LMB) (data not shown), a drug that specifically binds and inhibits CRM1 (Wolff et al., 1997), or dsRNA interference (RNAi) to knock down *crm1* expression (Fig. 1C). These data indicate that the cytoplasmic accumulation of YAN induced by RAS/MAPK activation is the result of CRM1-dependent nuclear export.

Nuclear export is necessary for downregulation of YAN in vivo

Because cytoplasmic accumulation of YAN has never been

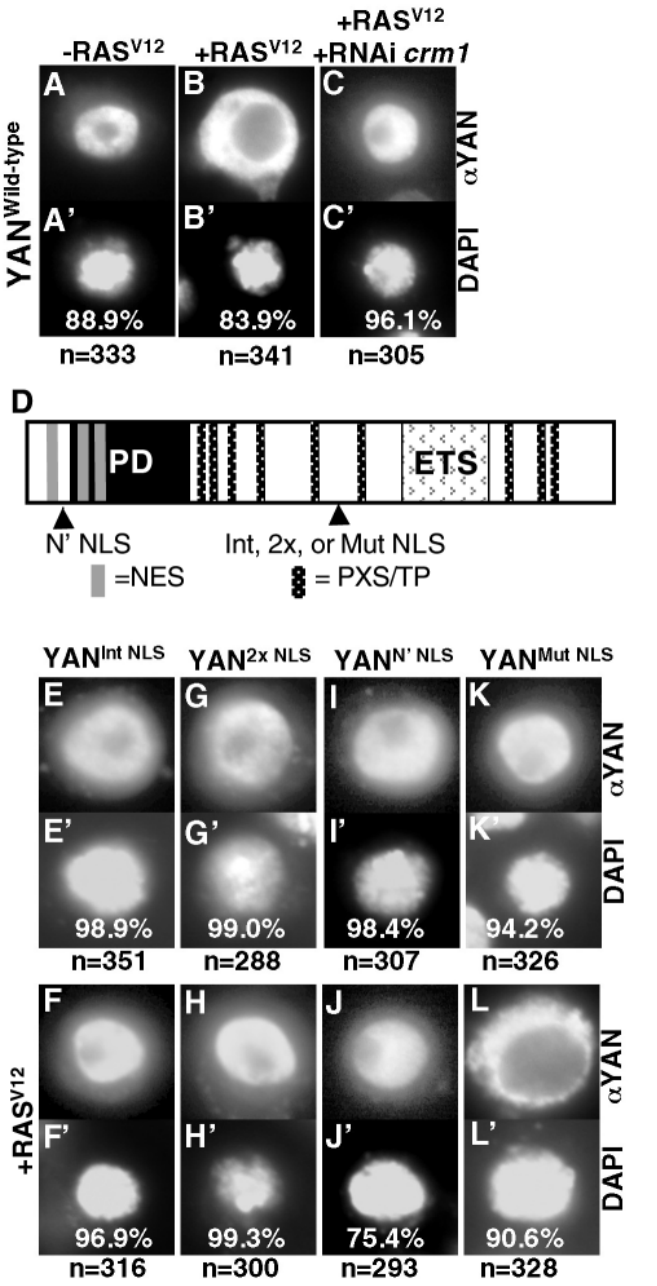

Fig. 1. Nuclear export of YAN is mediated by CRM1 and blocked by insertion of a NLS into YAN. (A-C,E-L) S2 cultured cells transfected with various YAN constructs and stained with anti-YAN. (A′-C′,E′-L′) DAPI staining of the same cells. (D) Schematic of YAN showing predicted domains and sites of SV40 Large T-antigen NLS insertions. For each experiment (A-C,E-L), the percentage of transfected cells exhibiting nuclear localization (A,C,E-K) or exclusively cytoplasmic localization (B,L) is indicated. *n*, number of cells scored in each experiment. (A-C′) YAN^{WT}; (E-F′) $YAN^{Int\ NLS}$; (G-H′) $YAN^{2x\ NLS}$; (I-J′) $YAN^{N'\ NLS}$; (K-L′) $YAN^{Mut\ NLS}$. (A,E,G,I,K) YAN localization in the absence of RAS^{V12}. (B,F,H,J,L) YAN localization in the presence of RAS^{V12}. (C) YAN localization in the presence of RAS^{V12} and RNAi of *crm1*. (C) YAN localization is restricted to the nucleus in the presence of RAS^{V12} and RNAi of *crm1*. (F,H) Internal NLS insertions completely inhibit nuclear export of YAN in the presence of RAS^{V12}, while the N-terminal insertion only partially prevents export (J). (L) Insertion of a nonfunctional NLS into YAN has no effect on export.

detected in developing *Drosophila* tissues (I. R., unpublished) (Lai and Rubin, 1992), it was possible that the nuclear export demonstrated in S2 cultured cells (Fig. 1A-C) did not reflect the actual downregulation mechanism used in vivo. To address this, the SV40 large T antigen nuclear localization signal (NLS) (Kalderon et al., 1984) was inserted into YAN. Insertions were made either near the N terminus ($YAN^{N' NLS}$) or in the middle of the protein ($YAN^{Int NLS}$ and $YAN^{2x NLS}$) (Fig. 1D). As a control, a mutated, and hence non-functional, version of the NLS (Kalderon et al., 1984) was inserted into the middle of the protein ($YAN^{Mut NLS}$). These constructs were placed under the control of the UAS promoter, which allows expression both in cell culture and in vivo when combined with an appropriate GAL4 driver (Brand and Perrimon, 1993).

We first demonstrated that the NLS insertions were capable of rendering YAN refractory to nuclear export in response to RAS/MAPK signaling in transiently transfected S2 cultured cells. In the presence of RAS^{V12}, the internal NLS insertions effectively overcame the export signals and completely restricted YAN to the nucleus (Fig. 1E-H). $YAN^{N' NLS}$ appears less potent, presumably owing to insertion in a less accessible region of the protein, and only partially restricted YAN to the nucleus (Fig. 1I,J). The control experiment, in which $YAN^{Mut NLS}$ behaved indistinguishably from wild-type YAN, localizing to the nucleus in unstimulated cells (Fig. 1K) and becoming cytoplasmic in RAS^{V12} stimulated cells (Fig. 1L), indicated that the insertion alone does not disrupt regulation of YAN localization. Given the reduced efficiency of the $YAN^{N' NLS}$ insertion relative to that of $YAN^{Int NLS}$ and $YAN^{2x NLS}$, only the internal insertions were used for in vivo analyses.

Having demonstrated that insertion of a NLS tag is sufficient to prevent nuclear export, transgenic flies expressing these constructs were generated and used to examine the role of nuclear export of YAN in vivo. For these experiments, ELAV GAL4 was used to drive expression in the central nervous system (CNS), a tissue whose differentiation requires precisely timed downregulation of YAN (Rebay and Rubin, 1995). We reasoned that if nuclear export is necessary for downregulation of YAN, restricting YAN to the nucleus should prevent this and result in a phenotype resembling YAN^{ACT}. Specifically, nuclear YAN expression should be detected in the region of the developing brain and ventral nerve cord of stage 11 embryos (Fig. 2A,A′) and CNS development should be inhibited as visualized by reduced expression of neuronal markers (Fig. 2A″) (Rebay and Rubin, 1995). Alternatively, if nuclear export is not required, then the NLS tagged YAN should be downregulated as effectively as overexpressed wild-type YAN, resulting in a lack of YAN staining in the presumptive ventral nerve cord and correspondingly normal CNS development (Fig. 2B,B′,B″).

Supporting the first model, expression of either $YAN^{Int NLS}$ (Fig. 2C,C′,C″) or $YAN^{2x NLS}$ (Fig. 2D,D′,D″) resulted in a YAN^{ACT} phenotype (Fig. 2A,A′,A″). Analogous results were obtained in the eye (data not shown), where downregulation of YAN is necessary for photoreceptor differentiation (Lai and Rubin, 1992), indicating an essential role for nuclear export in downregulating YAN in multiple cell types in vivo. The control construct, $YAN^{Mut NLS}$, exhibited wild-type YAN regulation (Fig. 2E,E′) and neuronal differentiation (Fig. 2E″). This NLS-mediated restriction of YAN to the nucleus, and subsequent inhibition of downregulation and differentiation, strongly suggests nuclear export plays a central role in downregulation of YAN in vivo.

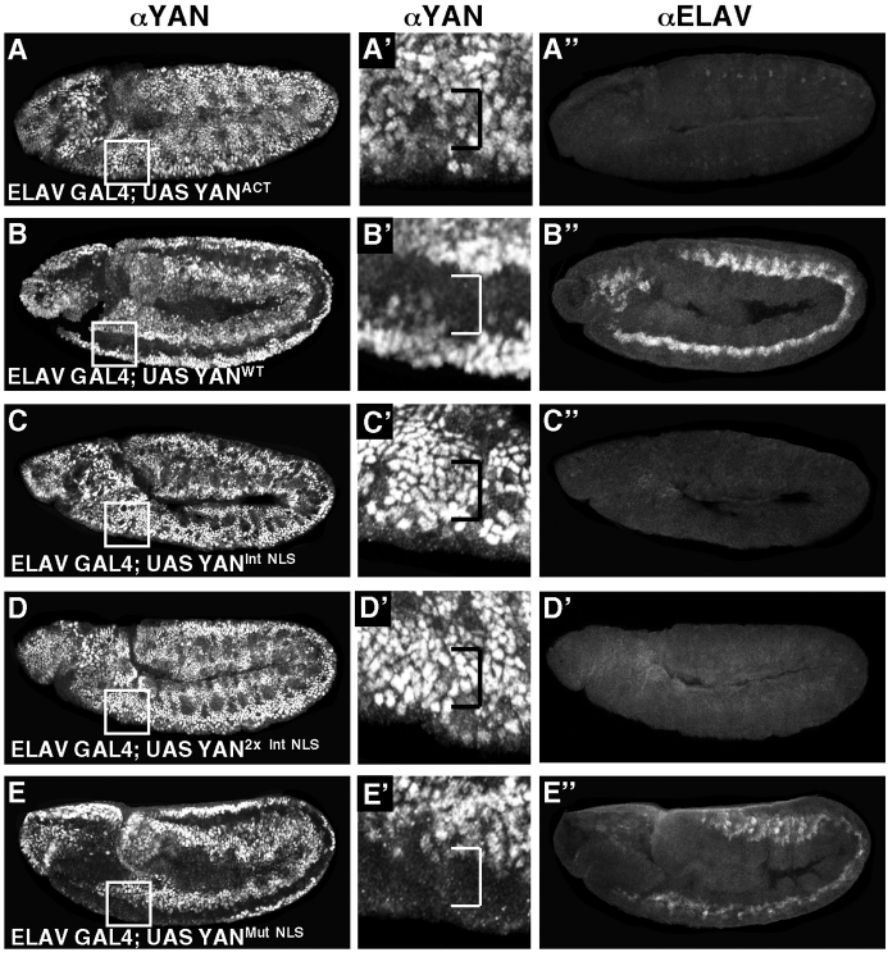

Fig. 2. NLS insertions restrict YAN to the nucleus in vivo. (A-E″) Confocal images of germband extended *Drosophila* embryos double labeled with anti-YAN (A-E,A′-E′) and anti-ELAV (A″-E″). (A′-E′) Higher magnification views of regions boxed in A-E with normal or failed YAN downregulation highlighted by bracket. ELAV GAL4 was used to drive expression of (A,A′,A″) UAS YAN^{ACT}; (B,B′,B″) UAS YAN^{WT}; (C,C′,C″) UAS $YAN^{Int NLS}$; (D,D′,D″) UAS $YAN^{2x Int NLS}$; (E,E′,E″) UAS $YAN^{Mut NLS}$. YAN^{WT} is downregulated normally in the ventral nerve cord (B,B′), allowing neuronal differentiation to proceed (B″). As with YAN^{ACT} (A,A′,A″), insertion of an NLS restricts YAN to the nucleus (C,C′,D,D′), thereby blocking CNS development (C″,D″).

The PD is necessary for regulating the subcellular localization of YAN

Having demonstrated a requirement for nuclear export in YAN downregulation in

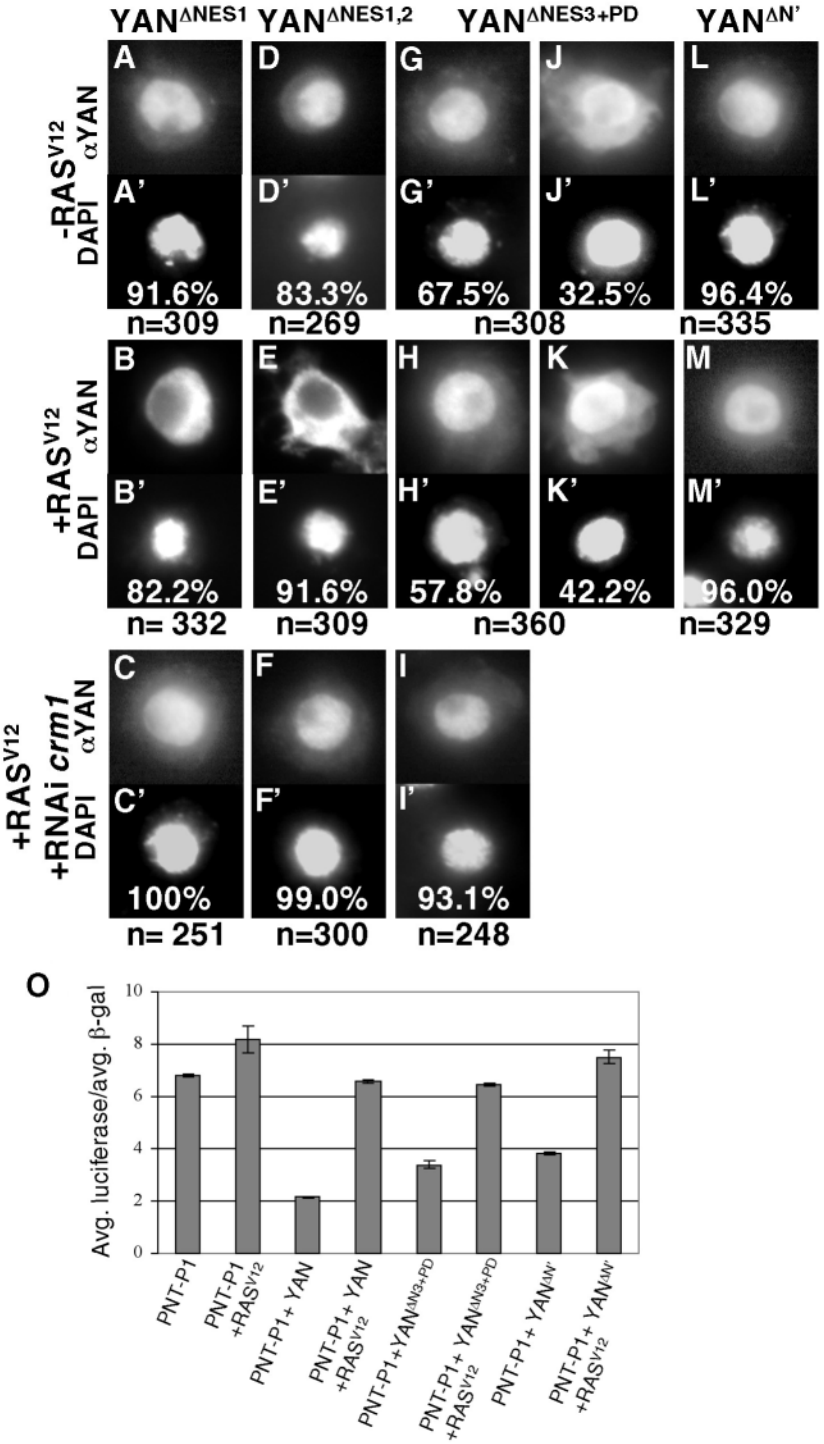

Fig. 3. CRM1-mediated nuclear export of YAN requires both the NESs and the PD. (A-M) S2 cultured cells transfected with various YAN deletion constructs and stained with anti-YAN. (A′-M′) DAPI staining of the same cells. For each experiment (A-M), the percentage of transfected cells exhibiting nuclear localization (A,C,D,F,G-I,L,M), both nuclear and cytoplasmic localization (J,K), or exclusively cytoplasmic localization (B,E) is indicated. *n*, number of cells scored in each experiment. (A-B,D-E) Deletion of the first or first and second NES has no effect on export. (G-H,J-K) Deletion of the third NES and majority of the PD results in inappropriate export in the absence of signaling, and impairs export in the presence of RAS^{V12}. (L-M) Deletion of the whole N terminus completely inhibits export. (C,F,I) RNAi-mediated knockdown of *crm1* restricts YAN to the nucleus in the presence of RAS^{V12}. (O) Transcription assays with $YAN^{\Delta N'}$ and $YAN^{\Delta N3+PD}$ show that both deletions repress transcription and are responsive to RAS^{V12}.

vivo, we sought to determine which domains of YAN are involved. Analysis of the YAN protein sequence (Lai and Rubin, 1992) reveals three N-terminal leucine-rich putative nuclear export sequences (NES) (Wen et al., 1995) that resemble canonical CRM1-binding sites (Fornerod et al., 1997) (Fig. 1D). Two of the putative NESs reside within the pointed domain (PD), suggesting this motif could be involved in regulating export.

A series of deletion constructs was made and assayed for nuclear export competence in S2 cultured cells. The deletion of the first NES ($YAN^{\Delta NES1}$) or the first and second NES ($YAN^{\Delta NES1,2}$) had no effect on regulated YAN localization (Fig. 3A,B and D,E respectively, when compared with Fig. 1A,B). Deletion of the third NES and the majority of the PD ($YAN^{\Delta NES3+PD}$) resulted in partial export in the absence of signaling and a slight increase in export upon RAS stimulation (Fig. 3G,J,H,K). However, strictly cytoplasmic localization was never seen with $YAN^{\Delta NES3+PD}$. Export of these constructs appeared to be regulated in the same manner as wild-type YAN, as inhibition of CRM1 resulted in the deletions being restricted to the nucleus (Fig. 3C,F,I). Finally, the deletion of the whole N terminus ($YAN^{\Delta N'}$), including all three NESs and the PD, localized to the nucleus and remained nuclear in the presence of RAS^{V12} (Fig. 3L,M). These results suggest that while individually the NESs may be redundant for nuclear export, together the NESs mediate export. The data also implicate the PD as necessary for regulated subcellular localization of YAN.

Because phosphorylation by MAPK has been shown to be a prerequisite for redistribution of YAN (Rebay and Rubin, 1995), it was important to rule out the possibility that the mislocalization of $YAN^{\Delta NES3+PD}$ and $YAN^{\Delta N'}$ reflected an inability of the proteins to be phosphorylated, rather than a defect in export. To test this, we used the previously published observation that phosphorylation of YAN in response to RAS/MAPK signaling abrogates the ability of YAN to repress PNT-P1-mediated activation of an ETS reporter construct (O'Neill et al., 1995). If YAN cannot be phosphorylated, as was shown for YAN^{ACT}, then transcriptional repression continues unabated even in the presence of RAS stimulation.

Therefore, to verify that $YAN^{\Delta NES3+PD}$ and $YAN^{\Delta N'}$ are responsive to RAS/MAPK signaling, transcriptional assays were performed. Both $YAN^{\Delta NES3+PD}$, which is partially exported in the absence of signaling, and $YAN^{\Delta N'}$, which is completely restricted to the nucleus, were capable of repressing

transcription, but not to the extent of wild-type YAN (Fig. 3O). This repression could be relieved by RAS^{V12}. The significant, albeit reduced, transcriptional repression exhibited by these constructs argues that the N-terminal deletions have not compromised the structure or function of the remainder of the protein. It also suggests that the PD may play a role in mediating transcriptional repression. Retention of normal RAS/MAPK responsiveness indicates that both proteins are likely to be phosphorylated and that their nuclear restriction reflects a specific failure in export. Thus phosphorylation of YAN by MAPK, although it abrogates transcriptional repression, is not sufficient to induce nuclear export; rather, nuclear export of YAN requires a functional N terminus, presumably to mediate dynamic interactions with CRM1 and possibly other co-factors in response to RAS/MAPK stimulation.

MAE is necessary for YAN downregulation in vivo

We have shown that loss of the PD and NES motifs results in inappropriate YAN localization. PDs are involved in protein-protein interactions (Chakrabarti and Nucifora, 1999; Carrere et al., 1998; Baker et al., 2001). MAE, a PD family member, has been shown in vitro to bind YAN via a PD-PD interaction, leading to phosphorylation of YAN at Serine127 (Baker et al., 2001), the phosphorylation site necessary for redistribution of YAN in S2 cultured cells (Rebay and Rubin, 1995). If promoting YAN downregulation were its primary function, MAE would be predicted to play a positive role in the RTK signaling cascade, although *mae* mutations have not been isolated in RTK pathway genetic interaction screens (e.g. Dickson et al., 1996; Karim et al., 1996; Rebay et al., 2000; Simon et al., 1991).

To confirm that MAE contributes to RTK signaling in vivo, we looked first for genetic interactions with known pathway components. Transgenic flies expressing RAS^{V12} under the control of the Sevenless promoter (Sev-RAS^{V12}) exhibit rough adult eyes (Karim et al., 1996) (Fig. 4B, compared with 4A). Heterozygosity for *mae*, with either a P-element insertion (*l(2)k06602*) or a deficiency uncovering the locus (*Df(2R)PC4*), dominantly suppressed the Sev-RAS^{V12} rough eye phenotype (Fig. 4C,D), consistent with the proposed function of MAE as a positive component of the pathway. Quantitation of this suppression by counting the number of R7 photoreceptors per ommatidium in tangential adult eye sections confirmed the interaction. Relative to the wild-type control which has 1.0 R7/ommatidium (Fig. 4E), Sev-RAS^{V12} exhibits 3.0 R7/ommatidium (Fig. 4F), while Sev-RAS^{V12}/*l(2)k06602* and Sev-RAS^{V12}/*Df(2R)PC4* exhibit 2.0 R7/ommatidium and 1.6 R7/ommatidium, respectively (Fig. 4G,H). Further supporting a positive role in the pathway, a reduction in dose of *mae* mildly enhanced the Sev-YAN^{ACT} rough eye phenotype (data not shown). The ability of *mae* to suppress Sev-RAS^{V12} and enhance Sev-YAN^{ACT} suggests that loss of *mae* function decreases signaling through the pathway and that MAE plays a positive role in RTK signaling in vivo.

We then asked whether the reduced RTK signaling associated with loss of

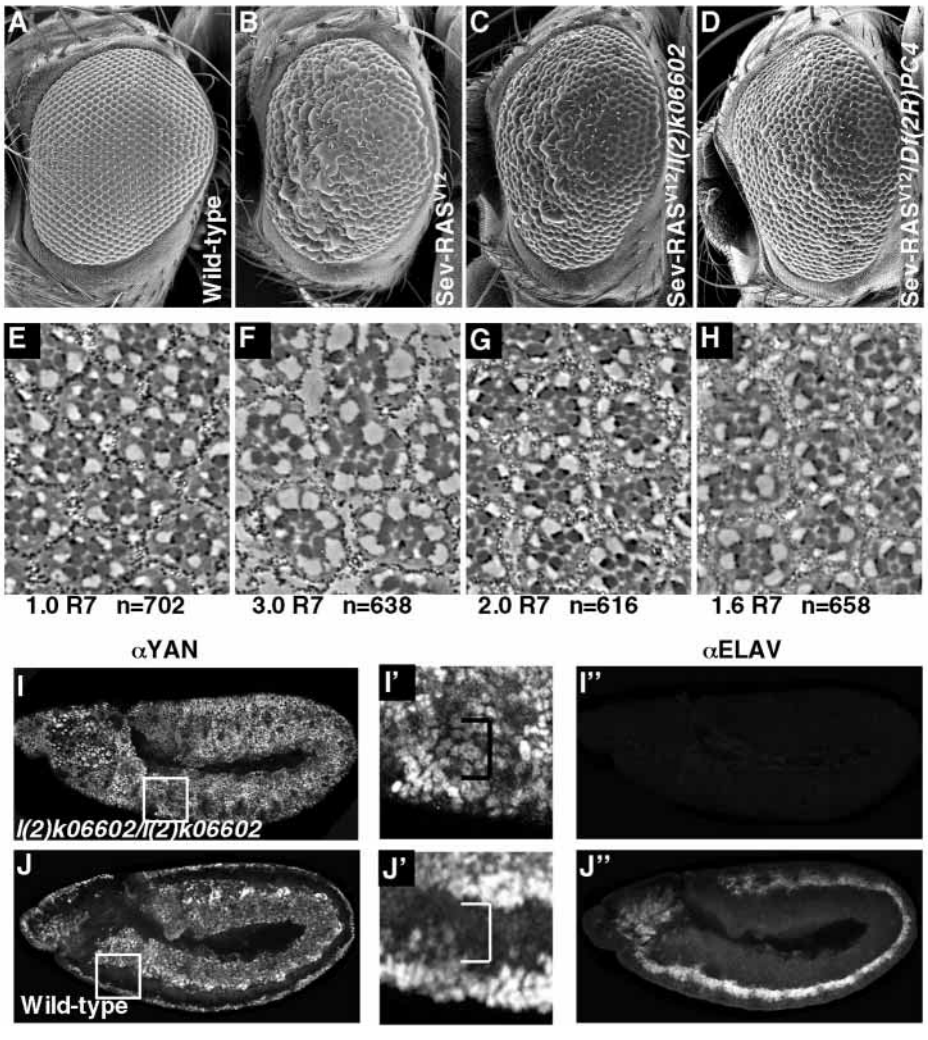

Fig. 4. MAE acts as a positive component of the RTK pathway and loss of *mae* function inhibits the downregulation of YAN. (A-D) Scanning electron micrographs of adult *Drosophila* eyes showing that loss of *mae* dominantly suppresses the rough eye phenotype of Sev-RAS^{V12}. (E-G) Tangential sections of adult *Drosophila* eyes. The average number of R7 photoreceptors per ommatidium is indicated below, with n referring to the total number of ommatidia scored. (A,E) Wild-type; (B,F) Sev-RAS^{V12}/+; (C,G) Sev-RAS^{V12}/*l(2)k06602*; (D,H) Sev-RAS^{V12}/*Df(2R)PC4*. Confocal images of germband extended embryos double labeled with anti-YAN (I,J, with high magnification of boxed region shown in I′,J′) and anti-ELAV (I″,J″). (I,I′,I″) show that in *mae* mutants, YAN fails to be downregulated in the CNS (I′, bracketed region) and ELAV expression is inhibited (I″). (I,I′,I″) *l(2)k06602/l(2)k06602*; (J,J′,J″) wild type.

mae function might result from improper YAN localization and downregulation. Initially we addressed this question in S2 cultured cells, where MAE has been shown to be endogenously expressed (Baker et al., 2001). RNAi of *mae* resulted in restriction of YAN to the nucleus in the presence of RAS^{V12} (Table 1), consistent with the model whereby MAE facilitates MAPK-mediated phosphorylation of YAN as a prerequisite for nuclear export. To assess the effect of *mae* loss of function in *Drosophila*, we examined YAN localization in embryos homozygous for either *l(2)k06602* (Fig. 4I,I′), *Df(2R)PC4* (data not shown) or transheterozygotes (data not shown). YAN is not downregulated in *mae* mutant embryos, which exhibit nuclear expression in the brain and ventral nerve cord (compare Fig. 4I,I′ with 4J,J′). Consistent with the presence of aberrant YAN expression in the CNS, neuronal differentiation was inhibited in *mae* mutants (compare Fig. 4I″ with 4J″). RNAi of *mae* performed in embryos produced identical phenotypes (data not shown). We therefore conclude that *mae* function is necessary to downregulate YAN in vivo.

MAE is required for nuclear export of YAN independent of its role in facilitating MAPK phosphorylation

Previous work has shown that MAPK-mediated phosphorylation of YAN is necessary for nuclear export, with Serine127 serving as the key phosphorylation site (Rebay and Rubin, 1995). MAE is thought to be necessary for phosphorylation of YAN at this site (Baker et al., 2001), and our results suggest that MAE is also required for nuclear export. We therefore wanted to determine whether the role of MAE in export was simply a secondary consequence of it being necessary for phosphorylation, or whether it reflected an independent requirement.

To address this, we needed to establish an experimental context in which nuclear export of YAN is uncoupled from the RAS/MAPK signal that normally triggers it. We reasoned that localization of YAN to the DNA was likely to be necessary for proper regulation of subcellular localization, perhaps by masking the N-terminal NES sequences from recognition by CRM1. Therefore, we introduced two point mutations into the ETS domain of YAN (W439G and K443G, $YAN^{Mut\ ETS}$) that have been shown previously to be important for DNA binding but not for nuclear localization (Kodandapani et al., 1996). $YAN^{Mut\ Ets}$, which is no longer able to bind DNA, might be accessible to CRM1, even in the absence of RAS/MAPK signaling, and might therefore be constitutively exported, providing us with a situation in which export was uncoupled from signaling.

We found that even in the absence of RAS^{V12} activation, $YAN^{Mut\ ETS}$ localized to the cytoplasm in S2 cultured cells, indicating that YAN must be bound to DNA to maintain its nuclear localization (Table 1). Furthermore, inhibition of CRM1-mediated export resulted in localization of $YAN^{Mut\ ETS}$ to the nucleus (Table 1), suggesting $YAN^{Mut\ ETS}$ initially localized properly to the nucleus but because of its inability to bind DNA was promptly exported. Thus, under conditions in which YAN is not phosphorylated by MAPK, CRM1-mediated nuclear export regulates localization of $YAN^{Mut\ ETS}$. Colocalization and coimmunoprecipitation experiments confirmed that the point mutations in $YAN^{Mut\ ETS}$ do not compromise its ability to bind MAE (data not shown).

Table 1. MAE is necessary for nuclear export of YAN

YAN localization	*n*	Nucleus	Nucleus + cytoplasm	Cytoplasm
YAN	347	88.2%	7.2%	4.6%
YAN + RAS^{V12}	318	2.8%	3.5%	93.7%
YAN + RAS^{V12} + RNAi *crm1*	305	96.1%	3.9%	0%
YAN + RAS^{V12} + RNAi *mae*	340	52.6%	26.2%	21.2%
$YAN^{Mut\ Ets}$	343	0.3%	4.4%	95.3%
$YAN^{Mut\ Ets}$ + RNAi *crm1*	323	74.5%	19.5%	6.2%
$YAN^{Mut\ Ets}$ + RNAi *mae*	324	50.9%	21.6%	27.5%

YAN localization in S2 cultured cells is indicated as the percentage of transfected cells exhibiting nuclear localization, both nuclear and cytoplasmic localization, or exclusively cytoplasmic localization.
n, number of cells scored in each experiment.

We exploited these findings to ask whether MAE plays a role in nuclear export separate from that proposed by Baker et al. (Baker et al., 2001) in facilitating phosphorylation. We found that RNAi of *mae* restricted $YAN^{Mut\ Ets}$ to the nucleus (Table 1). This suggests that MAE has a second function with respect to CRM1-mediated nuclear export of YAN, independent of its earlier role in promoting YAN phosphorylation in response to RAS/MAPK signaling.

RAS/MAPK signaling regulates MAE localization by modulating interactions with its binding partners YAN and PNT-P2

Our results indicate that MAE plays a significant role in the downregulation of YAN, both in cell culture and in vivo. To investigate the function(s) and regulation of MAE in more detail, we first asked whether the RAS/MAPK pathway might directly control the subcellular localization of MAE. To address this question, a MYC-epitope tagged MAE was generated and expressed in S2 cultured cells. We found that MAE was ubiquitously expressed throughout the cell in both the absence and presence of RAS^{V12} (Fig. 5C,D). Furthermore, inhibition of CRM1-mediated nuclear export had no effect on MAE subcellular localization (Fig. 5E,F), consistent with its predicted ability to diffuse freely through the nuclear pore based on its small (~19 kDa) size and lack of a recognizable NES. Therefore, the localization of MAE does not appear to be influenced directly by RAS/MAPK signaling, nor is it dependent upon CRM1-mediated export.

These results led us to hypothesize that any dynamic RAS/MAPK-mediated regulation of MAE was likely to be mediated through specific interactions with its binding partners, YAN and PNT-P2. Therefore, we looked for RAS^{V12}-induced changes in MAE localization in cells co-transfected with YAN and PNT-P2. Co-transfection of YAN with MAE alters MAE distribution. In the absence of RAS^{V12}, MAE was predominantly nuclear (Fig. 5G), because it is bound to YAN (Fig. 5A, lane 2), and then became both nuclear and cytoplasmic in the presence of RAS^{V12} (Fig. 5H). This suggests that MAPK phosphorylation of YAN may result in destabilization of the YAN-MAE complex, allowing MAE to reassume uniform distribution. Co-immunoprecipitation experiments supported this interpretation, as the amount of YAN bound to MAE appeared to be significantly reduced in RAS^{V12}-stimulated cells (Fig. 5A, compare lane 4 with lane 2; note that the total amount of YAN present is comparable with and without RAS^{V12}, lanes 1 and 3).

We speculated that destabilization of the YAN-MAE complex upon RAS/MAPK activation might require intervention from an additional YAN-binding partner, potentially CRM1. To address this possibility, we examined the effects of inhibiting CRM1-mediated export in RAS^{V12}-stimulated cells expressing YAN and MAE. Under these conditions, MAE remains nuclear, suggesting that interactions with CRM1 or some other associated factor, is needed to dissociate MAE from YAN (Fig. 5O). These results indicate that MAE localization is dependent on a dynamic balance between its own expression level, the expression level of YAN, the presence of additional YAN binding partners and RAS/MAPK signaling.

To characterize further the interaction between YAN and MAE, we analyzed MAE localization when co-transfected with several different mutants of YAN. It has been shown in vitro that MAE interacts with YAN via a PD-PD interaction (Baker et al., 2001). To confirm this, we examined MAE localization in the presence of $YAN^{\Delta N'}$ and found that MAE was ubiquitously expressed throughout the cell (Fig. 5I,J). Therefore, restriction of MAE to the nucleus by YAN requires the PD. We also looked at MAE localization in YAN^{ACT}-

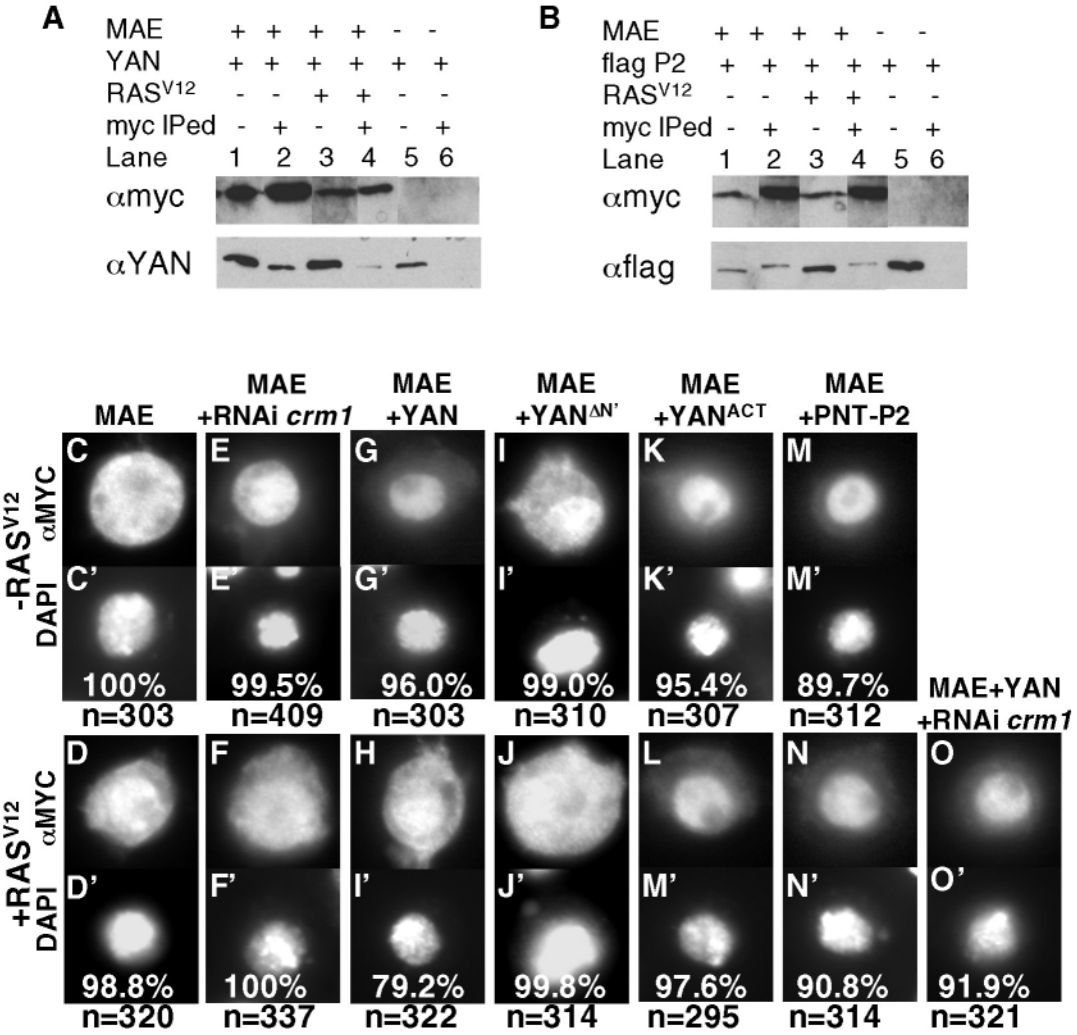

Fig. 5. MAE localization in S2 cells depends on the distribution of its binding partners. (A,B) Immunoblots of MYC-IPs visualized with anti-MYC (MAE), anti-YAN and anti-FLAG (PNT-P2). MAE complexes with YAN in the absence of RAS/MAPK signaling (A) and with PNT in the both the absence and presence of signaling (B). Lanes are from the same gel and immunoblot, but have been rearranged. Lanes 1, 3, 5 are non-IPed lysates; lanes 2, 4, 6 are the corresponding IPs. Specificity of the anti-MYC IP is demonstrated in lane 6 of A and B, where in the absence of MAE, YAN or PNT-P2 are not precipitated. (C-O) Anti-MYC staining of S2 cells transfected with MYC-*mae*. (C′-O′) DAPI staining of the same cells. (C,E,G,I,K,M) Absence of RAS^{V12}. (D,F,H,J,L,N,O) Presence of RAS^{V12}. For each experiment (C-O), the percentage of transfected cells exhibiting nuclear localization (G,K-O), or both nuclear and cytoplasmic localization (C-F,H-J) is indicated. *n*, number of cells scored in each experiment. (C,D) MAE; (E,F) MAE+RNAi *crm1*; (G,H) MAE+YAN; (I,J) MAE+$YAN^{\Delta N'}$; (K,L) MAE+YAN^{ACT}; (M,N) MAE +PNT-P2; (O) MAE+YAN+RNAi *crm1*. MAE is ubiquitously expressed in S2 cells (C,D), except when YAN or PNT-P2 is co-transfected. When MAE is co-transfected with wild-type YAN, MAE is nuclear in the absence of signaling (G) and becomes nuclear and cytoplasmic in the presence of RAS^{V12} (H). CRM1 does not mediate the export of MAE (E,F). However, when YAN is co-transfected with MAE, and CRM1-mediated export is inhibited by RNAi, MAE remains nuclear (O). MAE interacts with YAN via the PD, as MAE is ubiquitously expressed when $YAN^{\Delta N'}$ is co-expressed (I,J). Co-transfection of MAE with YAN^{ACT} restricts MAE to the nucleus in the absence and presence of RAS^{V12} (K,L). Similarly, PNT-P2 restricts MAE to the nucleus (M,N).

expressing cells. YAN^{ACT} cannot be phosphorylated by MAPK and therefore remains restricted to the nucleus in the presence of RAS^{V12}. Co-transfection of YAN^{ACT} restricted MAE to the nucleus in the absence and presence of RAS^{V12} (Fig. 5K,L), suggesting phosphorylation of YAN is necessary for redistribution of MAE.

Because YAN appears to play a significant role in regulating MAE localization, we next asked whether PNT-P2, the other known binding partner of MAE (Baker et al., 2001), might also be involved. Co-transfection of PNT-P2 and MAE resulted in restriction of MAE to the nucleus and formation of a MAE-PNT-P2 complex that can be co-immunoprecipitated in the absence and presence of RAS^{V12} (Fig. 5M,N; Fig. 5B, lanes 2,4). Together these results suggest that MAE localization is not subject to direct regulation by CRM1 and RAS/MAPK signaling, but is determined by the presence or absence of nuclear binding partners YAN and PNT-P2 in accordance with changing signaling conditions.

MAE inhibits both the ability of YAN to repress transcription and the ability of PNT-P2 to activate transcription

Baker et al. (Baker et al., 2001) have proposed that overexpression of MAE inhibits the ability of YAN to repress transcription and stimulates the ability of PNT-P2 to activate transcription. Because their work placed these *Drosophila* proteins in a potentially physiologically inappropriate mammalian cultured cell environment, we felt it was important to test the function of MAE in the *Drosophila* system used in our assays. With respect to regulation of YAN-mediated repression, our results concur with those of Baker et al. (Baker et al., 2001). In *Drosophila* S2 cells, overexpression of MAE inhibited YAN-mediated transcriptional repression, and slightly enhanced the RAS^{V12}-mediated removal of transcriptional repression (Fig. 6A).

However, our results disagree with the conclusion of Baker et al. (Baker et al., 2001) that MAE stimulates the ability of PNT-P2 to activate transcription. We found that overexpression of MAE completely inhibited PNT-P2 mediated activation of transcription (Fig. 6B). Therefore, MAE could have a role in downregulating, rather than stimulating, the ability of PNT-P2 to activate transcription.

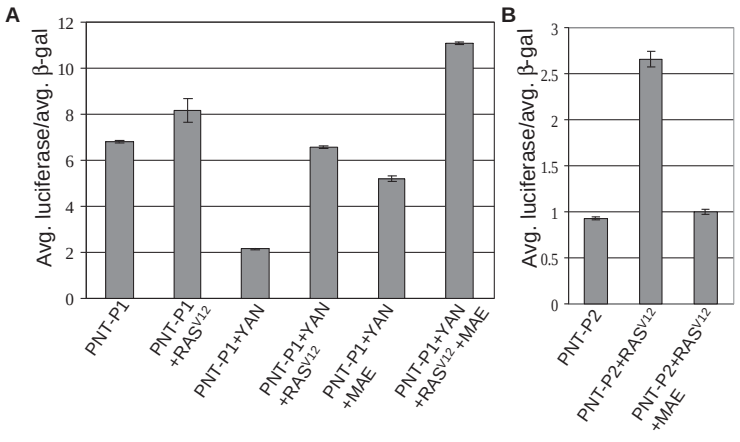

Fig. 6. MAE inhibits the ability of both YAN and PNT-P2 to regulate transcription. (A) Transcriptional repression assays with YAN. (B) Transcriptional activation assays with PNT-P2. Overexpression of MAE inhibits YAN-mediated transcriptional repression (A) and PNT-P2-mediated activation (B).

DISCUSSION

Precisely modulated competition between the two ETS-domain transcription factors POINTED and YAN plays a critical role in determining specific differentiative and proliferative responses to RTK signaling. We demonstrate that CRM1-mediated nuclear export of YAN is an essential step in its downregulation, and that this process requires a functional interaction between YAN and MAE. Our results suggest a second unexpected role for MAE in downregulating PNT-P2 to prevent uncontrolled signaling in response to RTK activation. Thus, we propose that MAE participates at multiple independent steps in the cellular mechanisms that fine-tune the levels of POINTED and YAN activity in accordance with changing RTK signaling conditions.

Regulation of YAN localization in the absence of RAS/MAPK activation: achieving a balance between nuclear retention and nuclear export

In unstimulated or undifferentiated cells, YAN localizes to the nucleus (Lai and Rubin, 1992; Rebay and Rubin, 1995). For both YAN and its mammalian ortholog TEL, the DNA-binding domain serves as a nuclear localization sequence (NLS) (I. R., unpublished) (Poirel et al., 1997). We have shown that upon RTK stimulation, YAN is actively exported from the nucleus via CRM1 recognition of its N-terminal NES motif. The presence of both NLS and NES motifs within YAN raises the question of how each domain is either recognized or masked under different signaling conditions.

Our results lead us to propose that proper YAN subcellular localization involves dynamic regulation of its DNA-binding affinity via modulation of protein-protein interactions in response to changing RTK signaling levels. Consistent with this model, we find that nuclear localization requires that YAN be bound to the DNA, as a mutation that abolishes DNA binding (Kodandapani et al., 1996), $YAN^{Mut\ ETS}$, results in CRM1-dependent cytoplasmic accumulation of YAN. The PD, an N-terminal protein-protein interaction motif, also plays a pivotal role in determining the subcellular localization of YAN, as loss of the PD ($YAN^{\Delta NES3+PD}$) results in partial CRM1-mediated export in the absence of signaling. In addition, $YAN^{\Delta NES3+PD}$ exhibits a 30% decrease in repression activity relative to wild-type YAN, suggesting a weaker or less productive interaction with DNA. Together these data suggest that PD-mediated protein-protein interactions may be crucial in facilitating productive DNA binding and/or masking inappropriate CRM1 recognition of the NESs.

Our finding that PD-mediated interactions are crucial for the transcriptional repression ability of YAN agrees with similar experiments with TEL (Lopez et al., 1999), but disagrees with the results of Baker et al. (Baker et al., 2001) who find that compromised PD function has no significant effect on the transcriptional repression of YAN. Presumably, this discrepancy reflects the use of the mammalian Cos7 cell line to study YAN (Baker et

al., 2001), as opposed to the more physiologically relevant *Drosophila* S2 cell line used in our experiments.

One explanation for how the PD of YAN might be involved in DNA-binding affinity, transcriptional repression and maintenance of nuclear localization comes from structural studies of the PD of TEL. This work suggests that DNA binding and transcriptional repression may be mediated by a PD-PD homo-oligomeric complex of TEL that wraps the target DNA around itself (Kim et al., 2001). Because the residues necessary for TEL oligomerization are conserved in YAN (Jousset et al., 1997), and YAN has been shown to self-associate via its PD (I. R., unpublished), it is possible that oligomerization of YAN could be critical for DNA binding/nuclear localization.

In addition to promoting homotypic YAN-YAN interactions, PD-mediated binding to heterologous proteins may also influence YAN localization and activity. MAE, the only protein known to interact with the PD of YAN (Baker et al., 2001), appears to serve such a function. Co-immunoprecipitation experiments confirmed that MAE can bind to YAN in the absence of signaling, and showed that the complex is destabilized in the presence of RAS/MAPK activation. However, because MAE inhibits YAN-mediated transcriptional repression, we expect that, in the absence of signaling, not all YAN will be bound to MAE. The finding that MAE can also be co-immunoprecipitated with PNT-P2, suggests a mechanism for sequestering MAE away from YAN to allow efficient repression and prevent inappropriate differentiation in the absence of signaling.

Regulation of YAN localization in response to RAS/MAPK activation: shifting the balance towards nuclear export

Upon activation of the RAS/MAPK cascade, dual phosphorylated MAPK enters the nucleus and phosphorylates YAN, triggering a cascade of events that ultimately leads to the removal of transcriptional repression. Recent work by Baker et al. (Baker et al., 2001) demonstrated that MAE is needed for MAPK-mediated phosphorylation of YAN at Serine127 in vitro, the same site previously shown to be critical for initiating YAN downregulation both in cell culture and in vivo (Rebay and Rubin, 1995). Our study sheds new light on the sequence of steps in this process.

We show that CRM1-mediated nuclear export is a necessary step in downregulation of YAN. How is this achieved? Our results support a model whereby in response to pathway stimulation, the PNT-P2-MAE complex is phosphorylated, releasing PNT-P2 to activate transcription and MAE to interact with YAN. Binding to MAE inhibits the transcriptional repression of YAN (this work), and may facilitate phosphorylation of serine127 by activated MAPK (Baker et al., 2001), although the order in which these two events happen remains to be determined. Our data suggest MAE then plays a third role in presenting YAN to CRM1, thereby promoting nuclear export.

In support of this model, loss of *mae* function, both in vivo and in cell culture, restricts YAN to the nucleus. However, as MAPK phosphorylation of YAN is a prerequisite for export (Rebay and Rubin, 1995) and requires MAE (Baker et al., 2001), our result could simply reflect a failure of YAN to be phosphorylated. Arguing against this, RNAi of *mae* also results in nuclear retention of $YAN^{Mut\ ETS}$, which normally localizes to the cytoplasm in a CRM1-dependent manner, even in the absence of RAS stimulation. Thus, in a situation where MAPK phosphorylation is not involved, MAE plays an active role in presenting YAN to CRM1. We therefore favor the interpretation that MAE has an essential function in regulating nuclear export, independent of its earlier postulated role in facilitating MAPK phosphorylation of YAN.

These same two events mediated by MAE, MAPK phosphorylation and CRM1 recognition of YAN, in turn lead to destabilization of the YAN-MAE complex. For example, inhibition of CRM1-mediated export results in MAE remaining nuclear when co-transfected with YAN, even upon RAS^{V12} stimulation. Because we have shown that MAE localization is not directly regulated by CRM1 or by RAS pathway activation, we interpret this result to indicate that CRM1 is needed to disrupt the YAN-MAE complex. It has recently been shown that in certain cases, phosphorylation of the cargo protein is necessary for CRM1 recognition (Ishida et al., 2002). In agreement with this, in the presence of RAS^{V12}, MAE remains nuclear when expressed with YAN^{ACT}, which has all the MAPK phosphoacceptor residues mutated to alanine. This leads to the model that phosphorylation of YAN, when in the YAN-MAE complex, leads to interaction with the exportin CRM1. This in turn disrupts the YAN-MAE complex, with YAN being actively exported by CRM1, and MAE being free to diffuse uniformly throughout the cell.

A negative feedback loop attenuates PNT-P2 activity in response to RTK signaling

The ultimate outcome of this complex series of events is abrogation of YAN-mediated repression of target genes and freeing the promoters for interaction with POINTED. In unstimulated cells, unphosphorylated PNT-P2 localizes to the nucleus in a complex with MAE, but is effectively out competed for binding to target gene promoters by YAN (Flores et al., 2000; Halfon et al., 2000; Xu et al., 2000). Upon activation of the RAS/MAPK cascade, phosphorylation of PNT-P2 transforms it into a potent transcriptional activator (O'Neill et al., 1994). Baker et al. (Baker et al., 2001) show in vitro experiments in which MAE binding to PNT-P2 leads to activation of transcription, and assume that this occurs via MAE promoting MAPK phosphorylation, and hence activation, of PNT-P2. However Seidel and Graves (Seidel and Graves, 2002) demonstrate that PNT-P2 contains a MAPK binding site, suggesting PNT-P2 interacts directly with MAPK without requiring a facilitator protein. Consistent with this second scenario, we find that MAE inhibits PNT-P2 transcriptional activation. However, it is formally possible that MAE could have dual and antagonistic roles with respect to PNT-P2 regulation, first stimulating its activity by promoting MAPK phosphorylation and later limiting its ability to activate transcription. Definitive validation of either model will require in vivo analysis of the role of MAE with respect to PNT-P2 regulation.

Superficially, this proposed role in antagonizing PNT-P2 function seems to disagree with the finding that loss of *mae* function suppresses the rough eye phenotype of $Sev\text{-}RAS^{V12}$. However, in the absence of MAE, YAN cannot be downregulated. Thus, the effect of loss of *mae* function on PNT-P2 regulation is irrelevant in this context, as the target

sites will still be occupied by YAN. However, the dual function of MAE as both a positive and a negative regulator of RTK signaling may explain the relatively weak suppression of Sev-RAS^{V12} and the fact that it has not been isolated in any of the numerous RTK pathway based genetic modifier screens.

In summary, our data lead to a model (Fig. 7) in which, in unstimulated cells, YAN binds with high affinity to the DNA (Fig. 7A) and blocks PNT-P2 from contacting and activating the promoters of downstream target genes (Fig. 7D). Upon stimulation by RAS, MAPK phosphorylation of YAN and PNT-P2 allows CRM1 to interact with and export YAN, in a process that disrupts YAN and MAE binding (Fig. 7C) and disrupts the PNT-P2-MAE complex, allowing PNT-P2 to bind to the DNA and activate transcription (Fig. 7E). Free MAE could then interact again with PNT-P2, resulting either in its removal from the DNA, inhibition of transcriptional activation or interaction with a phosphatase that returns it to an inactive state (Fig. 7F). Thus, a negative feedback loop would be created to prevent runaway signaling by PNT-P2. An alternative, and not necessarily mutually exclusive, mechanism with respect to PNT-P2, is that the interaction of MAE with PNT-P2 might prevent efficient phosphorylation by MAPK, thereby limiting the pool of activated PNT-P2 and keeping the signaling response in check. It is likely that additional co-factors that bind MAE, YAN and/or PNT-P2 will be required for fine-tuning activation and downregulation in response to changing RTK signaling conditions.

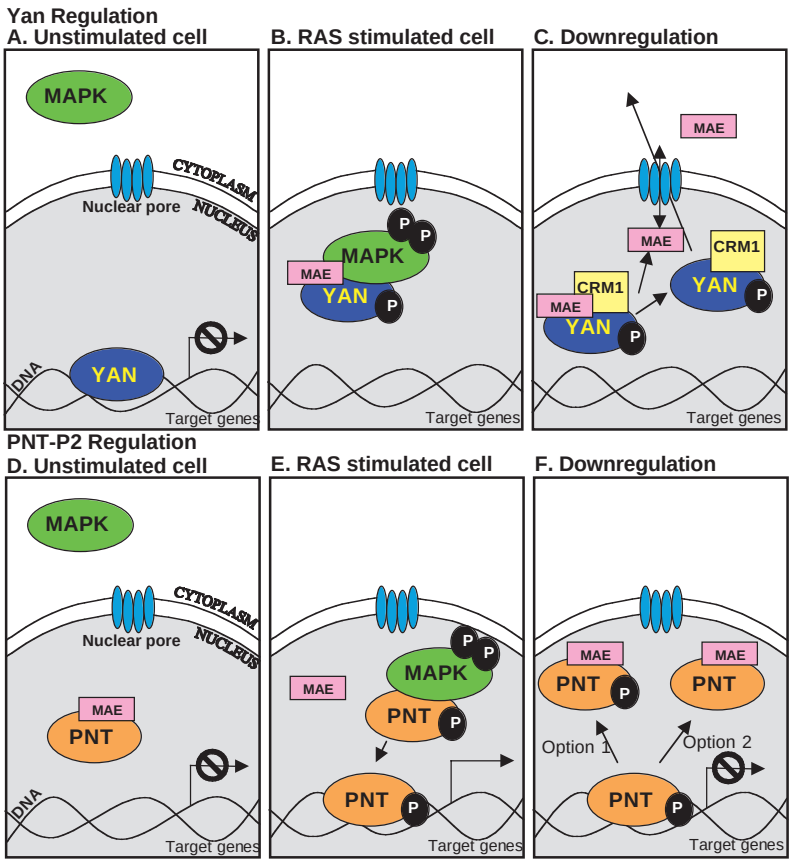

Fig. 7. (A-C) The model for the downregulation of YAN. (D,E) The model for the activation and subsequent downregulation of PNT-P2. (A) In the absence of signaling, YAN localizes to DNA, repressing transcription. (B) Upon RTK signaling, phosphorylated MAPK enters the nucleus, interacts with YAN-MAE complex and phosphorylates YAN. YAN is removed from the DNA, although the exact timing of this event is not yet clear. (C) The YAN-MAE complex then interacts with CRM1, causing release of MAE and CRM1 mediated export of YAN through the nuclear pore. (D) In the absence of signaling, PNT-P2 can bind to MAE and is prevented from activating transcription, either as a consequence of its interaction with MAE or because it is out competed by YAN, or both. (E) Upon RTK activation, phosphorylated MAPK enters the nucleus and phosphorylates PNT-P2. This allows PNT-P2 to bind DNA and activate transcription of the target genes now freed from YAN repression. (F) To prevent runaway signaling, a negative feedback loop may occur in which MAE binds to PNT-P2 and inhibits transcriptional activation. This could occur by MAE binding causing PNT-P2 to no longer bind DNA (1), or by MAE binding resulting in dephosphorylation of PNT-P2 (2), resulting in inhibition of transcriptional activation.

Evolutionarily conserved mechanisms of YAN downregulation

Precise regulation of RTK pathway activity appears crucial for achieving a proper balance between cellular proliferation, differentiation and survival in all metazoan animals. Excessive or continuous activation of the pathway has been linked to carcinogenesis in mammals, underscoring the importance of tightly controlled signaling. For example, numerous deletions and translocations involving TEL, the mammalian ortholog of YAN, have been associated with leukemias, and in some cases with solid tumors (reviewed by Rubnitz et al., 1999). Our studies indicate striking similarities between the regulation of TEL and YAN. Like YAN, TEL localizes to the nucleus (Poirel et al., 1997), where it functions as a transcriptional repressor (Lopez et al., 1999). YAN and TEL both require the PD for maintaining nuclear localization and transcriptional repression (YAN$^{\Delta N3+PD}$, this study) (Chakrabarti et al., 2000). Both proteins become phosphorylated in response to activation of signaling cascades (O'Neill et al., 1994; Poirel et al., 1997). Although the functional consequences of TEL phosphorylation remain to be investigated, our results predict that phosphorylation may downregulate TEL repression activity.

In the context of TEL downregulation, it is interesting to note that no mammalian orthologs of *mae* have been identified yet. However, a second mammalian TEL-like gene, referred to as TEL2 or TELB, has been isolated (Gu et al., 2001; Poirel et al., 2000). TEL2 also functions as a transcriptional repressor, is capable of oligomerizing with itself and with TEL, and may thus serve as a regulator of TEL (Poirel et al., 2000; Potter et al., 2000). Of particular interest with respect to our work defining the role of MAE, TEL2 encodes six splice variants, one of which, TEL2a, yields a protein with just the PD (Gu et al., 2001). TEL2a closely resembles the structure of MAE, and BLAST results show that the PD of MAE is most closely related to the PD of TEL2, with 39% identity and 51% similarity. Thus, it seems likely that TEL2a may regulate TEL

activity by a mechanism similar to that used by MAE for regulating YAN. With respect to the interactions we have demonstrated between PNT-P2 and MAE, it will be interesting to investigate whether TEL2a also interacts with and regulates other PD containing ETS family transcriptional activators, such as ETS1, the mammalian ortholog of PNT-P2.

Confocal and scanning electron microscope work was performed in the W. M. Keck Biological Imaging Facility. We thank the Bloomington Stock Center for providing *Drosophila* stocks, the Developmental Studies Hybridoma Bank for providing hybridoma lines, Rick Fehon for the gift of Mab 9E10, and A. Williams for assistance in generating the transgenic lines. We also thank the members of the Rebay laboratory for help and advice throughout the project. We thank R. Fehon, T. Jacks, T. Orr-Weaver, S. Silver and M. Voas for comments on the manuscript. I. R. is a recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences and is a Rita Allen Foundation Scholar. T. L. T. is supported by a Ludwig Fellowship. This work was supported in part by American Cancer Society Grant RPG-00-308-01-DDC.

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