Regulation of cell fate by phosphorylation: A tale of two transcription factors

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Post-translation modifications, particularly phosphorylation, are the main mechanisms by which signaling cascades regulate downstream transcription factors to execute changes in gene expression. We have focused on understanding the roles of phosphorylation in regulating the two transcription factors, YAN and Eyes absent (EYA).

We have been interested in determining the mechanisms of downregulating the *Drosophila* protein YAN, a transcriptional repressor of the ETS family of transcription factors that antagonizes receptor tyrosine kinase (RTK)/RAS/MAPK signaling. Under conditions of minimal RTK induction YAN outcompetes Pointed (PNT), an ETS transcriptional activator, for access to the DNA, resulting in transcriptional repression, thereby preventing inappropriate differentiation or proliferation. Upon RAS/MAPK stimulation, MAPK-mediated phosphorylation of YAN results in abrogation of YAN repressor activity, allowing PNT to activate transcription of formerly repressed genes. We show (Chapter 2) that MAE, which has been previously implicated in mediating the MAPK phosphorylation of YAN and PNT, plays a separate role in mediating the CRM1 dependent nuclear export of YAN, an essential step in the downregulation of YAN. In addition, MAE has been implicated in an inhibitory feedback loop that attenuates PNT activation. Further work (Chapter 3 and Appendix III) has shown that overexpression of MAE in S2 cells and the presumptive embryonic central nervous system inhibits nuclear export of YAN. However, overexpression in the eye does not exhibit YAN being aberrantly retained in the nuclei, but instead it results in an array of phenotypes that can be rescued by overexpression of PNT. Thus MAE appears to play a role in downregulation of PNT in vivo. Interesting, we find that *mae* transcription is regulated by YAN and PNT, adding further complexity to the signaling cascade. Whereby the regulator, MAE, becomes the regulated.

In addition to studying the mechanisms underlying the function and regulation of YAN, YAN has been used as a tool to identify other nuclear components downstream of RTK signaling. Through a genetic interaction screen *eyes absent (eya)* mutants were identified as enhancers of the rough eye phenotype of constitutively active YAN. EYA is a transcriptional coactivator in the Retinal Determination (RD) Network, which encompasses a signaling cascade of transcriptional regulators best known for their necessity during *Drosophila* eye development. Loss of function mutants exhibit an eyeless phenotype, while overexpression either alone or in conjunction with other RD members results in ectopic eye tissue. While one outcome of this signaling cascade is eye formation, null mutations within this network exhibit lethality, suggesting further roles during development. Homology between the EYA Domain, one of the evolutionarily conserved domains of EYA, and haloacid dehalogenases suggested that EYA, in addition to acting as a transcriptional coactivator, could be functioning as a phosphatase. We have shown that EYA is a protein tyrosine phosphatase (Chapter 4). Phosphatase activity is necessary for EYA function, as phosphatase mutants cannot rescue *eya* mutant phenotypes or induce ectopic eyes at a comparable level to wild-type EYA. We also show that EYA is tyrosine phosphorylated and serves as a substrate for itself. Continuing efforts to understand the roles of tyrosine phosphorylation in regulating EYA have revealed that EYA is likely to by tyrosine phosphorylated within the ED, and Y719 may be a site of phosphorylation (Chapter 5). Thus

EYA possesses two functions, as a transcriptional coactivator and as a protein tyrosine phosphatase, that are potentially regulated by tyrosine phosphorylation.

Phosphorylation of YAN has been shown to play a key role in its downregulation, while the effects of tyrosine phosphorylation on EYA remain to be determined. It is important to note that phosphorylation, in the case of YAN and likely in the case of EYA as well, is only one step in a complex sequence of events required for the proper developmental outcome to occur. It is especially intriguing that EYA, a known transcriptional regulator, possesses phosphatase activity, and can regulate its own phosphorylation. Thus we have a case in which an enzyme responsible for altering post-translational modifications, likely as a downstream effector of signaling events, also mediates the transcriptional response.

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Abstract

As transcription factors are the nuclear effectors of signaling cascades, mechanisms must exist to specifically regulate their activities in response to signaling events. Regulation can occur at the DNA, RNA, or protein level. Many transcription factors are present in cells and tissues before they are needed, allowing quick response to extracellular stimuli. In these cases the activity of the transcription factor is regulated at the protein level. A wide variety of post-translational modifications are known to play critical roles in regulating transcription factors, and are capable of altering the localization, stability, interactions, other post-translational modifications, and activity of a protein. The combinations of modifications that can occur on an individual protein yield immense regulatory possibilities. Aside from analyses of the effects of serine/threonine (S/T) phosphorylation, studies on post-translational modifications of transcription factors are only in the beginning stages.

The current paradigm that an enzyme activated in response to upstream signaling will localize to the nucleus and modify specific transcription factors is an over-simplification. Intriguingly, there appears to be an emerging theme in which the transcription factor itself possesses a second, enzymatic function. This creates an entirely new set of regulatory mechanisms that emanate locally from the transcriptional complexes themselves. Thus transcription factors are not passive on/off switches controlled by signaling pathways, but are active players in determining the activity, output, and regulation of transcriptional complexes. Here I will review the various post-translational modifications that are mediated by such dual function transcription factors and other signaling regulated enzymes, and provide specific examples of how such modifications regulate the activities of ETS transcription factors. Then I

will review the enzymatic functions of the transcriptional regulators EYA, DBP1, ERK5, p300, and $TAF_{II}250$.

Post-translational modifications influence transcription factor activity: A view from the ETS superfamily

ETS transcription factors have a wide range of roles throughout development including regulation of cell proliferation, differentiation, migration, apoptosis, and mesenchymal-epithelial interactions (Maroulakou and Bowe 2000). The majority of ETS transcription factors function as transcriptional activators, while some possess repressive activities (Mavrothalassitis and Ghysdael 2000), and others are capable of acting as both activators and repressors in a context dependent manner (Sharrocks 2001; Rohrbaugh et al. 2002; Wei et al. 2003; Yang et al. 2003).

The founding ETS transcription factor was identified as the oncogene transduced by the avian retrovirus E26, and thus ETS stands for E twenty-six (Karim et al. 1990; Laudet et al. 1999; Sharrocks 2001). There are approximately thirty ETS transcription factors in mammals, and multiple phylogenic analyses suggest these fall into eleven to thirteen groups (Laudet et al. 1999; Lim et al. 1999; Sharrocks 2001). There are eight ETS transcription factors in *Drosophila* (Hsu and Schulz 2000), providing a unique representation of the major mammalian groups. Thus, the functional redundancy that complicates the analysis of ETS transcription factors in mammals is not present in *Drosophila.*

An approximately eighty-five amino acid domain termed the ETS DNA binding domain or ETS domain characterizes ETS transcription factors. The ETS domain belongs to the superfamily of winged helix-turn-helix (HTH) DNA binding proteins. Structural analysis of various ETS transcription factors indicates that the DNA binding domain is made up of three α

helices and four β sheets. The third α helix contacts the major groove of the DNA where it interacts with the central GGAA/T motif, while additional contacts are made by the loop between β strands 3 and 4 (the "wing") and the loop between α helices 2 and 3 (Donaldson et al. 1996; Kodandapani et al. 1996; Sharrocks et al. 1997; Werner et al. 1997; Batchelor et al. 1998; Mo et al. 1998; Mo et al. 2000; Sharrocks 2001).

The core recognition sequence for ETS transcription factors, referred to as the ETS binding site (EBS), is GGAA/T. Sequences flanking this core EBS are variable and contribute to the specificity of individual ETS transcription factors (Bosselut et al. 1993; Shore et al. 1996; Sharrocks et al. 1997; Pio et al. 1999; Mo et al. 2000; Lelievre et al. 2001).

One-third of ETS transcription factors also contain a conserved amino-terminal domain called a pointed domain (PD). PDs belong to the Sterile Alpha Motif (SAM) family, and are involved in both homo and hetero-typic protein-protein interactions (Stapleton et al. 1999). Functions associated with PDs of ETS transcription factors include homooligomerization in the case of human TEL (Kim et al. 2001) and its *Drosophila* homolog YAN (J. Bowie, personal communication), heterodimerization, as exemplified by TEL - FLI-1 interactions (Kwiatkowski et al. 1998), and transrepression, documented for both TEL (Lopez et al. 1999) and YAN (Tootle et al. 2003a). PDs can also be the site of regulation by extracellular signaling pathways via MAPK mediated phosphorylation, as is the case for PNT-P2, ETS-1, ETS-2 and YAN (see below) (Lelievre et al. 2001).

Some ETS transcription factors also contain inhibitory domains that flank their ETS domain, and thus regulate their DNA binding affinity (Hagman and Grosschedl 1992; Jonsen et al. 1996; Skalicky et al. 1996; Cowley and Graves 2000; Greenall et al. 2001; Lelievre et al. 2001; Garvie et al. 2002; Wang et al. 2002). In the absence of DNA, the amino-terminal

inhibitory domain binds to the ETS domain and the carboxy-terminal inhibitory domain, blocking DNA binding. The inhibitory domains can be positively or negatively regulated by phosphorylation, as is the case for ETS-1 (see below), and positively or negatively regulated by protein-protein interactions (Lelievre et al. 2001; Sharrocks 2001).

ETS transcription factors exhibit overlapping expression patterns, and as they bind to similar or even identical DNA binding sites, their specificity must be regulated by other means. One common mechanism of regulating the specificity of ETS transcription factors is by their interactions with other transcriptional regulators (Lelievre et al. 2001; Sharrocks 2001). ETS transcription factors interact with other DNA binding transcription factors, such as AP-1 (JUN/FOS) (Bergelson and Daniel 1994; Wu et al. 1994), NFκB (Gri et al. 1998), and PAX family members (Plaza et al. 1994; Fitzsimmons et al. 1996), and the resulting complex then recognizes juxtaposed binding sites for the two transcription factors, increasing their respective DNA binding affinities and adding to their specificity (Li et al. 2000; Lelievre et al. 2001; Sharrocks 2001). In addition, interactions with non-DNA binding transcription factors and co-activators (Janknecht and Nordheim 1996; Yang et al. 1998a; Yamamoto et al. 1999; Yamamoto et al. 2002; Goel and Janknecht 2004) or co-repressors (Chakrabarti and Nucifora 1999; Guidez et al. 2000; Yang et al. 2001; Suzuki et al. 2003) can also contribute to DNA binding specificity, by altering the conformation of the ETS transcription factor (Lelievre et al. 2001; Sharrocks 2001).

ETS transcription factors are the nuclear targets of many extracellular signaling events. Thus, the other common mechanism of altering the specificity of ETS transcription factors is regulation by signaling cascades, often resulting in phosphorylation of ETS transcription factors. Phosphorylation can regulate the subcellular localization, protein-protein interactions, the DNA

binding ability, and the transcriptional activity of ETS transcription factors (see below for specific examples).

The specificity and thus the activity of ETS transcription factors are also affected by post-translational modifications besides phosphorylation. Below, I summarize what is known about the various post-translational modifications, phosphorylation, ubiquitination, sumoylation, acetylation, methylation, and glycosylation, and their affects on ETS transcription factors.

Phosphorylation

Phosphorylation is by far the best-studied post-translational modification, and many ETS transcription factors are regulated by phosphorylation. I will discuss general information on phosphorylation, discuss mitogen activated protein kinase (MAPK)-mediated serine/threonine (S/T) phosphorylation, overview the ETS transcription factors that are S/T phosphorylated, and provide specific examples of how phosphorylation regulates YAN and PNT-P2, ETS-1, and TEL. Lastly I will discuss tyrosine (Y) phosphorylation, a modification that has not been studied intensively in the context of nuclear transcription factors, but in light of work on EYA (Chapters 4 and 5), will likely prove relevant to this context.

General information about phosphorylation

Phosphorylation occurs by addition of a phosphate group to the hydroxyl group of serine (S), threonine (T), or tyrosine (Y) residues. The negative charge introduced by phosphorylation can affect the activity of the modified protein by inducing allosteric conformational changes, in addition to inducing repulsive and attractive forces (Holmberg et al. 2002). Thus single and multi-site phosphorylation can affect both protein-protein interactions and induce global changes in protein structure.

Two broad families of kinases mediate the phosphorylation of transcriptional regulators, S/T protein kinases, and Y kinases. Both types of kinases can be either receptor or membrane bound, or nonmembrane. In the majority of cases, transcription factors are modified by nonmembrane kinases. Like most post-translational modifications, phosphorylation is reversible. The enzymes responsible for dephosphorylation are phosphatases, which can be S/T, Y, or dual specificity phosphatases.

Phosphorylation and dephosphorylation can affect transcription factor function in a multitude of ways, including altering protein-protein interactions (either homo- or hetero- typic), subcellular localization, protein stability, DNA binding ability, and regulating transcriptional activity both positively and negatively (Whitmarsh and Davis 2000; Holmberg et al. 2002). Due to the large number of kinases, multiple signaling pathways can converge to regulate a particular transcription factor by differential phosphorylation at different or even the same residues (Holmberg et al. 2002). Thus multiple sites of phosphorylation add further potential for signal integration.

MAPK mediated S/T phosphorylation

While phosphorylation can occur at either S/T or Y residues, S/T phosphorylation as a means of regulating transcription factors appears more widespread and is better characterized. Mitogen activated protein kinase (MAPK) mediated S/T phosphorylation of transcription factors is a common mechanism by which extracellular signals regulate gene expression. There are three families of MAPKs: ERK, JNK, and p38 (Karin and Hunter 1995). Various receptors transduce the extracellular signals by utilizing both RAS dependent and independent means to activate a specific MAPK kinase kinase (MAPKKK), such as RAF, which in turn phosphorylates a MAPK kinase (MAPKK or MEK). MEKs, which are dual specific kinases, phosphorylate

MAPKs at both S/T and Y residues (Marshall 1994; Karin and Hunter 1995; Keyse 2000). Unphosphorylated MAPKs are tethered in the cytoplasm by MEKs, and phosphorylation of MAPKs result in release from this complex and subsequent nuclear localization, where MAPKs phosphorylate transcriptional regulators (Davis 1993; Marshall 1994; Karin and Hunter 1995; Keyse 2000; Kolch 2000). MAPKs thus provide the link between extracellular signaling and the corresponding changes in gene expression. It is thought that MAPKs are tethered in the nucleus by a protein whose expression is induced by the activated MAPK. This anchoring protein may be a phosphatase, retaining inactive MAPK in the nucleus to prevent reactivation by MEKs (Kolch 2000).

All MAPKs phosphorylate the same consensus sequence, with the strict consensus being P-X-S/T-P and the weak consensus being S/T-P (Davis 1993; Holland and Cooper 1999). However, each MAPK has different substrate specificities. ERK MAPKs are known to dock on the sequence LAQRRX_{4-5}L (Gavin and Nebreda 1999; Smith et al. 1999), while JNK docks on the sequence KX_2+X_4LXL, where + is R or K (Adler et al. 1992; Hibi et al. 1993; Holland and Cooper 1999). Delta domains, as these docking sites are referred to, are usually twenty residues away from the phosphorylation site. ETS transcription factors contain D domains, KX_2+X_3LXL, which are related to the delta domains (Bardwell and Thorner 1996; Yang et al. 1998b; Holland and Cooper 1999). The basic amino acids within both D and delta domains interact with the common docking (CD) domain in MAPKs, which is composed of a cluster of acidic amino acids at the C-terminus of the MAPK (Kolch 2000; Tanoue et al. 2001). CD domains were so named because they are the site of interaction with the activating MEKs, the MAPK's substrates, and the inactivating phosphatases (Kolch 2000; Tanoue et al. 2000). In addition to these docking domains, some ETS transcription factors contain a FXFP motif near the site of phosphorylation

that is involved in ERK binding (Holland and Cooper 1999; Jacobs et al. 1999; Kolch 2000). The D domain and the FXFP motif together have a combinatorial effect. D domains are recognized by both ERK and JNK MAPKs, while FXFP motifs are ERK specific (Holland and Cooper 1999). Both the kinase docking sites and the sequence context of the phosphorylation site contribute to the different specificities of the MAPKs (Yordy and Muise-Helmericks 2000).

S/T phosphorylation of ETS transcription factors

Many ETS transcription factors are known to be S/T phosphorylated (Table 1-1), including the PEA3 subfamily (PEA3, ER81, ERM) (Fitzsimmons et al. 1996; Janknecht 1996; Baert et al. 2002; Wu and Janknecht 2002), ERF (Le Gallic et al. 1999), GABPα (Imaki et al. 2003; Rosmarin et al. 2004), TCF subfamily (ELK-1, SAP1a, SAP1b, FLI-1, NET) (Yang et al. 1998b; Ducret et al. 2000; Wang and Prywes 2000; Yordy and Muise-Helmericks 2000), PU.1 (Pongubala et al. 1993; Yordy and Muise-Helmericks 2000), the subfamily made up of ETS-1, ETS-2, and POINTED-P2 (PNT-P2) (Wasylyk et al. 1998; Yordy and Muise-Helmericks 2000), LIN-1 (Tan et al. 1998; Sharrocks 2001), and the subfamily made up of YAN and TEL (Wasylyk et al. 1998; Yordy and Muise-Helmericks 2000). I will specifically discuss how YAN and PNT-P2, ETS-1, and TEL are regulated by phosphorylation

YAN and PNT-P2

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). *pnt* encodes two separate proteins (Klambt 1993), one which functions as a constitutive transcriptional activator, PNT-P1, and one which is a transcriptional activator that requires phosphorylation by MAPK in response to RTK/RAS signaling for activity, PNT-P2 (O'Neill et al. 1994). *yan* encodes a transcriptional repressor that competes with PNT for access to the

Table 1-1

ETS transcription factors	Kinase	Effect of phosphorylation	Reference
ER81	PKA	Reduced DNA binding affinity but increased transcriptional activation	Wu and Jancknecht, 2002
	MAPK	Increased transcriptional activation	Wu and Jancknecht, 2002 Jancknecht, 1996
ERM	PKA	Increased transcriptional activation due to increased DNA binding affinity	Baert et al., 2002
ERF	MAPK	Nuclear export	Le Gallic et al., 1999
GABPα	MAPKs: ERK, p38, JNK	Increased transcriptional activation	Rosmarin et al., 2004 Imaki et al., 2003
ELK-1	MAPKs: ERK, p38, JNK	Increased DNA binding affinity and ternary complex formation	Yang et al., 1998
NET	JNK	Nuclear export	Ducret et al., 1998
SAP1	MAPK	Increased transcriptional activation	Wang et al., 2000
PU.1	Casein kinase II	Potentiates protein-protein interactions	Pongubala et al., 1993
ETS-1	Casein kinase II	Inhibition of DNA binding	Wasylyk et al., 1998
ETS-1 and ETS-2	MAPK	Increased transcriptional activation	Yang et al., 1996
LIN-1	MAPK	Loss of transcriptional repression	Tan et al., 1998
YAN	ERK	Loss of transcriptional repression and nuclear export	O'Neill et al., 1994
	JNK	Nuclear export	T. Tootle, unpublished observation
	p38	Unknown	F. Hsiao, personal communication
TEL	ERK	Loss of transcriptional repression and nuclear export	Maki et al., 2004
	p38	Loss of transcriptional repression	Arai et al., 2002

Table 1-1

Table listing the ETS transcription factors known to be phosphorylated and the consequences of the phosphorylation.

regulatory regions of target genes (Gabay et al. 1996). The coordinate regulation of these two antagonistic transcription factors, YAN and PNT, plays a key role in determining specific differentiative and proliferative responses to RAS/MAPK signaling.

The first evidence that RAS/MAPK signaling regulates YAN came from studies of the development of the *Drosophila* eye. YAN is expressed in the nuclei of undifferentiated cells and appears to act antagonistically to proneural signals. As cells begin to differentiate, YAN expression is lost, suggesting YAN may function to maintain cells in an undifferentiated state (Lai and Rubin 1992). Similar studies showed that PNT-P2 is expressed in these same undifferentiated cells and its expression remains until these cells express markers indicative of neuronal differentiation (Brunner et al. 1994). PNT-P2 contains a single MAPK phosphorylation consensus site, $PLT_{151}P$, that is phosphorylated by MAPK in vitro (Brunner et al. 1994). Analysis of mutating T151 to alanine (T151A) suggested that phosphorylation at this site is necessary for PNT-P2 function, as this mutant can not rescue the *pnt-/-* phenotypes. T151A when expressed in a wild-type background, actually inhibits endogenous, wild-type PNT-P2 function, indicating that T151A is competing either directly or indirectly with the wild-type protein (Brunner et al. 1994). Thus MAPK negatively regulates the transcriptional repressor YAN and positively, by direct phosphorylation, regulates the activator PNT-P2.

Loss of *pnt* function results in a complete loss of neuronal differentiation in the developing *Drosophila* eye (O'Neill et al. 1994). Genetic analysis showed that PNT functions downstream of RAS signaling, and transcription assays revealed that activated RAS, RAS^{V12}, or partially activated ERK, ERK^{SEM}, increase PNT-P2's ability to activate transcription (O'Neill et al. 1994). The T151A mutant transcriptional activity is not stimulated by either method,

indicating that ERK-mediated phosphorylation at T151 activates PNT-P2's transcriptional activity (O'Neill et al. 1994).

Utilizing these same transcription assays it was shown that YAN represses the transcriptional activity of PNT-P1, and that this repression is negatively regulated by RAS^{V12} and ERK^{SEM}. YAN has nine MAPK phosphorylation consensus sites (Baker et al. 2001). In the presence of RAS^{V12} or ERK^{SEM}, YAN exhibits a slower electrophoretic mobility, indicating a post-translational modification. Phosphatase treatment results in restoration of normal mobility, revealing that YAN is phosphorylated in response to RAS/MAPK signaling (O'Neill et al. 1994). Thus the phosphorylation state of YAN has an inverse relationship to YAN's activity as a transcriptional repressor.

Mutating eight of the putative phosphorylation sites in YAN to alanine results in constitutively active YAN, YAN^{ACT}, meaning that even in the presence of RAS/MAPK signaling YAN^{ACT} still represses transcription and thus inhibits differentiation (Rebay and Rubin 1995). Expression of YAN^{ACT} in *Drosophila* cultured cells revealed constitutive nuclear localization, while wild-type YAN is nuclear and becomes cytoplasmic upon addition of RAS^{V12} (Rebay and Rubin 1995). Analysis of mutations in specific putative phosphorylation sites showed that mutating the first phosphorylation site, S127A, inhibits cytoplasmic localization of YAN in response to RAS/MAPK signaling, while mutating sites 2-8 has little effect on localization (Rebay and Rubin 1995). Therefore, this cytoplasmic localization of YAN in response to RAS/MAPK signaling is likely to require phosphorylation at the first phosphorylation site, S127. Transcription assays have revealed that while the first phosphorylation site, S127, is required for RAS/MAPK pathway responsiveness, phosphorylation at the other sites is important for

modulating this response (Rebay and Rubin 1995). Thus MAPK-mediated phosphorylation of YAN at multiple sites is required for proper downregulation of YAN.

The translocation of YAN from the nucleus to the cytoplasm in response to RAS/MAPK signaling seen in cultured cells has never been observed during *Drosophila* development, but this translocation suggested that after YAN is phosphorylated by MAPK, it is exported to the cytoplasm. Subsequent analysis has shown that nuclear export is essential for downregulating YAN activity in vivo, as trapping YAN in the nucleus inhibits differentiation (Chapter 2) (Tootle et al. 2003a). Nuclear export of YAN is mediated by the exportin CRM1, which recognizes the three leucine rich nuclear export sequences (NESs) at the amino-terminus of YAN (Chapter 2) (Tootle et al. 2003a). All these data together lead to the model in which nuclear YAN represses differentiation, upon RAS/MAPK signaling ERK phosphorylates YAN resulting in removal of its transcriptional repression, activation of PNT-P2, and nuclear export of YAN. As cytoplasmic YAN is not seen in during development, it is likely that YAN is rapidly degraded, a model supported by the many high scoring PEST sequences in YAN (Lai and Rubin 1992). PEST sequences are proline, glutamic acid, serine, threonine rich regions known to target proteins to the proteasome for degradation (Rechsteiner and Rogers 1996).

How ERK interacts with PNT-P2 and YAN is not known. While the docking sites for ERK binding to PNT-P2 and YAN have not been characterized, PNT-P2 contains a LXIXXF motif that is likely to function as the ERK docking site (Seidel and Graves 2002). This motif is located in the PD next to the phosphorylation site. However, PDs, in general, do not serve as ERK docking sites (Seidel and Graves 2002).

There is also evidence indicating of an alternate docking mechanism in which MAE (Modulator of Activity of ETS) mediates the interactions between ERK and YAN, and ERK and

PNT-P2. MAE interacts with YAN and PNT-P2 by PD-PD mediated interactions (Baker et al. 2001). Transcriptional analysis in mammalian cultured cells revealed that both MAE and RAS^{V12} are required to remove YAN mediated repression and to activate PNT-P2 transcriptional activation (Baker et al. 2001). MAE-YAN interaction prevents YAN from binding to DNA and MAE is necessary for ERK mediated in vitro phosphorylation of S127 on YAN (Baker et al. 2001). MAE has also been shown to play a phosphorylation independent role in mediating the nuclear export and downregulation of YAN (Chapter 2) (Tootle et al. 2003a). There is a large amount of data suggesting that MAE indeed mediates ERK-mediated phosphorylation and downregulation of YAN. However the data indicating that MAE is also involved in mediating the phosphorylation of PNT-P2 (Baker et al. 2001) is highly contested. Two other laboratories have independently shown that MAE inhibits PNT-P2 mediated transcriptional activation, indicating that MAE is involved in the downregulation of PNT-P2, not its activation (Chapter 2) (Tootle et al. 2003a; Yamada et al. 2003). In vivo analysis has shown that MAE, indeed, negatively regulates PNT-P2 function (Chapter 3) (Yamada et al. 2003).

Recent work has provided a mechanism for how MAE mediates the phosphorylation of YAN. YAN, like its mammalian ortholog TEL, forms a PD-PD head to tail oligomer, and this oligomerization is required for transcriptional repression (Kim et al. 2001; J. Bowie, personal communication). MAE binding to the PD of YAN results in depolymerization of YAN, which should expose the critical S127 phosphorylation site, and thus would facilitate the ERK mediated phosphorylation of YAN (J. Bowie, personal communication). The residues implicated in mediating such polymerization are not conserved in PNT-P2 or ETS-1 (Kim et al. 2001). Thus if MAE facilitates ERK- mediated phosphorylation of PNT-P2 it is occurring by a different mechanism.

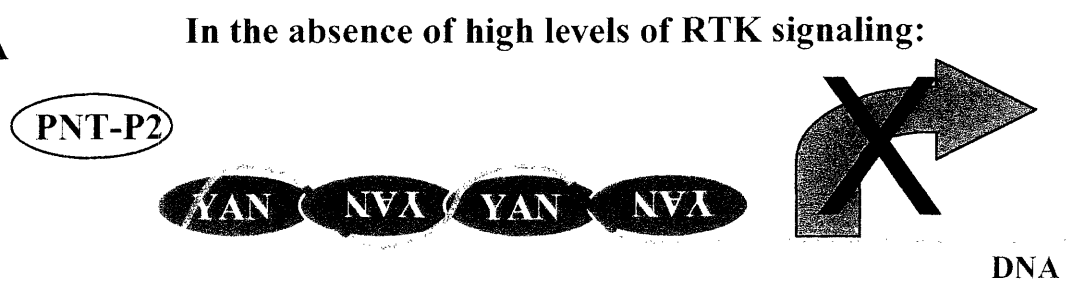
In the absence of high levels of RAS/MAPK signaling, YAN forms an oligomer (J. Bowie, personal communication), is bound to the DNA, repressing transcription and maintaining the cell in an undifferentiated state (Lai and Rubin 1992). Upon RAS/MAPK signaling, MAE facilitates the ERK-mediated phosphorylation of YAN and YANs removal from the DNA (Baker et al. 2001), most likely be depolymerizing YAN (Figure 1-1A). PNT-P2 is phosphorylated by ERK, binds DNA, and activates transcription, sending the cell down the road to differentiation (O'Neill et al. 1994). It remains possible that MAE is involved in the phosphorylation of PNT-P2 (Baker et al. 2001). Phosphorylated YAN in a complex with MAE then interacts with CRM1, resulting in dissociation of MAE and nuclear export of YAN (Tootle et al. 2003a). In a negative feedback loop, MAE inhibits PNT-P2 mediated transcriptional activation by an unknown mechanism (Tootle et al. 2003a; Yamada et al. 2003) (Figure 1-1B). This provides the cell with a precisely controlled mechanism by which to achieve the appropriate balance between transcriptional repression and activation according to the level of signaling.

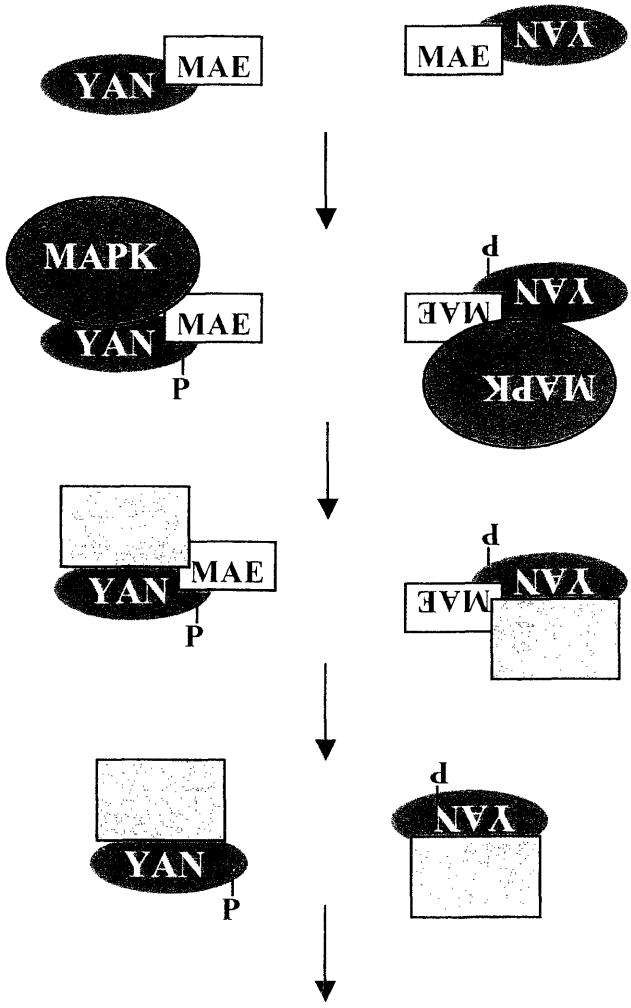
ETS-1

ETS-1 is the mammalian ortholog of PNT-P2. Like PNT-P2, ETS-1 is directly phosphorylated by ERK (Treisman 1996). Phosphorylation of ETS-1 at T38, analogous to T151 in PNT-P2, is required for ETS-1 to function with other transcription factors, including AP-1 and PIT-1, to activate RAS responsive elements (RREs) (Yang et al. 1996; Yordy and Muise-Helmericks 2000). Utilizing an EBS-reporter it was shown that RAS signaling-mediated phosphorylation of ETS-1 is necessary for transcriptional activity, but has no affect on protein localization or stability (Yang et al. 1996). This phosphorylation could be affecting ETS-1's DNA binding ability or protein-protein interactions with unknown coactivators. However, phosphorylation does not affect ETS-1's ability to interact with the coactivators p300/CBP

Figure 1-1

A



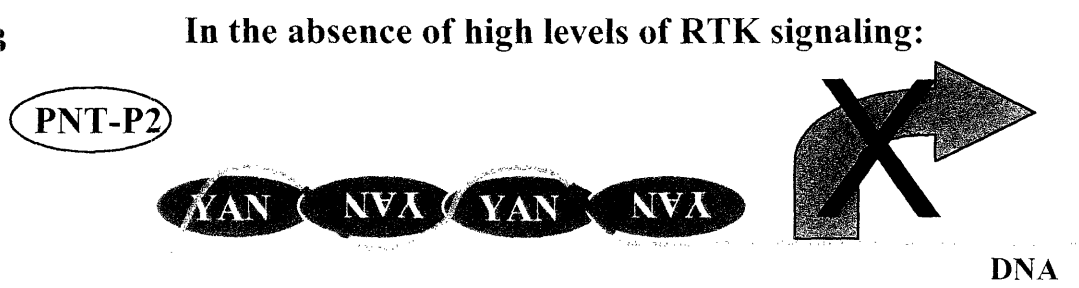


Nuclear export and degradation of YAN.

Transcriptional activation by phosphorylated PNT-P2.

Figure 1-1

B


In the absence of high levels of RTK signaling:
PNT-P2
YAN
YAN
YAN
YAN
DNA

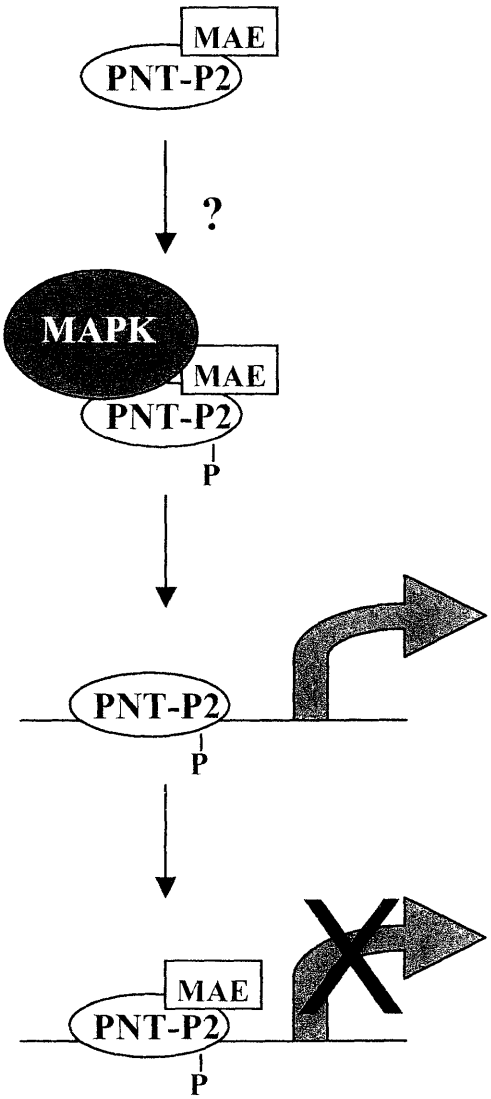

Upon high levels of RTK signaling:
MAE
PNT-P2
?
MAPK
MAE
PNT-P2
P
PNT-P2
P
MAE
PNT-P2
P

Figure 1-1

Model for how YAN and PNT are regulated in response to high levels of RTK signaling. A. The mechanisms by which YAN is downregulated in response to RTK signaling. In the absence of high levels of signal, YAN represses transcription by forming a homooligomer and wrapping the DNA around itself. Upon RTK signaling, MAE is activated or allowed by some unknown mechanism to interact with YAN, resulting in inhibition of YAN oligomerization. This MAE-YAN complex is likely to remove YAN from the DNA, and then MAPK can interact with the MAE-YAN complex, phosphorylating YAN. This results in YAN interacting with CRM1, ultimately leading to the nuclear export of YAN and its presumed degradation. B. The mechanisms by which PNT-P2 is activated and subsequently downregulated in response to RTK signaling. Upon high levels of signal, PNT-P2 is phosphorylated by MAPK, this may or may not be mediated by interactions with MAE. Phosphorylated PNT can then bind to the DNA and activate transcription. PNT-P2 is quickly downregulated by interaction with MAE, resulting in removal of transcriptional activation. The mechanism by which MAE downregulated PNT-P2 remains to be elucidated.

(Jayaraman et al. 1999). Both PNT-P2 and ETS-1 are positively regulated by direct phosphorylation by MAPK. Conversely, phosphorylation of ETS-1 by calcium calmodulin dependent protein kinase II (CAMK II) on six sites, D/E-S-F/Y-D/E, near the ETS DNA binding domain results in inhibition of DNA binding (Wasylyk et al. 1997; Yordy and Muise-Helmericks 2000; Sharrocks 2001). PNT-P2 lacks these consensus sites, and therefore is unlikely to be similarly regulated by calcium signaling (Wasylyk et al. 1997). Both PNT-P2 and ETS-1 are positively regulated by direct phosphorylation by MAPKs, while ETS-1 is also negatively regulated by phosphorylation in response to calcium signaling. It will be interesting to see whether other phosphorylation events negatively regulate the activity of PNT-P2.

TEL

TEL is the mammalian ortholog of YAN and the two appear to be regulated by similar mechanisms. TEL is phosphorylated constitutively at S22 and inducibly at S257. Like YAN, hyperphosphorylated TEL is exported from the nucleus in a CRM1 and PD dependent manner, but unlike YAN, TEL is not likely to be degraded as TEL is a very stable protein (Wood et al. 2003). p38 binds directly to and phosphorylates TEL, reducing its ability to repress transcription (Arai et al. 2002). Similarly, YAN is also phosphorylated by p38 in vitro (F. Hsiao, personal communication). ERK also phosphorylates TEL, at S113 and S257, removing TEL's transcription repression by decreasing its DNA binding ability (Maki et al. 2004). Mutating S113 and S257 to E (E213/257) in TEL, to mimic the negative charge of phosphorylation, results in the complete loss of transcriptional repression due to TEL's loss of DNA binding ability (Maki et al. 2004). Interestingly, E213/257 functions as a dominant negative, inhibiting wild-type TEL mediated transcriptional repression. As TEL can homooligomerize, it is likely that E213/257 oligomerizes with wild-type TEL, inhibiting DNA binding of the oligomer. Both

YAN and TEL are regulated by specific phosphorylation events that lead to removal of their transcriptional repressive activities and their nuclear export.

As MAE has been shown to play critical roles in mediating the downregulation of YAN, 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 (Poirel et al. 2000; Gu et al. 2001). As TEL2 functions as a transcriptional repressor, and is capable of oligomerizing with itself and with TEL, TEL2 may serve as a regulator of TEL (Poirel et al. 2000; Potter et al. 2000). Of particular interest with respect to MAE, TEL2 encodes six splice variants, one of which, TEL2a, yields a protein with just the PD (Gu et al. 2001). TEL2a structurally resembles 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. Therefore it seems likely that TEL2a could regulate TEL activity by a similar mechanism to what has been shown for MAE regulating YAN (Tootle et al. 2003a).

Y phosphorylation

While Y phosphorylation is a widespread mechanism of post-translational modification of membrane bound and cytoplasmic proteins (Hubbard and Till 2000), there is little evidence of tyrosine phosphorylation regulating transcription factors. STATs are the only transcription factors whose activities are known to be regulated by Y phosphorylation (Darnell 1997; Brivanlou and Darnell 2002; Calo et al. 2003). STATs are Y phosphorylated on a single Y in the cytoplasm by receptor tyrosine kinases (RTKs) and RTK associated non-RTKs. Y phosphorylation results in homo- or hetero-dimerization of STATs via reciprocal SH_2 domain-phosphotyrosine interactions, leading to nuclear localization and transcriptional activation due to increased DNA binding activity (Darnell 1997; Brivanlou and Darnell 2002; Kisseleva et al.

2002; Calo et al. 2003). Nuclear Y dephosphorylation results in inactivation of STATs (Darnell 1997).

Some non-RTKs have been shown to function in the nucleus. For example, c-ABL tyrosine kinase phosphorylates the CTD of RNA polymerase II, and such Y phosphorylation appears to function equivalently to S/T phosphorylation (Baskaran et al. 1999). Therefore, c-ABL-mediated tyrosine phosphorylation may contribute to the transition from initiation of transcription to elongation. Additionally, c-ABL is activated by DNA damaging agents, and regulates the activity of the proapoptotic transcription factor p73 by tyrosine phosphorylation (Agami et al. 1999; Gong et al. 1999a). Therefore, tyrosine phosphorylation is likely to play more direct roles in regulating transcription than presently understood.

Intriguingly v-SRC, a non-RTK, relieves TEL mediated repression by facilitating the nuclear export of TEL (Lopez et al. 2003). This removal of repression depends on v-SRC kinase activity and the amino-terminus of TEL, as the splice form TEL-M1 but not TEL-M43 is regulated in this manner. The amino-terminal region of TEL-M1 is not tyrosine phosphorylated as mutations of the tyrosine residues do not affect v-SRC mediated nuclear export (Lopez et al. 2003). It remains to be determined if v-SRC interacts with the amino-terminus of TEL-M1, resulting in the phosphorylation of tyrosine residues outside of this region.

Although no ETS transcription factor to date has been shown to be Y phosphorylated, it seems probable that Y phosphorylation may be a more widespread mechanism of transcriptional regulation than currently appreciated.

Ubiquitination/Ubiquitlyation

While there is little information on ubiquitination of ETS transcription factors, it is important to discuss the general mechanism and roles of ubiquitin modification, as it has recently been implicated in playing critical roles in regulating transcription factor activity (Figure 1-2). Brief examples of how ubiquitination regulates specific, non-ETS transcription factors, will be discussed, as it is likely that similar regulation of ETS transcription factors occurs.

There are two separate ubiquitin modification pathways: classic ubiquitin-dependent proteolysis, and ubiquitin mediated activation of transcription factors which can be proteolysis dependent or independent (Conaway et al. 2002). I will first briefly review the general information about ubiquitin and the enzymes responsible for the modification before discussing the roles of ubiquitination in regulating transcription factors.

General information on ubiquitination

Ubiquitin is a 9kDa globular protein that is covalently conjugated to lysine (K) residues of other proteins. In the majority of the studied cases ubiquitination targets these proteins for degradation by the multisubunit ATP dependent protease, the proteasome (Verger et al. 2003). E1 ubiquitin activating enzymes form a thioester bond with the carboxy-terminal glycine of ubiquitin in an ATP dependent process. The ubiquitin conjugating enzyme (UBC/E2) accepts ubiquitin from the E1 enzyme by a trans-thiolation reaction with the carboxy-terminus of ubiquitin. The transfer of ubiquitin from the E2 to the ε-amino group of a specific lysine (K) on the target protein is catalyzed by the E3 ubiquitin protein ligase (Desterro et al. 2000; Weissman 2001; Conaway et al. 2002; Pickart 2004). There are multiple E3 enzyme families, including HECT domain E3s, which directly transfer ubiquitin to the target protein, and RING finger and U box domain E3s (Hatakeyama et al. 2001; Hatakeyama and Nakayama 2003), which mediate

Figure 1-2

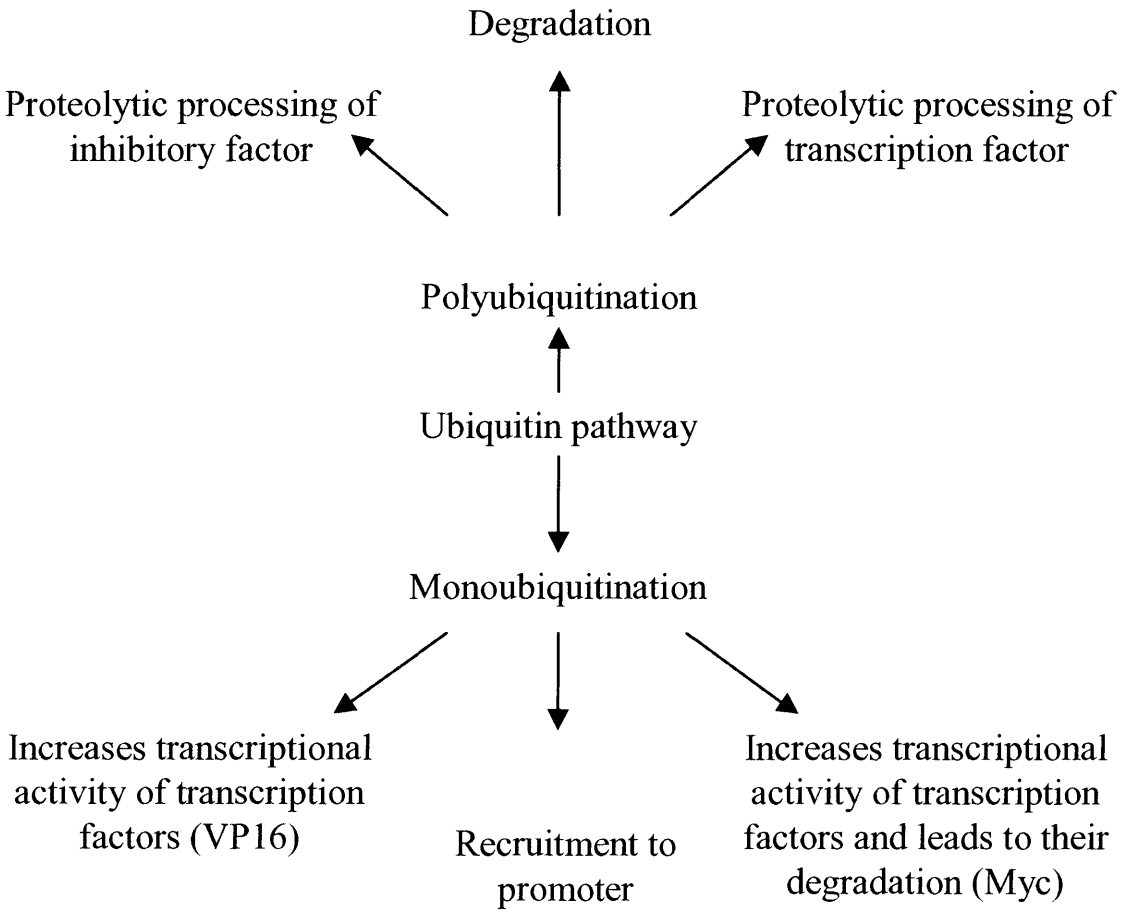


Figure 1-2

Diagram of the many functions the ubiquitin pathway plays in regulating transcription factors.

the transfer of ubiquitin from the E2 enzyme to the target protein (Weissman 2001; Greer et al. 2003). Specificity is largely determined by the E3 enzyme, alone or with the E2 enzyme (Weissman 2001). E4 enzymes polyubiquitinate proteins at sites of monoubiquitination. Ubiquitin-specific processing enzymes (UBPs), a type of deubiquitinating enzyme (DUB), cleave ubiquitin from proteins and disassemble multi-ubiquitin chains (Weissman 2001; Greer et al. 2003).

Degradation as a means of regulating transcription factors

Ubiquitin mediated degradation plays a large role in regulating transcription factor function. One regulatory strategy is to destroy transcription factors when they are no longer needed and thus ubiquitination is involved in their downregulation (Rodriguez et al. 2000; Muratani and Tansey 2003). Another strategy is to couple transcription factor activity tightly to proteolysis, allowing tight control over transcription (Muratani and Tansey 2003). Ubiquitination can also activate transcription factors by leading to the proteolytic processing of the transcription factor itself (Aza-Blanc et al. 1997), or the proteolysis of an inhibitory interacting protein (Palombella et al. 1994; Conaway et al. 2002; Muratani and Tansey 2003). Similarly ubiquitination can positively or negatively affect protein-protein interactions, in turn altering the transcriptional activity of the target factor (Conaway et al. 2002; Muratani and Tansey 2003). Most interesting is the role of ubiquitination in regulating transcriptional activation domains (TADs).

The half-life of a transcription factor is inversely correlated with the potency of its TAD. The TADs with the strongest activity are ubiquitinated and degraded by the proteasome the most rapidly (Molinari et al. 1999; Conaway et al. 2002). Intriguingly, transcription factors are most efficiently targeted for ubiquitin-mediated degradation when bound to DNA. This suggests there

is a close association between the degradation and general transcription machinery. In a growing number of cases the TAD is also the domain that serves as the degradation signal, or degron, that is recognized by the ubiquitin-mediated degradation machinery (Salghetti et al. 2000; Salghetti et al. 2001; Conaway et al. 2002; Muratani and Tansey 2003). TAD dependent ubiquitination of transcription factors not only targets the protein for destruction but is also required for transcriptional activation (Conaway et al. 2002).

Not all TADs are also degrons. Pulse-chase experiments revealed that activation domains in general do not signal for proteolysis (Salghetti et al. 2000). Most of the TADs that also serve as degrons are composed of a high percentage of acidic amino acids. Interestingly, degrons from nontranscriptional regulators can also function as TADs; these are not acidic domains but are known to be phosphorylated (Salghetti et al. 2000). Thus either the negative charge of the phosphorylation mimics that acidic TAD or the acidic TAD mimics the negative charge of phosphorylation. It is notable that two domains with very different purposes can perform the same functions, and indicates the importance of linking potent transcriptional activators to ubiquitin-mediated proteolysis.

Non-degradation roles of ubiquitination in transcriptional regulation

The first evidence that ubiquitination activates transcription factors came from the study of the viral synthetic activator VP16. VP16 dependent ubiquitination by SCF^{Met30} E3 ligase is required for both VP16 turnover and transcriptional activity (Salghetti et al. 2001; Conaway et al. 2002). Fusion of a single ubiquitin moiety to VP16 rescues the requirement of SCF^{Met30} E3 ligase in transcription, but has no effect on protein turnover (Salghetti et al. 2001). This suggests that monoubiquitination is enough to activate VP16. VP16 can bind to promoters in the absence of SCF^{Met30} E3 ligase, indicating that SCF^{Met30} E3 ligase may be targeted to VP16 on the DNA

(Salghetti et al. 2001). Thus, in addition to transcription factor TADs serving as degradation signals, components of the degradation machinery are recruited to promoters, emphasizing the need for the association of these two seemingly different processes, transcription and degradation. Analysis of VP16 in mammalian cells reveals that both ubiquitination and a functioning proteasome are required for efficient transactivation, indicating that transcription is associated with proteolysis (Zhu et al. 2004). Therefore, both recruitment of the E3 ligase to the promoter and the subsequent monoubiquitination of the transcription factor, and proteasome activity are required for VP16 transcriptional activation.

Evidence linking ubiquitination to transcription in higher eukaryotes comes from a study on the transcriptional coactivator CIITA, a MHC class II transactivator. CIITA is the master regulator of all members of the MHC class II gene complex and is involved in initiation, propagation and regulation of adaptive immune responses (Greer et al. 2003). Monoubiquitination of CIITA enhances the recruitment of CIITA to the complex on the promoter and thus facilitates transcriptional initiation. Additional ubiquitination results in polyubiquitination and degradation (Greer et al. 2003). Acetyltransferases and deacetyltransferases are involved in controlling the ubiquitination of CIITA (Greer et al. 2003). The acetyltransferases, CBP and P/CAF, enhance the ubiquitination and function as coactivators of CIITA; but it remains to be determined whether this is due to acetylation of CIITA or histones. There is evidence that p300/CBP can function as E4 ligases to mediate polyubiquitination (See above, dual function transcriptional regulators) (Grossman et al. 2003). Alternatively, acetylated histones might recruit ubiquitinating enzymes to chromatin. Ubiquitination plays two different roles in regulating CIITA, activating transcription and

mediating degradation. This example illustrates how various post-translational modifications and the enzymes that mediate them can coordinately affect transcription factor function.

Myc is another example of a transcription factor that is regulated by ubiquitination, both for protein turnover and transcriptional activity. Myc, with a half-life of about 30 minutes, is an unstable protein. Skp2, an F-box protein that is part of an E3 ligase, associates with the TAD of Myc, controlling Myc levels by ubiquitin-mediated degradation, and activating induction of Myc responsive genes (Jin and Harper 2003). Skp2 and Cul1, another part of the E3 ligase, co-immunoprecitate with Myc on the cyclin D2 promoter, as do 19S proteasome subunits and the α2 subunit of the 20S proteasome (Jin and Harper 2003). While the 19S subunit is known to possess transcriptional activity (see below), recruitment of subunits of the 20S proteosomal core is novel. It is possible that the entire proteasome is at the promoter. It is evident from these few examples that many components of the ubiquitination machinery and the 26S proteasome are implicated in having direct roles in mediating transcription.

Degradation machinery or general transcription factor?

As mentioned above, many components of ubiquitin-mediated degradation are recruited to promoters, where they appear to function beyond simply modifying transcription factors (Figure 1-3). For example, E3 ubiquitin ligases function as part of the RNA polymerase II machinery. Rsp5/hPRF1 (Imhof and McDonnell 1996) and E6-AP (Nawaz et al. 1999) function as coactivators of nuclear receptors (Conaway et al. 2002), interact with TADs, and ubiquitinate the largest subunit of RNA polymerase II (Salghetti et al. 2000). As phosphorylation of RNA polymerase II leads to its ubiquitination and phosphorylation is required for transcriptional elongation (Beaudenon et al. 1999; Mitsui and Sharp 1999), it is likely that ubiquitination plays a role in the transition from initiation of transcription to elongation (Muratani and Tansey 2003).

Figure 1-3

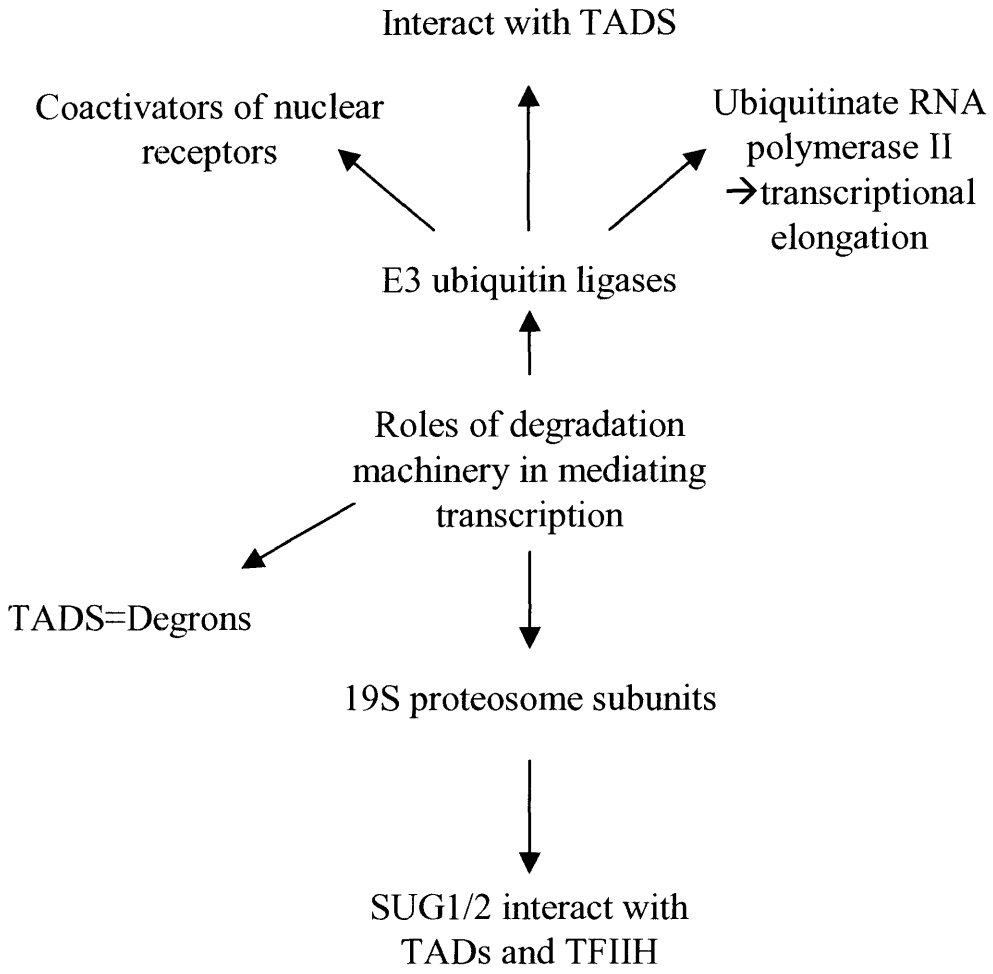


Figure 1-3

Diagram of known connections between proteins/domains involved in mediating both degradation and transcription.

In addition, $TAF_{II}250$, a component of the general transcription machinery, possesses monoubiquitinating ability (see the above discussion of dual function transcriptional regulators) (Pham and Sauer 2000; Conaway et al. 2002).

Subunits of the 19S regulatory complex of the 26S proteasome have been implicated in transcriptional regulation (Ferdous et al. 2001; Conaway et al. 2002; Gonzalez et al. 2002; Muratani and Tansey 2003). At least five 19S subunits are recruited to actively transcribed genes in yeast (Swaffield et al. 1992; Muratani and Tansey 2003). Two such subunits, Sug1, an AAA-type ATPase, and Sug2, interact with TADs (Swaffield et al. 1995; Russell et al. 1996; vom Baur et al. 1996; Wang et al. 1996; Masuyama and MacDonald 1998) as well as the general transcription factors TBP (Melcher and Johnston 1995; Makino et al. 1999) and TFIIH (Weeda et al. 1997) (Salghetti et al. 2000; Muratani and Tansey 2003). The roles of Sug1 and Sug2 in transcription are independent of their proteasomal roles, and are most likely involved in transcriptional elongation (Conaway et al. 2002; Muratani and Tansey 2003).

The large number of proteins possessing roles in mediating both ubiquitin-mediated proteasomal degradation and transcription indicates the importance of linking these two activities. Highly acidic TADs, which also function as degrons, signal efficient transcriptional elongation (Muratani and Tansey 2003). As open chromatin is associated with ubiquitinated histones, the transcriptional activators may function to recruit the components of the degradation machinery to modify histones, in addition to modifying basal transcription factors, and ultimately the activators themselves (Salghetti et al. 2000). Ubiquitination leads to association of the transcription factor, the proteasome, and general components of transcription (Zhu et al. 2004). Degradation of the activator would allow rapid reprogramming of transcription or alternatively allow transcriptional elongation to proceed. When proteolysis of the transcription factor is

complete, subunits of the proteasome are likely to engage in the elongation step of transcription (Zhu et al. 2004).

Ubiquitination of ETS transcription factors

ELK-1 is the only ETS transcription factor that has been shown to be ubiquitinated and the functional consequences of this modification are not understood (Fuchs et al. 1997) (Table 1-2). In addition, it has been suggested that ER81 undergoes ubiquitin-mediated degradation (Goel and Janknecht 2003) and ELF-1 is degraded by the proteasome (Juang et al. 2002a). YAN, in response to phosphorylation by MAPK, appears to be degraded (Lai and Rubin 1992; Rebay and Rubin 1995; Tootle et al. 2003a). As YAN contains many high scoring PEST sequences, which can serve as signals for ubiquitin-mediated proteasomal degradation, it is likely that YAN is ubiquitinated. It is likely that many more ETS transcription factors are ubiquitinated, leading to both degradation of the protein and activation of transcription, depending on the context.

Sumoylation

It is becoming apparent that sumoylation is a widespread mechanism of post-translational modification. I will briefly review the mechanism and enzymes responsible for sumo-conjugation, overview which ETS transcription factors are or are likely to be sumoylated (Table 1-2), and discuss in greater depth how sumoylation regulates the two ETS transcription factors TEL and ELK-1.

General information on sumoylation

SUMO is an 11kDa ubiquitin-like small polypeptide modifier that covalently attaches to K residues, within the consensus sequence of ψ-K-X-E (ψ=large hydrophobic residue like I/V). Sumoylation can affect the stability, activity, and localization of its targets (Muller et al. 2001;

Table 1-2

ETS transcription factor	Lysine modification	Effect of modification	Reference
ELK-1	Ubiquitinated	Unknown	Fuchs et al., 1997
	Sumoylated	Switches ELK-1 to transcriptional repressor	Yang et al., 2003
	Acetylated?	Interacts with p300/CBP	Nissen et al., 2001
ER81	Ubiquitinated?	Degraded	Goel and Janknecht, 2003
	Acetylated	Increased DNA binding, transcriptional activity, and protein stability	Goel and Janknecht, 2003 Goel and Janknecht, 2004
ELF-1	Ubiquitinated?	Degraded by proteasome	Juang et al., 2002
	Sumoylated?	Interacts with UBC9	Hahn et al, 1997
YAN	Ubiquitinated?	Likely to be degraded	Lai and Rubin, 1992 Rebay and Rubin, 1995
	Sumoylated?	Consensus sequence	Wood et al., 2003
TEL	Sumoylated	Subcellular localization: nuclear bodies and nuclear export	Chakrabarti et al., 2003 Wood et al., 2003
NET	Sumoylated?	Consensus sequence	Wood et al., 2003
ETS-1	Acetylated	Negatively regulates protein-protein interactions, downregulating ETS-1's transcriptional activity	Czuwara-Ladykowska et al., 2002
	Sumoylated?	Interacts with UBC9	Hahn et al., 1997
PU.1	Sumoylated?	Interacts with UBC9	Hahn et al., 1997
	Acetylated	Interacts with p300/CBP	Yamamoto et al., 1999

Table 1-2

Table listing the known and predicted lysine modifications and the effects of such modifications on ETS transcription factors.

Seeler and Dejean 2001; Gill 2003; Verger et al. 2003). SUMO and ubiquitin are only 18-20% identical, yet structural studies reveal a common three dimensional structure (Bayer et al. 1998; Muller et al. 2001). However, there is an amino-terminal extension found on SUMO that is not present in ubiquitin (Muller et al. 2001). Yeast and *Drosophila* have only one *sumo* gene, while mammalian species possess three genes (Seeler and Dejean 2001). SUMO-2 and SUMO-3, like ubiquitin, can form multimeric chains on target proteins, while SUMO-1 mono-modifies proteins (Saitoh and Hinchey 2000; Tatham et al. 2001; Gill 2003; Verger et al. 2003).

Sumoylation occurs by an analogous process to ubiquitination. Like ubiquitin, SUMO is synthesized as an inactive precursor that is activated by a carboxy-terminal cleavage event mediated by ubiquitin-like protein processing enzyme (ULP) or SUMO-specific proteases (Muller et al. 2001). This cleavage exposes a glycine residue at the carboxy-terminus that forms an isopeptide bond between SUMO and the ε-amino group of the K residue on the target protein (Muller et al. 2001). The SUMO-E1 enzyme is a heterodimer of AOS1 and UBA2. UBA2 is similar in sequence to the carboxy terminus of UBA1, the E1 ubiquitin-conjugating enzyme, while AOS1 is related to the amino-terminus of UBA1 (Johnson et al. 1997; Desterro et al. 1999; Gong et al. 1999b; Muller et al. 2001). The E1 enzyme, in an ATP-dependent process, forms a high-energy thioester bond between the carboxy-terminal residue of SUMO and the E1 enzyme (Seeler and Dejean 2001).

Activated SUMO is then transferred to the sole SUMO-E2 conjugating enzyme, UBC9. A conserved cysteine residue within UBC9 forms the thioester linkage with SUMO (Seeler and Dejean 2001). UBC9 is similar in sequence to ubiquitin E2 enzymes, except for the important difference that the surface which binds the negatively charged surface of SUMO is positively charged, while the surfaces of ubiquitin E2 enzymes are either negatively charged or neutral

(Giraud et al. 1998; Liu et al. 1999; Muller et al. 2001). As a result of this UBC9 can not bind ubiquitin. UBC9 can directly transfer activated SUMO to the ε-amino group of the specific K in the target protein, however a specific E3 ligase may be required in vivo (Verger et al. 2003).

There are two classes of SUMO-E3 ligases, the Protein Inhibitor of Activated STAT (PIAS) family and RanBP2/Nup358. PIAS members have RING-like domains with similarities to the RING finger of ubiquitin E3 ligases, while RanBP2 is a component of the nuclear pore complex with no similarity to other E3 ligases (Verger et al. 2003). As with ubiquitination, the E3 ligases are thought to confer the specificity of the modification.

SUMO modification is a dynamic and reversible process. Removal of conjugated SUMO from its protein targets is catalyzed by SUMO proteases, whose localization determines their substrate specificity in vivo (Gong et al. 2000; Nishida et al. 2000; Nishida et al. 2001; Best et al. 2002; Hang and Dasso 2002; Gill 2003). These proteases are the same enzymes that process immature SUMO (See above) (Verger et al. 2003).

Sumoylation of ETS transcription factors

The ETS transcription factors TEL and ELK-1 are sumoylated in vivo. As UBC9 is the only E2 SUMO-conjugating enzyme identified, it is likely that most, if not all, of its known interactors are sumoylated. The ETS members that have been shown to interact with UBC9 are ETS-1, FLI-1, ELF-1, PU.1, and NET (Hahn et al. 1997) (Table 1-2). UBC9 interacts directly with ETS-1 and increases its the transcriptional activation activity, while having no effect on protein stability (Hahn et al. 1997). Future work should elucidate whether these and other ETS transcription factors are sumoylated, and how such modification affect their activities.

TEL

TEL, the human ortholog of *Drosophila* YAN, is a transcriptional repressor of the ETS family of transcription factors. Similar to YAN, TEL has an amino-terminal PD, that is involved in both homo- and hetero- typic protein-protein interactions (Jousset et al. 1997; Kwiatkowski et al. 1998), and a carboxy-terminal ETS DNA binding domain.

The E2 conjugating enzyme UBC9 interacts with the PD of TEL but not with the PD of YAN (Chakrabarti et al. 1999). At the time it was believed that the main function of UBC9 was as another E2 enzyme involved in ubiquitin-mediated degradation. However, mutating the residues within UBC9 that would be important for ubiquitin conjugation and protein degradation did not affect the interaction between UBC9 and TEL (Chakrabarti et al. 1999). Instead of the interaction with UBC9 leading to degradation of TEL, it was found that UBC9 directly inhibits TEL mediated repression (Chakrabarti et al. 1999). This interaction is transient and is not required for TEL's DNA binding activity (Chakrabarti et al. 1999).

Evidence was emerging that UBC9 was involved in the conjugation of SUMO-1 to target proteins. Initial analysis of the effects of SUMO-1 on the subcellular localization of TEL revealed no obvious effect (Chakrabarti et al. 1999). However, it was later shown that the PD of TEL interacts with SUMO-1 via yeast two-hybrid analysis and in cell culture (Chakrabarti et al. 2000). In 10% of the cells SUMO-modified TEL localizes to nuclear bodies termed TEL-bodies, which are transient structures formed during S phase (Chakrabarti et al. 2000). TEL-bodies are distinct from other known nuclear bodies containing SUMO-1, such as PML bodies. The PD of TEL is required for nuclear localization and SUMO-1 modification, and K99 within the PD is the predominant SUMO-1 modification site (Chakrabarti et al. 2000). PDs are not the only domains capable of interacting with UBC9 as two of the five ETS members that interact with UBC9 do not contain PDs.

SUMO may regulate the subcellular localization of TEL by facilitating nuclear export. Endogenous TEL localizes to both the nucleus and the cytoplasm, with only a small percentage being SUMO conjugated. Analysis revealed that TEL is actively exported from the nucleus in a CRM1 and PD dependent manner (Wood et al. 2003). The PD of TEL is known to mediate dimerization, and mutations inhibiting dimerization localize to the nucleus, indicating homodimerization is needed for nuclear export. Dimerization mutants are not sumoylated, suggesting dimerization may be needed for SUMO conjugation (Wood et al. 2003). It is unclear whether the role of dimerization in mediating the nuclear export of TEL is simply due to its necessity for sumoylation or whether dimerization is otherwise required for export. TEL K99R, which can not be sumoylated, can not be exported from the nucleus or localize to TEL-bodies, but can interact via its PD with the corepressor Sin3A and even functions as a better transcriptional repressor than wild-type TEL (Wood et al. 2003). These data suggest that SUMO modification contributes to the downregulation and nuclear export of TEL and that TEL bodies may be loading sites for nuclear export.

Hyperphosphorylated TEL is predominantly cytoplasmic, indicating phosphorylation may also contribute to the nuclear export of TEL (Wood et al. 2003). Thus both phosphorylation and sumoylation are involved in mediating the nuclear export of TEL. Intriguingly the other ETS members known to be regulated by phosphorylation-mediated nuclear export, NET and YAN, contain putative SUMO acceptor sites (Wood et al. 2003). This suggests that phosphorylation and sumoylation may generally work in concert to mediate the downregulation nuclear export of transcription factors. Future work will hopefully determine whether this is the case, and whether, indeed, NET and YAN are also sumoylated.

The order in which TEL is phosphorylated and sumoylated is unclear, as is whether one modification is required for the next to occur or whether they have no effect on each other. It is important to understand the relationships of these modifications and how they, together, mediate the nuclear export and downregulation of TEL.

Chromosomal rearrangements of the *tel* locus, along with deletion of the second *tel* allele, are frequently found in cases of human leukemias, as well as solid tumors (Maroulakou and Bowe 2000). Mouse knockout analysis reveals that while TEL is not essential for initiation of embryonic angiogenesis, TEL is required for the development and maintenance of complex vasculature (Bartel et al. 2000). In addition TEL is essential for adult hemotopoiesis (Bartel et al. 2000). The leukemia associated fusion protein TEL/acute myeloid leukemia 1 (AML1) transcription factor, is sumoylated and localizes to nuclear speckles during S phase (Chakrabarti et al. 2000), while AML1 alone normally localizes to larger, distinct nuclear bodies. Thus one consequence of the chromosomal rearrangement resulting in the TEL/AML1 fusion protein is altered nuclear localization of AML1 (Chakrabarti et al. 2000). TEL/AML1 fusions can also localize to the cytoplasm, while wild-type AML1 is predominantly nuclear (Wood et al. 2003), adding another possible reason for such a fusion to lead to leukemia development. The resulting mislocalization of the transcription factor results in alterations in gene expression, ultimately causing the disease state.

Sumoylation of TEL is involved in regulating its subcellular localization and thus its transcriptional activity. SUMO-TEL localizes to nuclear TEL bodies and results in nuclear export of TEL. Thus TEL is removed from the DNA, removing its repression. By these same mechanisms TEL PD fusions with transcription factors, caused by chromosomal translocations,

result in altered localization of these factors, removing them from the DNA, and thus aberrantly downregulating them, ultimately resulting in disease.

ELK-1

ELK-1 belongs to the ternary complex factor (TCF) subfamily of the ETS transcription factors. TCFs act through a ternary nucleoprotein complex composed of a TCF, a serum response factor (SRF), and a serum response element (SRE), which is composed of the DNA binding sites for these two factors (Sharrocks 2002; Shaw and Saxton 2003). In response to growth signals and cellular stress, MAPK signaling leads to the phosphorylation of TADs of TCFs and induction of their activities as transcriptional activators (Sharrocks 2002; Shaw and Saxton 2003). In the absence of MAPK signaling, the ETS domain along with an inhibitory domain, called the R motif in ELK-1, suppresses the activity of the TAD, maintaining the TCF in an inactive state (Sharrocks 2002; Shaw and Saxton 2003).

Alanine scanning mutagenesis of the R motif in ELK-1 revealed that the conserved residues K249 and E251 are important for the repressive activity of this domain (Yang et al. 2003). These residues are within a potential SUMO modification site (Verger et al. 2003). Sequence analysis revealed an additional SUMO consensus site within the R motif, K230. Blocking sumoylation by mutating the SUMO modification sites (K230R/K249R), expressing dominant negative UBC9, or expressing the SUMO specific protease SSP3 increases ELK-1 transcriptional activity in the absence of MAPK activation, suggesting that sumoylation plays a role in repressing the basal level the ELK-1 transcriptional activity (Yang et al. 2003). Activation of the ERK MAPK pathway synergizes with the above conditions, indicating that the ERK and SUMO pathways function antagonistically to control ELK-1 transactivation potential (Yang et al. 2003). Of the two potential sumoylation sites, K249 is the major modification site in

vitro and in vivo (Yang et al. 2003). Activation of the ERK MAPK pathway leads to an increase in the level of phosphorylation and a decrease in the level of sumoylation of ELK-1 (Yang et al. 2003). Thus the activity of the autonomous repression domain of ELK-1, the R motif, is controlled by the SUMO pathway, which is in turn regulated by MAPK-mediated phosphorylation of the TAD of ELK-1.

The repressive properties of the R motif are due to its recruitment of the histone deacetylases 1 and 2 (HDAC-1 and HDAC-2). Blocking sumoylation by the K249R ELK-1 mutation removes the repression mediated by the HDACs (Yang and Sharrocks 2004). Chromatin immunoprecipitation reveals that the ELK-1 K249R mutation leads to reduced HDAC-2 recruitment to promoters (Yang and Sharrocks 2004). These data suggest that sumoylation of ELK-1 is required for HDAC recruitment to the promoter and subsequent transcriptional repression. Stimulation of the ERK MAPK pathway reduces SUMO-1 and HDAC-2 promoter occupancy and enhances the level of histone H4 acetylation at the promoter (Yang and Sharrocks 2004). Thus phosphorylation is the switch that changes ELK-1 from a repressor to an activator.

Additional studies on ELK-1 sumoylation have revealed that SUMO has intrinsic repressive properties (Yang et al. 2003). This repression is not affected by activation of the MAPK pathway outside the context of ELK-1. Therefore, sumoylation of transcription factors may be a general mechanism of mediating transcriptional repression.

ELK-1 can function as both a transcriptional activator and repressor in the same cells. Sumoylation of ELK-1 results in recruitment of corepressors, resulting in Elk-1 repressing transcription. MAPK-mediated phosphorylation of ELK-1 inhibits sumoylation and results in ELK-1 functioning as a transcriptional activator.

It is intriguing that like TEL, ELK-1 is regulated by phosphorylation and sumoylation and these two modification appear to affect each other. In the case of TEL, both function to mediate the downregulation of TEL, while in the case of ELK-1, phosphorylation and sumoylation act antagonistically. It is likely that, as discussed above, other ETS transcription factors are dually regulated by these two modifications. Further work is needed determine the interplay between phosphorylation, sumoylation, and other post-translation modifications with respect to regulation of transcription factor function.

Acetylation

Although acetylation is best known for its involvement in regulating histones and thus the state of chromatin, it is a common modification involved in regulating transcription factors. I will review the enzymes which mediate this modification, overview the protein-protein interaction domains which recognize acetylated lysines, discuss the roles of specific acetyltransferases as transcriptional coactivators, overview the ETS transcription factors which are acetylated (Table 1-2), and discuss how ER81 and ETS1 are regulated by acetylation.

General information on acetylation

Acetyltransferases transfer an acetyl group from acetyl-Coenzyme A (acetyl-CoA) to the ε-amino group of a specific K on the target protein (Sterner and Berger 2000). Modification by acetylation was originally thought to be restricted to histones. K acetylation of histone tails partially neutralizes their positive charge, weakening histone-DNA interactions and resulting in chromatin "opening" (Sterner and Berger 2000; Wang et al. 2000).

There are several families of acetyltransferases, including p300/CBP, GNAT (P/CAF /GCN5), $TAF_{II}250$, p160 steroid receptor coactivator (SRC) family (SRC1/p160/NCoA-1,

ACTR/Rac3/AIB1/ TRAM-1/pCIP, TIF2/GRIP1/NCoA-2), and MYST (MOZ, Ybf2/Sas3, Sas2, Tip60) (Bannister and Miska 2000; Sterner and Berger 2000). As very few Ks within a particular protein are acetylated, it is likely that acetyltransferase have specificity. A possible consensus sequence for acetylation is GK (Kouzarides 2000). Acetyltransferases are known to self-modify, possibly regulating their cellular localization (Creaven et al. 1999; Bannister and Miska 2000; Kalkhoven et al. 2002), and their activity may be regulated by extracellular signaling events (Kouzarides 2000).

Like most post-translational modifications, acetylation is reversible and the enzymes responsible are deacetylases. All known deacetylases are histone deacetylases, of which there are two families. The first family composed of HDAC1, HDAC2, and HDAC3 is related to yeast deacetylase RPD3, and contains a highly conserved catalytic domain. The other family is related to yeast HDA1 and is composed of HDAC4, HDAC5, and HDAC6 (Kouzarides 2000). It is not known whether there is specificity and site selection by deacetylases. HDACs have been shown to deacetylate transcription factors (Kouzarides 2000). In addition, as HDACs can associate with various transcriptional corepressors, their interactions with transcription factors could result in recruitment of these corepressors to promoters along with deacetylation or "closing" of the chromatin (Pazin and Kadonaga 1997).

Bromodomains, which recognize and bind acetylated Ks, are found in many chromatin associated proteins and all acetyltransferases. NMR studies revealed that bromodomains function as acetyl-K binding domains by forming a specific hydrogen-bond between the oxygen of the acetyl carbonyl group and the side chain of the amide nitrogen of the conserved asparagines within the domain (Hudson et al. 2000; Zeng and Zhou 2002). In general bromodomains recognize acetylated Ks that are neighbored by aromatic or hydrophobic residues.

Individual bromodomains possess specificity, as the GCN5 bromodomain recognizes the acetylated K, and the Y/F at +2 to the acetylated K(Hudson et al. 2000), while P/CAF recognizes the Y at –3 and the Q at +4 (Mujtaba et al. 2002; Zeng and Zhou 2002). These domains function to tether acetyltransferases to specific chromosomal sites and assemble multi-protein complexes.

Acetyltransferases which function as coactivators

Many acetyltransferase families function as transcriptional coactivators, including p300/CBP, P/CAF and nuclear receptor coactivators. p300/CBP are structural and functional homologs that were originally characterized as transcriptional coactivators (Janknecht and Hunter 1996) and were later found to possess intrinsic acetyltransferase activity (Bannister and Miska 2000; Sterner and Berger 2000). As coactivators they act as scaffolds between the transcription factors and the general transcription machinery. Many transcription factors interact with the same site on p300/CBP, indicating there could be competition for limiting amounts of this coactivator (Janknecht and Hunter 1996). p300/CBP acetyltransferase activity is required for its function as a transcriptional coactivator (Benkirane et al. 1998; Vanden Berghe et al. 1999; Sterner and Berger 2000). The central regions of p300/CBP contain a bromodomain and p300/CBP interact with other acetyltransferases, GCN5, P/CAF, and nuclear receptor coactivators (Bannister and Miska 2000; Sterner and Berger 2000). Therefore, p300/CBP can acetylate transcription factors and the general transcription machinery in addition to recruiting other coactivators/acetyltransferases to the promoter and thereby amplifying its effects.

P/CAF, as mentioned above, can form a complex with p300/CBP to regulate transcription, and can itself function as a coactivator in an acetyltransferase dependent manner (Benkirane et al. 1998; Sterner and Berger 2000). P/CAF contains a bromodomain, which is required for its full acetyltransferase activity (Sterner and Berger 2000).

The nuclear receptor coactivators SRC-1 and ACTR are known to possess acetyltransferase activity, while this function has never been shown for TIF2. Both SRC-1 and ACTR interact with nuclear hormone receptors and stimulate their transcriptional activation (Chen et al. 1997a; Ding et al. 1998; Sterner and Berger 2000). They also interact with p300/CBP and P/CAF, and are acetylated by p300/CBP. In most cases acetylation of acetyltransferases have positive effects on their activities, however acetylation is known to inhibit ACTR's function as a coactivator (Chen et al. 1999b; Sterner and Berger 2000).

These three families of acetyltransferases fall into the category of factor acetyltransferases (FATs). FATs acetylate architectural DNA binding elements, general transcription machinery, and site specific DNA binding factors (Bannister and Miska 2000). Acetylation can regulate protein-DNA interactions, as well as protein-protein interactions, possibly by affecting protein conformation similar to how it affects histone conformation (Bannister and Miska 2000; Sterner and Berger 2000).

Acetylation of ETS transcription factors

The ETS transcription factors ER81 and ETS1 are known to be acetylated. In addition ELK1 and PU.1 are known to interact with p300/CBP (Nissen et al. 2001 and Yamamoto et al. 1999, respectively), and it remains to be determined whether these interactions lead to acetylation of these transcription factors (Table 1-2). As the majority of ETS transcription factors are activators it is likely that many more interact with coactivator acetyltransferases, and may themselves be acetylated.

ER81

Acetylation of ER81 increases protein-protein interactions, protein-DNA interactions, transcriptional activity, and protein stability. Additionally ER81 activity is not only regulated by acetylation, but is also regulated by phosphorylation and ubiquitin-mediated degradation.

The first indication that ER81 might be modified by acetylation was the discovery of interactions between ER81 and p300/CBP (Papoutsopoulou and Janknecht 2000; Bosc et al. 2001; Wu and Janknecht 2002). p300/CBP function as adaptors between transcription factors and the general transcription machinery; in addition p300/CBP possess intrinsic acetyltransferase activity. Therefore p300/CBP may directly regulate chromatin structure and/or may modify other proteins such as the transcription factors and general transcriptional machinery associated with it. Later work has shown that in addition to p300/CBP, P/CAF, and members of the p160 steroid receptor coactivator (SRC) family, SRC-1, ACTR and GRIP-1, interact with and acetylate ER81 (Goel and Janknecht 2003 and Goel and Janknecht 2004, respectively).

ER81 and p300/CBP associate both in vitro and in vivo, where this association leads to increased ER81 transcriptional activity (Papoutsopoulou and Janknecht 2000). P/CAF also interacts with ER81 in vivo, and the region of interaction between P/CAF and ER81 partially overlaps with the region of interaction between p300/CBP and ER81 (Goel and Janknecht 2003). However, P/CAF-ER81 interaction does not inhibit p300/CBP binding. Therefore, all three proteins, ER81, p300 and P/CAF, may form a complex, leading to transcriptional activation.

ER81 is acetylated in its amino-terminal TAD at K33 and K116 (Goel and Janknecht 2003). Acetylation at K116 enhances ER81's affinity for DNA, most likely due to a conformational change allowing the ETS domain to bind DNA better (Goel and Janknecht 2003). Acetylation also increases the potency of ER81's amino-terminal TAD (Goel and Janknecht

2003). This may be due to recruitment of cofactors or chromatin remodeling complexes that interact with acetylated lysines through bromodomains. Additionally, acetylation of either K33 or K116 also increases the in vivo half-life of ER81 (Goel and Janknecht 2003). A common mechanism for acetylation increasing protein stability is by modifying the Ks that are to be ubiquitinated, and thus preventing protein degradation (Freiman and Tjian 2003). However this is not the case for ER81, suggesting that acetylation at K33 and K116 prevents the ubiquitination of other lysines by either inducing a conformational change, or allowing or preventing interaction with proteins that shield ER81 from or target to ubiquitin ligases (Goel and Janknecht 2003).

In addition to being modified by acetylation, ER81 is also phosphorylated due to RAS signaling downstream of the HER2/Neu receptor tyrosine kinase (Bosc et al. 2001). Interestingly, p300 expression leads to increased ER81 phosphorylation but phosphorylation does not affect p300/CBP binding to ER81 (Papoutsopoulou and Janknecht 2000). Mutation of either the two acetylation sites or the five phosphorylation sites reduces HER2/Neu mediated activation of ER81, while mutation of both practically eliminates all activity (Goel and Janknecht 2003). Therefore, both phosphorylation and acetylation are required for maximal transcriptional activation by ER81. HER2/Neu and the downstream components RAS and RAF, induce the acetyltransferase activity of p300 but not P/CAF by direct phosphorylation, and thus increases the in vivo acetylation of ER81 (Goel and Janknecht 2003). HER2/Neu signaling leads to phosphorylation of ER81 and p300, activating p300's acetyltransferase activity and leading to acetylation of ER81, ultimately resulting in increased ER81-dependent transcriptional activation.

Phosphorylation leads to the acetylation of ER81, which results in enhancement of both protein-protein and protein-DNA interactions, increased transcription activity, and increased

protein stability. ER81 is a prime example for how different post-translational modifications, in this case acetylation and phosphorylation, of transcription factors, and other protein classes for that matter, should not be looked at in exclusion but rather should be studied together to determine whether there is synergism or antagonism between the different modifications.

ETS1

Turnover of extracellular matrix (ECM) proteins is necessary for embryogenesis, the female reproductive cycle, angiogenesis, and tissue repair. Abnormal turnover of ECM proteins is observed in adults during tumorigenesis and arthritis (Trojanowska 2000). TGFβ signaling induces the expression of ECM proteins, while ETS transcription factors ETS1 and the E1AF/PEA3 subfamilies play roles in inducing ECM turnover by activating transcription of serine proteases (uPA) and matrix metalloproteinases (MMP-1, 3, 9) (Altieri et al. 1995; Westermarck et al. 1997; Watabe et al. 1998; Trojanowska 2000).

p300/CBP are recruited by ETS1 to the *mmp-1* promoter, contributing to its transcriptional activation. The interaction and cooperation between ETS-1 and p300/CBP are not affected by phosphorylation (Jayaraman et al. 1999). Two separate regions of p300/CBP, which do not possess acetyltransferase activity, independently interact with and coactivate ETS-1. This indicates that p300/CBP enhancement of ETS-1 mediated transcriptional activation does not require acetyltransferase activity.

TGFβ and ETS1 act antagonistically to regulate ECM proteins. For instance, TGFβ signaling inhibits *mmp-1* expression, while ETS1 can overcome this inhibition and activate *mmp-1* expression (Czuwara-Ladykowska et al. 2002). Like ETS1, TGFβ transcriptional stimulation is enhanced by p300, but unlike ETS-1, this stimulation requires p300's acetyltransferase activity; this enhancement is inhibited by ETS1 (Czuwara-Ladykowska et al. 2002). As ETS-1 is

known to interact with p300/CBP, it is likely that ETS-1 inhibition of TGFβ-dependent transcriptional activation is due to ETS-1 sequestering away p300/CBP.

TGFβ stimulation leads to rapid and prolonged acetylation of ETS1, but has no affect on the phosphorylation of ETS1 (Czuwara-Ladykowska et al. 2002). Acetylation of ETS-1 results in dissociation of the p300/CBP-ETS1 complex. Thus, under normal cellular conditions TGFβ signaling leads to acetylation of ETS-1, resulting in release of p300/CBP which can then interact with transcription factors downstream of TGFβ signaling, SMADs, to activate transcription.

The abnormally high levels of ETS-1 expressed in fibroblasts (Czuwara-Ladykowska et al. 2002) result in an altered cellular response to TGFβ signaling, specifically resulting in the sequestrating of p300/CBP by ETS-1 and subsequently inhibiting TGFβ dependent transcription. Thus increased ETS-1 expression in these cells is likely to be a contributing factor in the pathology of tumor progression and arthritis.

Acetylation in the case of ETS-1 negatively affects protein-protein interactions, resulting in the downregulation of ETS-1 mediated transcriptional activation. The competition for limiting amounts of p300/CBP exhibited by ETS-1 and TGFβ signaling components is a mechanism of regulation that is used repetitively to regulate transcription factor activity.

Methylation

While no ETS transcription factors are yet known to be methylated, I will briefly review the mechanism and roles of methylation in regulating other transcription factors. Future work is likely to reveal that ETS transcription factors are regulated by methylation.

General information on methylation

Methylation of non-histone proteins occurs at arginine (R) residues. There are three forms of methylarginine, N^G-monomethylarginine (MMA), N^GN^G (asymmetric) dimethylarginine (aDMA), and N^GN^G (symmetric) dimethylarginine (sDMA), modification of guanidino nitrogen atoms (McBride and Silver 2001). aDMA occurs on R in RGG, RXR, and RG motifs, while MMA only occurs at RGG tripeptides (Najbauer et al. 1993; Rawal et al. 1995; McBride and Silver 2001). While R methylation does not change the charge of the residue, it increases steric hindrances and decreases hydrogen bonding capability (McBride and Silver 2001).

R methyl transferases are likely to function as dimers and contain a S-adenosyl methionine (AdoMet) binding motif and a less conserved carboxy-terminal domain that is involved in substrate recognition (Weiss et al. 2000; Zhang et al. 2000; McBride and Silver 2001; Rho et al. 2001; Komoto et al. 2002). All R methyl transferases are capable of monomethylating, type I can asymmetrically dimethylate, and type II can symmetrically dimethylate (McBride and Silver 2001). The majority of R-methylation is mediated by the subfamily of R methyl transferases including hPRMT1 (yeast HMT1/RMT1). Most PRMTs are type I enzymes, while hPRMT5/JBP1 is a type II enzyme (McBride and Silver 2001). Coactivator associated arginine methyltransferase 1 (CARM1) functions as a secondary coactivator which interacts directly with primary coactivators of the p160 family, which possess acetyltransferase activity (see above). CARM1 methyl transferase activity is required for coactivator function (Chen et al. 1999a). It is interesting that acetyltransferases p300/CBP and P/CAF also function as secondary coactivators, suggesting that primary coactivators in general may serve to recruit enzymes to post-translationally modify nearby proteins, including histones and transcriptional regulators. As discuss above (Dual function transcriptional regulators), it is

emerging that some transcription factors themselves possess enzymatic activity to mediate such post-translation modifications.

Unlike the other post-translational modifications, R methylation has not been shown to be reversible, indicating that the regulation of this modification has to occur at the level of the R methyl transferase (McBride and Silver 2001). However, analysis of histone methylation suggests that although no demethylating enzymes have been identified, methylation is likely to be reversible (Ma et al. 2001; Davie and Dent 2002).

R methylation is an important regulator of mammalian skeletal muscle differentiation (Chen et al. 2002), erythroid differentiation (Bakker et al. 2004), and is likely to play a role in neuronal differentiation (Cimato et al. 2002). Mouse knockout experiments have revealed that loss of PRMT1 results in death at embryonic day 6.5 (Pawlak et al. 2000), and loss of CARM1 results in decreased size and perinatal death (Yadav et al. 2003). This analysis has also shown that CARM1 is essential for estrogen responsive transcriptional activation, definitively showing that R methylation is involved in transcriptional regulation (Yadav et al. 2003).

R methylation is known to affect protein-protein interactions and nuclear import/export (McBride and Silver 2001). SAM68, a SRC kinase adaptor protein, has SH3 and WW domains flanked by RG repeats that are aDMA, resulting in inhibition of SH3 domain binding but having no effect on WW domain interactions (Bedford et al. 2000). Thus R methylation can differentially regulate protein-protein interactions. PRMT1 has a positive role in interferon (IFN) α and β signaling, and specifically methylates STAT1 in response to interferon stimulation. R methylation of STAT1 results increased interaction with its inhibitor PIAS1, an E3 SUMO-ligase, leading to decreased DNA binding (Mowen et al. 2001), and decreased interaction with the nuclear tyrosine phosphatase TcPTP, leading to increased protein stability

(Zhu et al. 2002). This suggests that methylation can affect other post-translation modifications on the modified protein. Interestingly the coactivator and acetyltransferase CBP is R methylated in vivo. This methylation is important for CBP coactivator function in relation to steroid hormone dependent transcription, but not for other promoters (Chevillard-Briet et al. 2002). Aberrant regulation of methylation has been associated with cardiovascular disorders and autoimmune diseases, including multiple sclerosis and lupus erythematosis (McBride and Silver 2001).

No ETS transcription factors have been shown to be methylated. However, as methylation is just beginning to be understood as a means of regulating transcription factors, it is likely that future research will reveal that ETS transcription factors are regulated by such modifications.

Glycosylation

Originally thought to only be a modification found on membrane associated proteins, glycosylation is emerging as a post-translational modification of both cytoplasmic and nuclear proteins. I will discuss the interplay of glycosylation and phosphorylation, review the enzymes which mediate it, overview the ETS transcription factors modified by glycosylation, and discuss how glycosylation regulates ELF-1.

Glycosylation vs. phosphorylation

Complex N- and O-linked glycosylation occur on membrane bound and secreted proteins that are synthesized in the endoplasmic reticulum (ER) or Golgi apparatus (Vosseller et al. 2001; Wells et al. 2001). Contradicting the dogma that carbohydrate modifications can only occur in the ER and Golgi apparatus, it was found that many nuclear and cytoplasmic proteins are

modified at multiple S or T hydroxyl groups by a simple monosaccharide modification of a single β-N-acetylglucosamine (O-GlcNAc) moiety (Torres and Hart 1984; Hart 1997; Comer and Hart 2000; Wells et al. 2001; Zachara and Hart 2002). O-GlcNAc modification is found in all eukaryotes studied, but not in prokaryotes (Zachara and Hart 2002). While there is no consensus motif for O-GlcNAc attachment, many of the sites are identical to those recognized by S/T protein kinases (Hart 1997; Comer and Hart 2000; Zachara and Hart 2002).

Phosphorylation and O-GlcNAc occur at the same or adjacent hydroxyl moieties. Every protein modified by O-GlcNAc is known to be phosphorylated and to form reversible multimeric protein complexes by associations that are regulated by phosphorylation (Hart 1997; Comer and Hart 2000; Vosseller et al. 2001). O-GlcNAc may mediate protein-protein interactions either directly or indirectly; however, there is no known domain which specifically binds to O-GlcNAc (Vosseller et al. 2001). Phosphatase inhibitors and kinase activators decrease the levels of O-GlcNAc modification, while kinase inhibitors increase these levels (Griffith and Schmitz 1999; Vosseller et al. 2001). These data indicate that O-GlcNAc and phosphorylation play competing and antagonistic roles. Most curious is that many of the O-GlcNAc modification sites are within high scoring PEST sequences (Comer and Hart 1999; Zachara and Hart 2002). PEST sequences are associated with signaling proteasome mediated degradation either constitutively, or by a phosphorylation induced mechanism. O-GlcNAc may neutralize the effect of the PEST sequence by preventing phosphorylation and subsequent degradation (Comer and Hart 1999; Zachara and Hart 2002). As both modifications can occur at the same residues, it complicates interpretations of phosphorylation site mutants as mutations of such residues prevent both types of modification.

General information on glycosylation

The enzyme responsible for O-GlcNAc modification, uridine diphospho-N-acetylglucosamine polypeptide β-N-acetylglucosaminyl transferase or O-GlcNAc transferase (OGT), localizes to the cytoplasm and the nucleus (Hart 1997; Comer and Hart 2000; Vosseller et al. 2001; Kamemura and Hart 2003). OGT represents a novel enzyme, with no obvious family members, that is 80% homologous between humans and *C. elegans* (Zachara and Hart 2002). OGT has eleven tetratricopeptide repeats (TPRs) at its amino terminus which mediate both trimerization of the catalytic subunits and interactions with other proteins (Hart 1997; Kreppel et al. 1997; Kreppel and Hart 1999; Comer and Hart 2000; Zachara and Hart 2002; Iyer and Hart 2003; Kamemura and Hart 2003). There is some evidence suggesting that these repeats are also important for substrate selectivity. OGT is itself modified by O-GlcNAc and tyrosine phosphorylation (Kreppel et al. 1997; Kreppel and Hart 1999; Comer and Hart 2000; Zachara and Hart 2002), and purifies with an unknown S/T phosphatase (Zachara and Hart 2002).

O-GlcNAc is a dynamic and reversible modification, with a turnover rate similar to that of phosphorylation (Comer and Hart 2000). O-GlcNAcase, a cytoplasmic and nuclear β-N-acetylglucosaminidase, functions optimally at a neutral pH and exhibits selectivity towards O-linked GlcNAc. O-GlcNAcase is phosphorylated and purifies as part of a larger complex (Wells et al. 2002; Zachara and Hart 2002). Known inhibitors of this enzyme increase the levels of O-GlcNAc modified proteins (Comer and Hart 2000). The proximity of both OGT and O-GlcNAcase to their substrates allows rapid regulation of existing proteins (Vosseller et al. 2001).

O-GlcNAc modification is likely to have protein and even modification site specific influences (Vosseller et al. 2001). Known targets of this widespread modification include nuclear pore proteins, chromatin associated proteins, RNA polymerase II and its transcription

factors, hormone receptors, phosphatases and kinases (Zachara and Hart 2002). It is intriguing that many chromatin associated proteins, particularly histones, are glycosylated. Like modification by acetylation, glycosylation may regulate the transition from transcriptionally inactive or "closed" chromatin to transcriptionally active or "open" chromatin (Kelly and Hart 1989; Comer and Hart 1999).

Practically all of the RNA polymerase II transcription factors that have been studied in detail are modified by O-GlcNAc. There is evidence indicating O-GlcNAc plays roles in nuclear transport, regulating protein turnover, and regulating transactivation activities of transcription factors (Hart 1997; Comer and Hart 2000). It may mediate assembly of transcriptional complexes and may even directly regulate protein translation (Comer and Hart 2000).

Mouse knockout studies have shown that OGT is required for cell viability, indicating that O-GlcNAc modifications play essential roles (Comer and Hart 2000; Shafi et al. 2000; Zachara and Hart 2002). Loss of O-GlcNAcase is also toxic to cells (Fang and Miller 2001; Zachara and Hart 2002). Aberrant O-GlcNAc modifications have been associated with type II diabetes (Yki-Jarvinen 1998; Comer and Hart 2000; Akimoto et al. 2001; Vosseller et al. 2001), neurodegenerative diseases including Alzheimer's disease (Griffith and Schmitz 1995; Comer and Hart 2000), and even cancer (Zachara and Hart 2002).

Glycosylation of ETS transcription factors

ELF-1 is the only ETS transcription factor known to be glycosylated and is one of the few proteins known to be phosphorylated and glycosylated at the same time (Juang et al. 2002a). Many ETS transcription factors are known to be S/T phosphorylated, and as glycosylation can occur at the same site as phosphorylation, it is probable that many more ETS transcription factors are regulated by glycosylation.

ELF-1 mediates the induction of various genes in hematopoietic cells, including *cd4*, *gm-csf*, IgH enhancer gene, and *tcr* α and ζ chains (Tsokos, 2003 #99]. Predicted to be 68 kDa, ELF-1 has the apparent molecular weights of 80 and 98 kDa. This apparent molecular weight is not due to a high ratio of charged amino acids and therefore indicates that post-translational modifications are likely to be the cause of the increased weight.

The 80 kDa form of ELF-1 is cytoplasmic, while the 98 kDa form is nuclear (Juang et al. 2002a). The retinoblastoma (Rb) protein is known to sequester ELF-1 in the cytoplasm (Wang et al. 1993) and in agreement with this, Rb binds the 80 kDa form of ELF-1 more so than the 98 kDa form (Juang et al. 2002a). Upon phosphorylation of Rb, ELF-1 is no longer bound to Rb and is translocated to the nucleus (Wang et al. 1993). Thus the conversion of the 80 kDa form to the 98 kDa form decreases interactions with Rb and contributes to nuclear localization. The 98 kDa form of ELF-1 is phosphorylated and the kinases responsible are likely to belong to the protein kinase C family (Juang et al. 2002a). Phosphorylation of both the cytoplasmic scaffold Rb and ELF-1 itself leads to nuclear localization of ELF-1.

As phosphorylation alone can not make up for the ELF-1 size discrepancy, glycosylation was analyzed. Both forms of ELF-1 are O-GlcNAc, but the 98 kDa form has eight-fold more O-GlcNAc modifications. The ability of ELF-1 to bind to one of its target promoters, the TCR ζ-chain promoter, requires both O-GlcNAc and phosphorylation modifications (Juang et al. 2002a), indicating these modifications occur at different sites. The nuclear, 98 kDa form of ELF-1 is the main form bound to DNA. Proteasome inhibitors lead to increased cytoplasmic levels of the 98 kDa form, indicating that this form of ELF-1 is constantly degraded through the proteasome pathway. These inhibitors had no affect on the amount of the 80 kDa form of ELF-1 (Juang et al. 2002a). Thus glycosylation may also contribute to the degradation of ELF-1. Both

phosphorylation and glycosylation contribute to the nuclear localization and DNA binding affinity of ELF-1.

Patients with the autoimmune disease systemic lupus erythematosus (SLE) exhibit defective expression of TCR ζ-chain protein and mRNA (Liossis et al. 1998), a known transcriptional target of ELF-1. 83% of SLE patients have normal cytoplasmic levels of the 80 kDa form, while one-third of the patients have decreased levels of the nuclear 98 kDa form of ELF-1 (Juang et al. 2002b). Nuclear extracts from the latter patients exhibit defective DNA binding and decreased TCR ζ-chain expression. It is thought that the decreased amount of active ELF-1 is due to decreased levels of phosphorylation (Juang et al. 2002b). It is also likely the glycosylation plays a role in the disease state.

O-GlcNAc modication of ELF-1 leads to increased DNA binding, and may be involved in regulating protein-protein interactions and nuclear localization. ELF-1 is unique in that it is known to be both glycosylated and phosphorylated at the same time, indicating that the sites of modification can be different. This is another example of how multiple post-translational modifications are used to regulate various aspects of transcription factor function.

Concluding remarks

Extracellular signaling events ultimately regulate gene expression to promote a specific outcome. Therefore, signaling pathways must regulate the activities of transcription factors. Transcription factors function downstream of many different signaling cascades, and thus mechanisms must exist for different signaling events to differentially regulate transcription factors. A common method of regulating transcription factor function is by post-translational modifications, including phosphorylation, ubiquitination, sumoylation, acetylation, methylation

and glycosylation (Figure 1-4). Post-translational modifications can regulate both the activity and the specificity of transcription factors. This regulation of specificity is particular important for the ETS transcription factor family, as the more than 30 mammalian members of the family all recognize the same core DNA sequence.

Just from the above examples of how various post-translational modifications individually affect ETS transcription factor function, it is apparent that specific modifications should not be looked at in isolation as one modification can affect another, either positively or negatively. A particular modification may lead to a subsequent modification by altering the subcellular localization, the conformation of the protein, or protein-protein interactions. By these same mechanisms one modification can prevent another. Competition for a particular residue is another means by which modifications negatively regulating each other. Ubiquitination, sumoylation, and acetylation all occur at lysine residues, and thus modification by one of these will prevent other modifications at the same site. Similarly glycosylation and phosphorylation can target the same serine/threonine residues. It is important to note that in some cases there is competition for a particular residue, while in other cases, although they target the same amino acid, they modify different sites within a protein. In the latter case these modifications can regulate modifications at other sites within the target protein by the ways discussed above.

Often when one studies a transcription factor and how it is regulated one look at one step in the process. One needs to be aware that many mechanisms exist to regulate the functions of the protein, including the many types of post-translation modifications. A protein can be modified at multiple sites by a particular post-translation modification and by more than one type of post-translation modification at a time. The number of sites for each modification and the

Figure 1-4

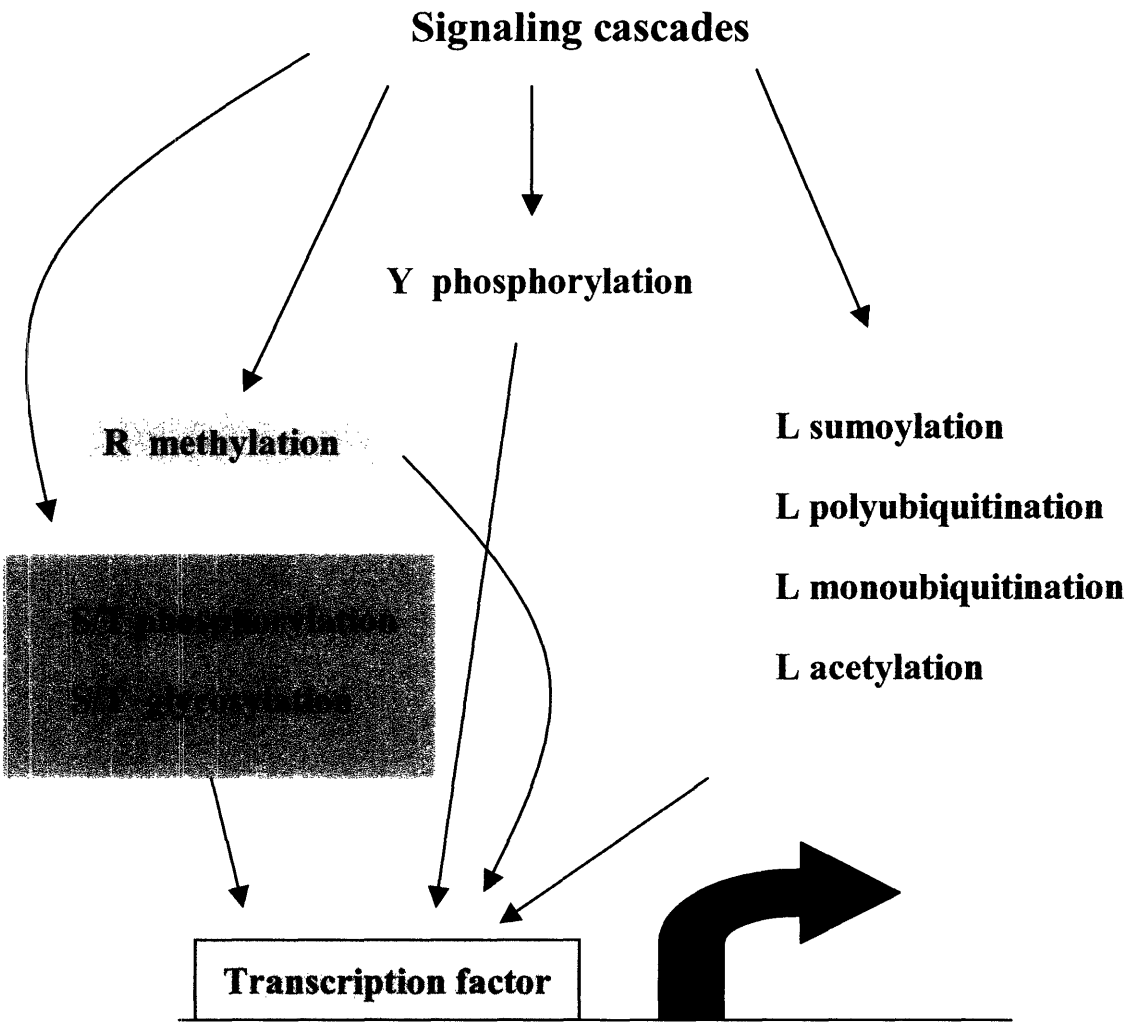


Figure 1-4

Schematic of how signaling events regulate post-translational modifications of transcription factors and thus regulate their activities.

combinations of modifications possible is extremely high, yielding immense regulatory opportunities that the cell is most likely taking advantage of. Additionally, the timing and order in which modifications occur adds other levels of regulation.

The order, timing, and combinations in which the multitude of post-translational modifications can occur provide the cell with an enormous amount of regulatory options. Although many years of research have yielded insights into how ETS transcription factors are regulated by post-translational modifications, we are probably only beginning to understand how these transcriptional regulators are controlled by these modifications. Broader analysis of how and why these factors are post-translationally modified in conjunction with how such modifications affect other modifications is needed. Particularly intriguing are the growing number of transcriptional regulators that possess the enzymatic activities to mediate these modifications.

Dual function transcriptional regulators

Evident from the examples above, post-translation modification play critical roles in regulating transcription factors, and the combinations of modifications that can occur on individual transcription factors yield immense regulatory possibilities. It is emerging that a small, but growing number of transcriptional regulators possess enzymatic activity and mediate post-translational modifications. I will review the enzymatic functions of the transcriptional regulators EYA, DBP1, ERK5, p300, and $TAF_{II}250$.

Transcription factors as phosphatases

EYA

EYA is an evolutionarily conserved transcriptional coactivator in the Retinal Determination (RD) Network and a protein phosphatase. The RD network encompasses a signaling cascade of transcriptional regulators best known for their necessity during *Drosophila* eye development. Additionally the RD network is deployed in both vertebrates and invertebrates in many developmental contexts outside of eye development. The RD network functions in a hierarchical manner in that Twin on Eyeless (TOY) turns on the expression of Eyeless (EY), which in turn leads to expression of EYA and Sine oculis (SO), who turn on Dachshund (DAC) (Figure 1-5) (Chen et al. 1997b; Pignoni et al. 1997; Czerny et al. 1999; Hauck et al. 1999).

EYA was originally characterized as the founding member of a novel family of transcriptional regulators, and contains two evolutionarily conserved domains, the carboxy-terminal EYA domain (ED), and the tyrosine rich EYA domain 2 (ED2) (Figure 1-6A) (Xu et al. 1997; Zimmerman et al. 1997). The ED2 resides within a larger proline, serine, threonine or PST rich region that is necessary for transcriptional activation (Silver et al. 2003). The ED has

Figure 1-5

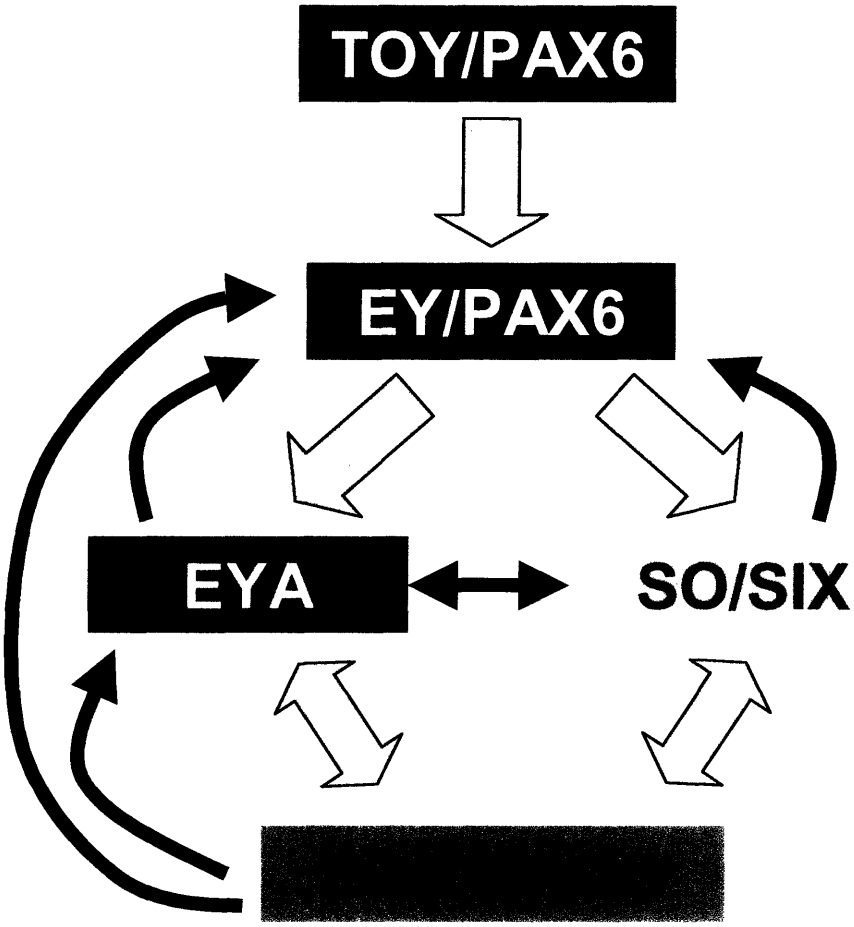
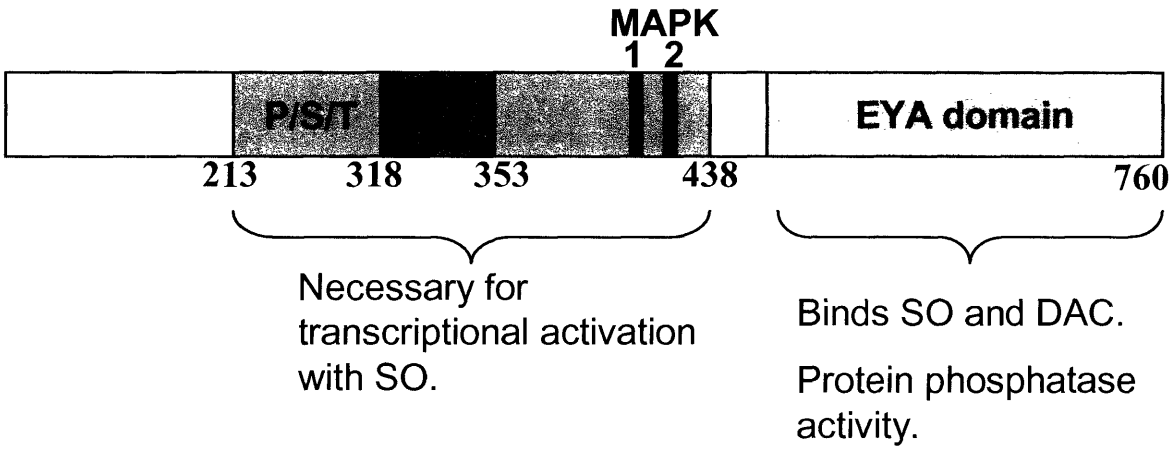


Figure 1-5

Schematic of the Retinal Determination Network cascade.

Figure 1-6

A


MAPK
1 2
P/S/T
EYA domain
213
318
353
438
760
Necessary for transcriptional activation with SO.
Binds SO and DAC.
Protein phosphatase activity.

B

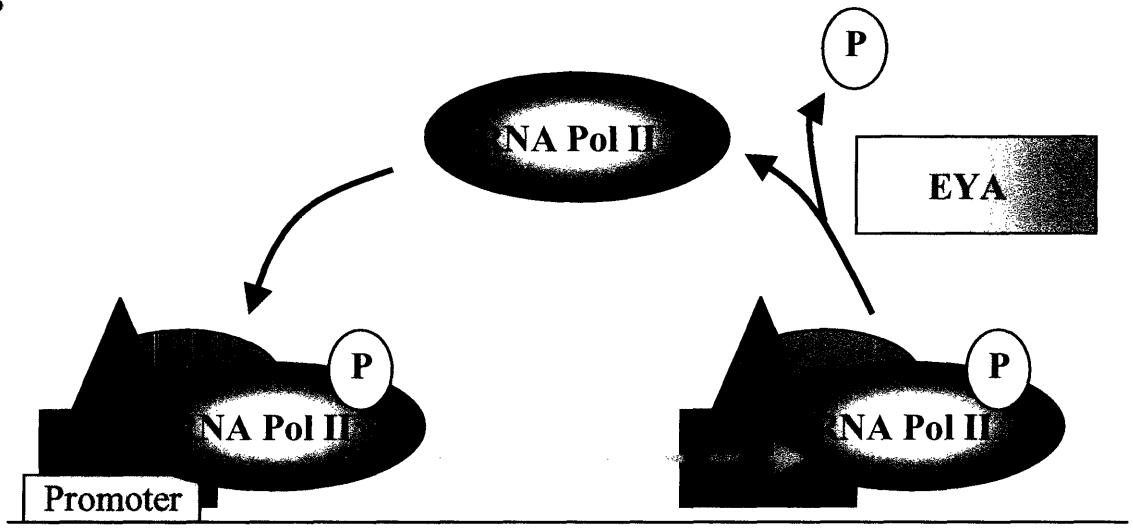

P
NA Pol II
EYA
P
NA Pol II
P
NA Pol II
Promoter

Figure 1-6

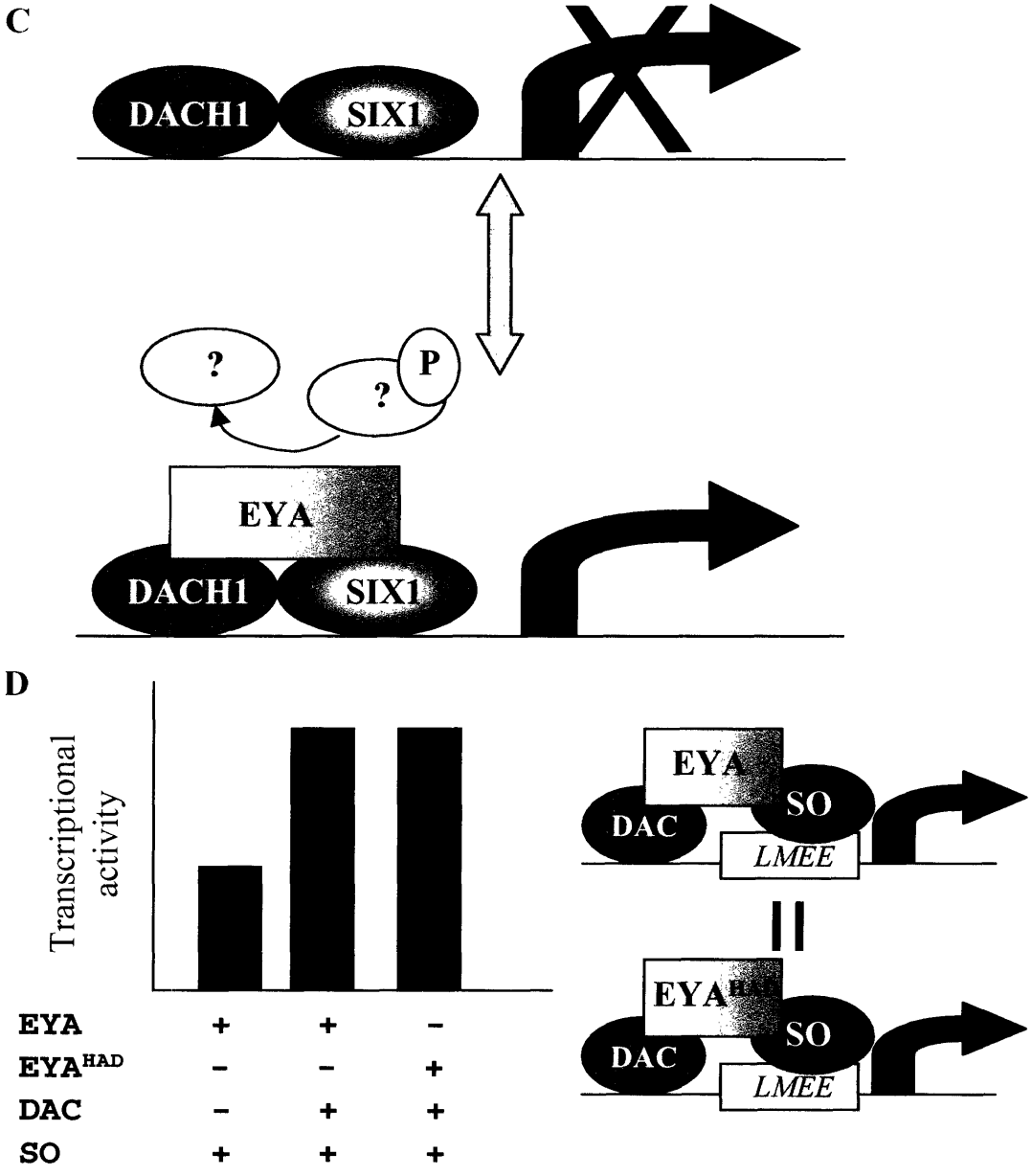

C
DACH1
SIX1
?
?
P
EYA
DACH1
SIX1
D
Transcriptional activity
EYA + + −
EYA^HAD − − +
DAC − + +
SO + + +
EYA
DAC
SO
LMEE
EYA
DAC
SO
LMEE

Figure 1-6

EYA's role as a protein phosphatase. A. Diagram of the EYA protein, pointing out the known domains and their respective functions. B. How EYA's activity as a protein phosphatase may be involved in regulating the activity of RNA polymerase II. RNA polymerase II binds to the DNA as part of the preinitiation complex. Phosphorylation on the CTD repeats of RNA polymerase II contributes to the transition from initiation to elongation of transcription. Dephosphorylation of RNA polymerase II, possibly mediated by EYA, leads to its removal from the DNA, allowing it to be recycled and bind to the promoter for another round of transcription. C. Schematic illustrating that DACH1 and SIX1 together repress transcription, addition of phosphatase active EYA switches this complex from functioning as a repressor to an activator. D. Graphic representation and schematic showing that DAC can enhance transcription from a native promoter mediated by the EYA-SO transcription factor, independent of EYA's phosphatase activity.

been shown to directly mediate protein-protein interactions with SO and DAC (Pignoni et al. 199 and Chen et al. 1997b, respectively). In the case of SO, the interaction with EYA results in the formation of a bipartite transcription factor (Ohto et al. 1999), with the homeodomain of SO providing the DNA binding activity and the PST rich region of EYA providing the transactivation (Silver et al. 2003). As DAC has recently been shown to possess DNA binding ability (Kim et al. 2002), it is possible that DAC acts like SO and recruits the transactivator EYA to the DNA, forming an active transcription factor. However the interaction between the ED of EYA and DAC is highly contested (Ikeda et al. 2002; Silver et al. 2003), suggesting that if such an association occurs it is likely to be context specific. Recent work indicates that under certain conditions SO, EYA, and DAC may form a tripartite factor to regulate transcription (Li et al. 2003;S. Silver, personal communication; see discussion below).

Multiple groups have shown that EYA, in addition to functioning as a transcriptional coactivator, is the defining member of a new family of protein phosphatases. The catalytic domain, the ED, was defined by homology to haloacid dehalogenases, a family of enzymes possessing a broad range of activities (Thaller et al. 1998; Collet et al. 1999). There is some discrepancy as to what type of protein phosphatase EYA is, as one group, utilizing full-length EYA, found that EYA is a dual specific phosphatase that can dephosphorylate both phospho-serine/threonine (S/T) and phospho-tyrosine (Y) (Li et al. 2003) residues, while two groups show that ED alone constructs are only capable of dephosphorylating phospho-tyrosine residues (Rayapureddi et al. 2003; Tootle et al. 2003b). It is possible that the amino-terminus of EYA is necessary for EYA to function as a dual specific phosphatase. Mutating residues known to be important for HAD enzyme catalytic function, results in a severe reduction or complete loss of EYA's phosphatase activity. In vivo analysis has shown that EYA's phosphatase activity is

required for *Drosophila* eye development (Chapter 4) (Rayapureddi et al. 2003; Tootle et al. 2003b). Therefore, EYA possesses two functions, one as a transcriptional coactivator and one as a protein phosphatase, both of which are required in vivo.

EYA is the first eukaryotic transcriptional activator shown to possess protein phosphatase activity. The only two known substrates of EYA are itself (Chapter 4) (Tootle et al. 2003b), and RNA polymerase II (Li et al. 2003). EYA dephosphorylates itself on tyrosine residues, while it dephosphorylates S/T residues on RNA polymerase II. The functional relevance of these dephosphorylation events are not known.

Analysis of EYA has yielded some insight into how tyrosine phosphorylation and dephosphorylation may regulate EYA's activities. Tyrosine phosphorylation could influence one or both of EYA's functions by affecting its protein-protein interactions, conformation, subcellular localization, or stability. EYA phosphatase mutants, collectively called EYA^{HAD}, exhibit higher levels of tyrosine phosphorylation and have reduced function in vivo (Chapter 4) (Tootle et al. 2003b). Some EYA^{HAD} mutants exhibit decreased transcriptional activity with SO in cell culture based transcription assays utilizing a reporter driven by a SO responsive segment of the *lozenge* promoter (*LMEE*-reporter) (S. Silver, personal communication). Both of these effects could solely be due to the loss of dephosphorylation of other substrates of EYA, but could also be partially due to the loss of dephosphorylation of EYA. Thus tyrosine phosphorylation of EYA may be a means of negatively regulating its activities. Both *Drosophila* EYA (Chapter 4) (Tootle et al. 2003b) and Mouse EYA3 (MmEYA3) are tyrosine phosphorylated (Chapter 5) when expressed in *Drosophila* cultured cells, suggesting that tyrosine phosphorylation is likely to have functional and conserved consequences.

Additionally EYA is positively regulated by MAPK-mediated serine/threonine phosphorylation, and it is possible that EYA can dephosphorylated itself on both Y and S/T residues. There are two MAPK phosphorylation sites (P-X-S/T-P) about 80 bp upstream of the ED; these sites are strictly conserved in EYA1, the first site is strictly conserved and the second site is only S/T-P in EYA2 and EYA4, while only the second site is S/T-P in EYA3 (Hsiao et al. 2001). Analysis of phosphorylation mutants by utilizing the ectopic eye induction assay indicates that phosphorylation at these sites positively regulates EYA's activity in the *Drosophila* eye, while analyses with multiple transcriptional reporter systems reveals that direct phosphorylation of EYA results in increased transactivation (Silver et al., 2003; S. Silver personal communication). It is possible that EYA negatively regulates its own activity by altering its S/T phosphorylation levels.

EYA dephosphorylates S/T residues in the carboxy-terminal domain (CTD) repeats of RNA polymerase II (Li et al. 2003). Phosphorylation of the CTD repeats of RNA polymerase II is necessary for the transition from preinitiation of transcription to elongation (Dahmus 1996). Dephosphorylation is thought to play a key role in the recycling of the polymerase, quickly releasing it from the DNA when it has finished transcribing the gene and allowing it to rebind to the promoter and transcribe the gene again (Majello and Napolitano 2001), a property important for transcriptional activation. Therefore, one of EYA's functions as a coactivator may be to dephosphorylate RNA polymerase II to allow recycling of the polymerase (Figure 1-6B).

It is possible that the only targets of EYA's phosphatase activity are itself and RNA polymerase II, however most phosphatases exhibit a fairly broad range of substrates. As SO and DAC family members are known to interact with EYA, they may also be targets of EYA's protein phosphatase activity. Murine DACH1, a homolog of DAC, complexes with SIX1, a

homolog of SO, and functions as both a transcription co-activator and co-repressor in mammalian cultured cells. The switch from repressor to activator appears to be mediated by EYA and requires its phosphatase activity (Li et al. 2003) (Figure 1-6C). This is the first evidence that EYA's phosphatase activity has a direct role in regulating transcription, however, the proteins dephosphorylated in this context are unknown.. Alternatively, utilizing the *LMEE*-reporter discussed above, *Drosophila* DAC has been shown to synergize with the EYA-SO transcription factor to further stimulate transcription, and this synergism is not dependent on EYA's ability to act as a phosphatase (Figure 1-6D) (S. Silver, personal communication). These different findings may be due to the nature of the reporters used or the cellular context of the assays. Thus, just as the EYA-DAC interactions are likely to be context specific, so are the effects of EYA's phosphatase activity. Additionally human SIX1 (HSIX1) is differentially S/T phosphorylated throughout the cell cycle by casein kinase II (Ford et al. 2000). Similarly *Drosophila* SO is known to be phosphorylated (E. Davies, personal communication). Therefore, SO family members may also be targets of EYA's protein phosphatase activity.

How the two functions of EYA are utilized throughout development, and whether one function depends on or inhibits the other is not known. Evolutionarily it appears that EYA originally possessed only phosphatase activity, as plant homologs are comprised of only the ED, while planarian to human homologs have substantial amino-terminal extensions. EYA has evolved to possess two functions as a transcriptional coactivator and as a nuclear protein phosphatase known to be in close association with the DNA. The two known substrates of EYA, itself and RNA polymerase II, are both intimately involved in regulating transcription, and their respective activities are known to be regulated by phosphorylation. Indeed phosphorylation is the most common means by which signaling events regulate transcription factor function (see

below). Therefore it is very probable that other unknown substrates of EYA's protein phosphatase activity are also involved in transcriptional regulation.

Two groups have independently found that EYA is specifically a protein tyrosine phosphatase (Rayapureddi et al. 2003; Tootle et al. 2003b). Tyrosine phosphorylation of transcription factors is a poorly studied area (see below). Therefore, it will be important to determine the substrates of EYA's protein tyrosine phosphatase activity and how EYA's transcription coactivator function is affected by tyrosine phosphorylation. These studies will yield insight into the mechanisms and breadth of the use of tyrosine phosphorylation and dephosphorylation as a means of regulating transcription.

DBP1

DBP1, DNA-binding protein phosphatase 1 from tobacco, regulates the transcription of pathogen-defense related genes, including CEVI1, and possesses phosphatase activity (Carrasco et al. 2003). The amino-terminus of DBP1 has homology to the DNA binding motif of the general transcription factor TFIIB. In vitro assays have shown that DBP1 has sequence specific DNA binding activity, and antisense experiments reveal that DBP1 is needed for proper gene expression of CEVI1 (Carrasco et al. 2003). In addition, the carboxy-terminus of DBP1 has homology to PP2C phosphatases and has intrinsic Mg^{2+} dependent protein phosphatase activity (Carrasco et al. 2003). The function of this phosphatase activity is not known. DBP1 is the first DNA binding protein shown to possess protein phosphatase activity.

To date there are two transcriptional regulators, DBP1 and EYA, which possess intrinsic protein phosphatase activity. Both are nuclear proteins that are highly associated with DNA, making it very likely, as discussed above, that their respective phosphatase activities are

involved in regulating transcription. This is not surprising, as phosphorylation appears to be the most common mechanism by which signaling cascades regulate downstream transcription factors. As the phosphatase activities of DBP1 and EYA have only recently been elucidated, it is possible that many more transcriptional regulators also possess such activities.

Kinases as transcription factors

ERK5

The MAPK ERK5 or Big MAP Kinase 1 (BMK1) has an amino-terminal kinase domain and a 400 amino acid carboxy-terminal domain that interacts with the transcription factor myocyte enhancer factor 2 (MEF2). Interestingly, this carboxy-terminal domain also appears to function as a transcriptional activation domain (TAD) (Figure 1-7A) (Kasler et al. 2000). For example, the carboxy-terminal domain (664-789 a.a.) of ERK5 is rich in acidic amino acids and can function as a TAD when fused to the GAL4-DNA binding domain (Figure 1-7B). Similarly when the MEF2 DNA binding domain is fused to this region of ERK5, the resulting protein can activate endogenous MEF2 target genes (Figure 1-7C). Intriguingly, ERK5 also directly phosphorylates MEF2 at serine 387, resulting in enhancement of MEF-2-mediated transcriptional activation (Figure 1-7D) (Kato et al. 1997). Therefore ERK5 is both a kinase and a transcriptional coactivator, with both activities implicated in regulating MEF2 function.

Again there is evidence of how transcriptional activity is highly influenced by phosphorylation. ERK5 is a S/T kinase that is also a transcriptional coactivator, and above I have discussed two distinct transcriptional regulators that possess phosphatase activity. These examples suggest it may be important to exert local control over post-translational modifications, particularly phosphorylation, as a means of quickly regulating transcription. It is well

Figure 1-7

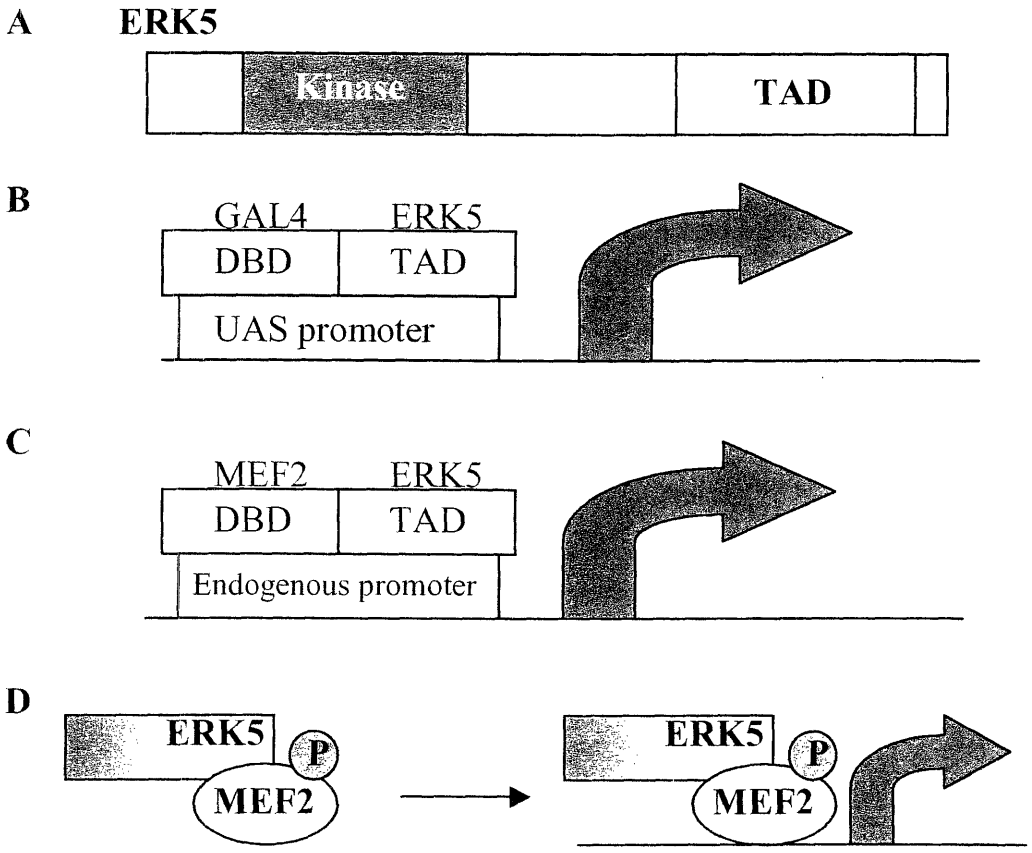


Figure 1-7

ERK5 is both a kinase and a transcriptional coactivator. A. Diagram of the ERK5 protein, illustrating that it possess both a kinase domain and a transcriptional activation domain (TAD). B. Schematic showing that if the TAD of ERK5 is fused to the GAL4 DNA binding domain (DBD), the fusion protein can activate transcription. C. Schematic showing that if the TAD of ERK5 is fused to the DNA binding domain of the transcription factor MEF2, the fusion protein activates transcription from endogenous target promoters. Thus, this region of ERK5 is indeed a TAD. D. Schematic illustrating how ERK5 enhances MEF2-mediated transcriptional activation by directly phosphorylating MEF2 and by serving as a transcriptional coactivator.

established that by having transcriptional regulators present but inactive due to a modification or lack there of, activity shifts can be achieved quickly by altering these modifications in response to specific signaling cascades. The rate of response to a signaling event may be further increased by having the protein responsible for the modification also playing a role in mediating the transcriptional response, thus requiring one less protein to be recruited (essentially eliminating the middleman).

Transcription factors as acetyltransferases and parts of the ubiquitin pathway

p300

p300, a known acetyltransferase, is also involved in ubiquitin-mediated degradation (Grossman et al. 2003). This is particularly interesting because both acetylation and ubiquitination occur on lysine residues, and often modify the same lysines within a particular protein. Intriguingly, p300 differentially regulates the transcription factor p53, a key mediator of cell cycle arrest and apoptosis in response to DNA damage, by both acetylating and ubiquitinating p53 on the same lysine residues (Figure 1-8). In the absence of DNA damage, p300 facilitates p53 polyubiquitination, resulting in p53 being targeted to the proteasome for degradation. In contrast, following DNA damage, p300 acetylates and coactivates the transcription factor p53 (Grossman 2001). In addition p300 cooperates with the E1A oncoprotein, a multifunctional protein originally identified in as Adenovirus 5 early region 1A, to stabilize p53, preventing its degradation (Chiou and White 1997; Querido et al. 1997; Zhu et al. 2001). Thus p300 plays dual roles in regulating the transcription factor p53 by both acetylating p53 and contributing to its transcriptional activation, and by facilitating p53's degradation when it is no longer needed.

Figure 1-8

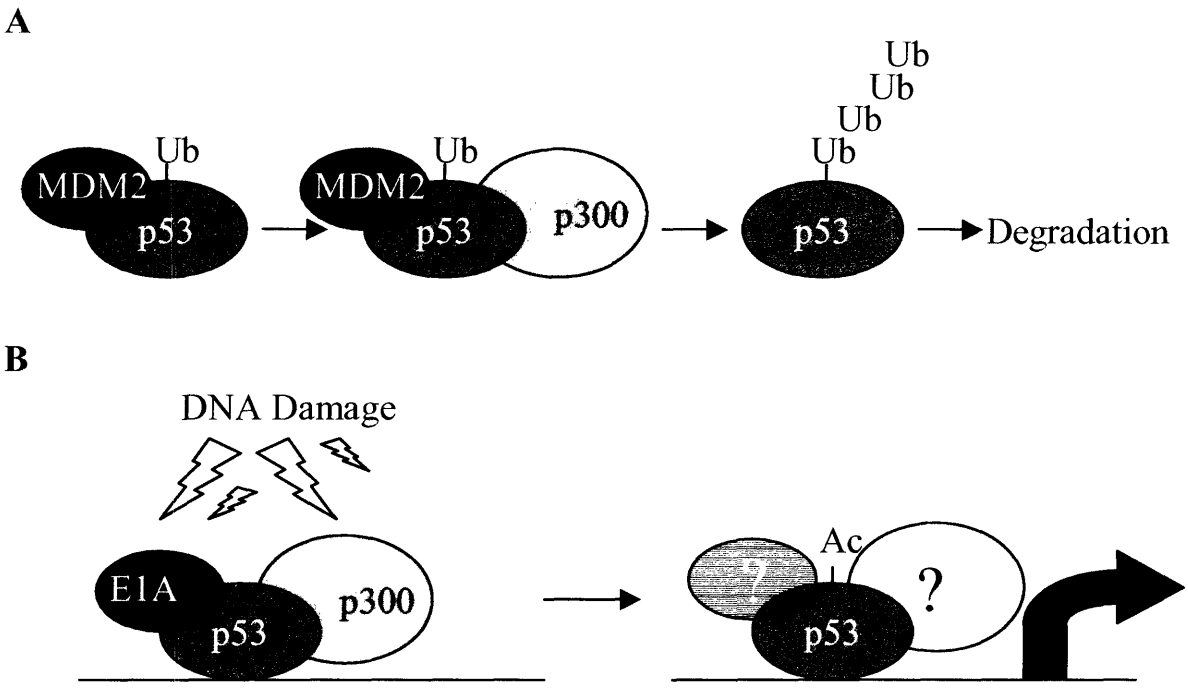


Figure 1-8

p300 mediates both the degradation of p53 and the activation of p53-mediated transcription by its two enzymatic activities, as an acetyltransferase and an E4 enzyme. A. Schematic illustrating how in the absence of DNA damage, MDM2, an ubiquitin ligase, monoubiquitinates p53. p300 can then polyubiquitinate p53, resulting in p53 being targeted for degradation. B. Upon DNA damage, E1A and p300 stabilize the p53 protein, and p300 then acetylates p53, resulting in increased p53-mediated transcriptional activation.

Purified p300 and endogenous p300 have intrinsic ubiquitin ligase activity (Grossman et al. 2003). See below for a review of the ubiquitin modification pathway. The amino-terminal 595 amino acids possess potent ubiquitin ligase activity, while amino acids 671-1196 have much weaker activity (Grossman et al. 2003). The amino-terminus has no homology to known ubiquitin ligases but does have a cysteine/histidine rich sequence resembling a HECT domain (Grossman et al. 1998). HECT domain E3 ubiquitin ligases transfer ubiquitin from the E2 enzyme to their active site cysteine forming a thio-ester that then functions as the donor for the amide bond formation with the substrate. The HECT-like domain of p300 has been shown to interact with Mouse Double Minute 2 (MDM2), an ubiquitin ligase that mono- but not poly-ubiquitinates p53 (Rodriguez et al. 2000; Lai et al. 2001), and this association has been implicated in p53 stability (Grossman et al. 1998). Thus the cysteine/histidine rich region of p300 may directly bind ubiquitin and transfer it directly to the substrate protein.

In the absence of DNA damage MDM2 monoubiquitinates p53 at numerous lysines and p300 can act as an E4 enzyme, which recognizes and polyubiquitinates sites of monoubiquitination, and polyubiquitinate p53 at these sites of monoubiquitination, resulting in p53 degradation (Figure 1-8A) (Grossman et al. 2003). In response to cellular stresses, like DNA damage, E1A directly interacts with p300 and inhibits p300 from polyubiquitinating p53 (Grossman et al. 2003), thereby stabilizing the protein. By an unknown mechanism p300 can also acetylate and activate p53, possibly by an interaction between p53 and the E1A-p300 complex (Figure 1-8B).

p53 is acetylated and ubiquinated at the same lysine residues (Ito et al. 2002), and one modification on a particular lysine prevents other modifications from occurring at this residue. This allows strict regulation of the modified protein's activity, much like an on/off switch. In the

case of p53, ubiquitination is the on switch for degradation and the off switch for transcriptional activity, while acetylation is the on switch for transcriptional activation and the off switch for degradation. Intriguingly, monoubiquitination of transcription factors, like MDM2-mediated monoubiquitination for p53, has been associated with transcription activation (see below). This suggests that MDM-2 monoubiquitination of p53 may play a role in p53-mediated transcriptional activation in addition to its role in facilitating p53 degradation.

$TAF_{II}250$

$TAF_{II}250$ is the largest subunit of the general transcription factor TFIID and possess a wide range of functions, including acting as an acetyltransferase, a kinase, and an E1/E2 enzyme in the ubiquitin pathway. I will first review the general role of TFIID in transcription, and then discuss the three enzymatic functions of $TAF_{II}250$.

TFIID is a complex of the TATA-binding protein (TBP) and ten TBP associated factors (TAFs) (Struhl and Moqtaderi 1998). TFIID recognizes the promoters of all protein-coding genes, either through the sequence specific binding of the TBP or by the interactions of its various TAFs with downstream promoter elements called initiators, and subsequently recruits the rest of the preinitiation complex to the promoters, including RNA polymerase II (Verrijzer and Tjian 1996; Struhl and Moqtaderi 1998; Sterner and Berger 2000). The TBP of TFIID mediates a basal level of transcription, while the TAFs are required for activation of transcription (Verrijzer and Tjian 1996).

$TAF_{II}250$ homologs from yeast ($yTAF_{II}130$), *Drosophila* ($dTAF_{II}230$), and human all possess intrinsic acetyltransferase activity (Mizzen et al. 1996). All three homologs exhibit specificity and acetylate histones H3 and H4 (Mizzen et al. 1996). Amino acids 1-1140 of

$TAF_{II}250$ are important for acetyltransferase activity, with amino acids 885-1140 being critical (Mizzen et al. 1996). $TAF_{II}250$ proteins have no sequence homology to known acetyltransferases and therefore define a new family of acetyltransferases. Like other acetyltransferases, $TAF_{II}250$ homologs from humans and *Drosophila* have bromodomains; but these domains are dispensable for in vitro acetyltransferase activity (Mizzen et al. 1996).

$TAF_{II}250$ acetyltransferase activity is negatively regulated by interactions with $TAF_{II}55$, another component of TFIID. This results in inhibition of $TAF_{II}250$ dependent MHC class I gene transcription (Gegonne et al. 2001). Therefore modulation of $TAF_{II}250$'s acetyltransferase activity has direct affects on the transcriptional output of specific promoters.

A temperature sensitive mutation in the acetyltransferase domain of $TAF_{II}250$ (hamster cell line t13) arrests cells in G_1. The mutant protein has reduced acetyltransferase ability and exhibits decreased transcription of certain genes including G_1 cyclins, but can still interact with TBP and other TAFs at the restrictive temperature (Dunphy et al. 2000). This indicates that $TAF_{II}250$ acetyltransferase activity is required for cell cycle progression and is involved in the regulation of transcription of proliferative control genes, but is not required for the transcription of all protein encoding genes (Figure 1-9A).

Other TAF_{II}s are components of acetyltransferase complexes, SAGA (SPT-ADA-GCN5-acetyltransferase) (Grant et al. 1998), and the P/CAF complexes (Struhl and Moqtaderi 1998), in addition to the TFIID complex. Not all of the TAF_{II}s found in TFIID are in the other complexes, in particular $TAF_{II}250$ and $TAF_{II}130$ are not in the SAGA and P/CAF complexes. All three complexes share some TAF_{II}s, but they each have a different acetyltransferase enzyme and different DNA binding proteins (Struhl and Moqtaderi 1998). This indicates that the different acetyltransferase complexes are likely to target different promoters. These complexes all

Figure 1-9

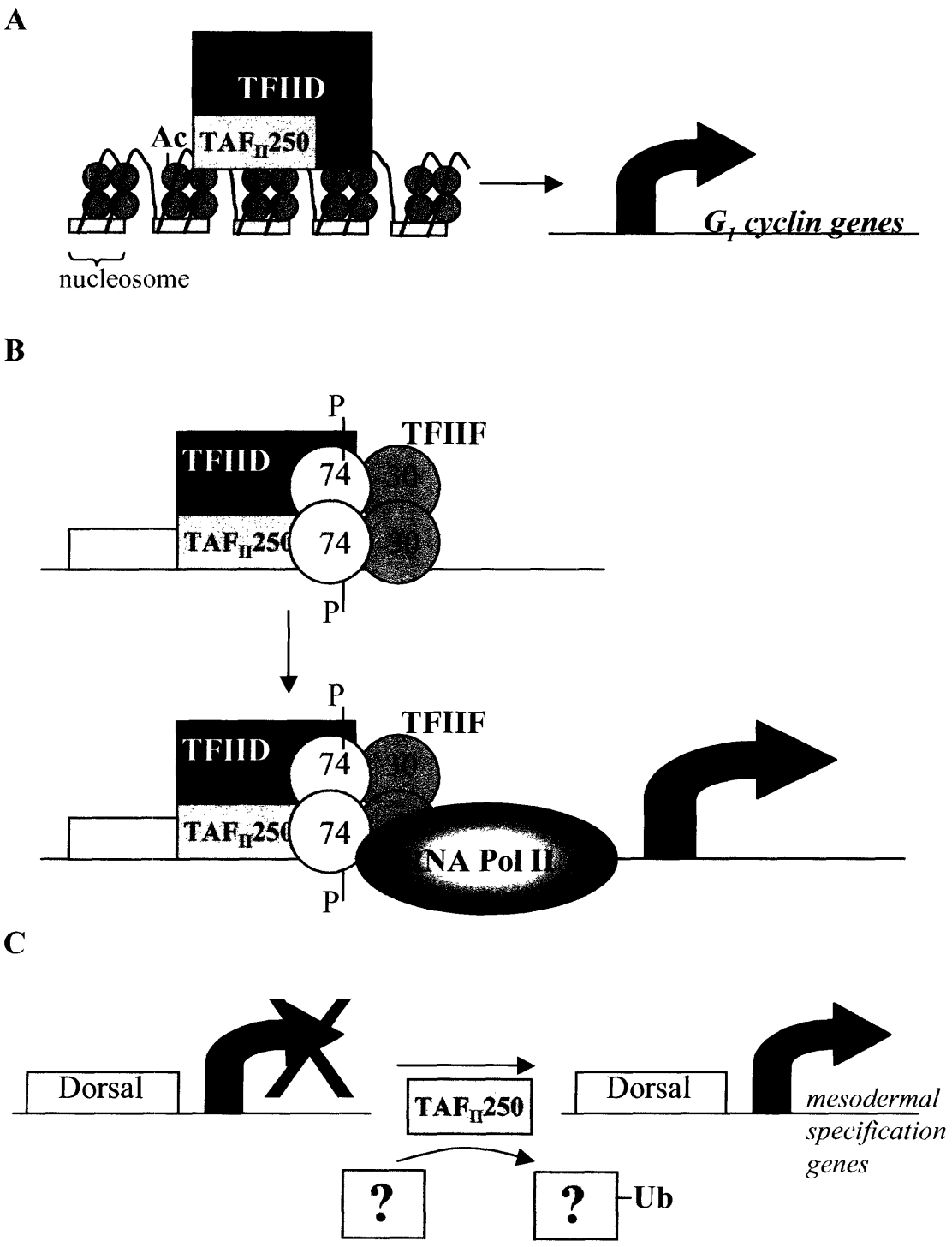

A
TFIID
Ac
TAF$_{II}$250
nucleosome
G_1 cyclin genes
B
P
TFIIF
TFIID
74
TAF$_{II}$250
74
P
P
TFIIF
TFIID
74
TAF$_{II}$250
74
NA Pol II
P
C
Dorsal
TAF$_{II}$250
Dorsal
mesodermal specification genes
?
?
Ub

Figure 1-9

$TAF_{II}250$ possesses three activities, acetyltransferase, kinase, and E1/E2 enzyme activity, which differentially regulate transcriptional activation from distinct sets of promoters. A. Schematic illustrating that $TAF_{II}250$ is thought to mediate transcription via its acetyltransferase activity by directly acetylating chromatic associated proteins, like histones, and resulting in an "open" chromatic state. B. Schematic showing that $TAF_{II}250$ kinase activity is important for the phosphorylation of the RAP70 subunits of TFIIF, resulting in recruitment of RNA Polymerase II, and thus transcriptional activation. C. Schematic illustrating that $TAF_{II}250$ E1/E2 monoubiquitinating activity is important for Dorsal-mediated transcriptional activation. The target of this ubiquitinating activity is unknown, but could include histones, general transcription machinery, or even the transcription factor.

promote acetyl-CoA dependent transcriptional activation from promoters occupied by nucleosomes, suggesting such complexes function to regulate chromatin structure. It is interesting that structurally the various TAF_{II}s resemble histones, and thus the histone-like folds of these TAF_{II}s in the various acetyltransferase complexes may play important roles in interacting with nucleosomes.

In addition to possessing acetyltransferase activity, $TAF_{II}250$ has two kinase domains, one at the amino-terminus (1-434 a.a.) and one at the carboxyl terminus (1423-1893 a.a.) (Dikstein et al. 1996). Both domains can autophosphorylate $TAF_{II}250$, and are required for efficient serine phosphorylation of RAP74. RAP74 is the largest subunit of TFIIF, a tetramer of two RAP30 and two RAP74 molecules (Dikstein et al. 1996). This phosphorylation is not a non-specific activity of $TAF_{II}250$, as it does not phosphorylate other general transcription factors or common phosphoproteins. Phosphorylation of RAP74 by $TAF_{II}250$ occurs in the context of the TFIID complex (Dikstein et al. 1996), and thus is likely to play a role in mediating the formation of the preinitiation complex. The RAP74 subunits interact directly with RNA polymerase II to recruit it to the promoter, and remain associated with the polymerase during elongation (Rossignol et al. 1999). Hyperphosphorylated TFIIF is associated with transcription activation and therefore, phosphorylation of RAP74 may be one mechanism of regulating transcription (Figure 1-9B).

Mutations in the amino-terminal kinase domain of $TAF_{II}250$ result in inhibition of autophosphorylation but do not block all kinase activity. These $TAF_{II}250$ mutants can still be incorporated into TFIID complexes but have reduced ability to rescue a temperature sensitive acetyltransferase $TAF_{II}250$ mutant cell line (ts13), and exhibit decreased transcription from *cyclin A* and *cdc2* promoters (O'Brien and Tjian 1998). This indicates that both the

acetyltransferase and kinase functions of $TAF_{II}250$ are required for the regulation of some promoters.

Gene profiling of acetyltransferase versus kinase $TAF_{II}250$ mutants revealed that these activities in general regulate nonoverlapping gene sets (O'Brien and Tjian 2000). Expression of 18% of the genes analyzed is disrupted by acetyltransferase mutations, while the expression of 6% of the genes is affected by mutations within the amino-terminal kinase domain. Only 1.3% of the genes analyzed were affected by both mutations (O'Brien and Tjian 2000). Therefore, both activities of $TAF_{II}250$ are important for transcriptional regulation, and appear to be differentially utilized depending on the promoter.

In addition to $TAF_{II}250$ functioning as both an acetyltransferase and a kinase, it is also involved in ubiquitination (Pham and Sauer 2000; Hicke 2001). *Drosophila* embryo extracts analyzed for enzymes that ubiquitinate histones identified $dTAF_{II}250$. $dTAF_{II}250$ specifically ubiquitinates histone H1 in the absence of an E1 enzyme, indicating that $TAF_{II}250$ possesses E2 activity. This along with the fact that $TAF_{II}250$ can conjugate with ubiquitin by a thiolester bond indicates that $TAF_{II}250$ possesses both E1 and E2 enzymatic activities (Pham and Sauer 2000; Hicke 2001). In agreement with this, $TAF_{II}250$ has sequence similarities to both E1 and E2 enzymes. In vitro ubiquitination assays also revealed that $TAF_{II}250$ monoubiquitinates histone H1, and the catalytic domain is encoded within amino acids 768-1218.

$TAF_{II}250$'s E1/E2 activity is required for activation of transcription from specific promoters. For example, $dTAF_{II}250$ mediates the activation of the maternal activator Dorsal, which in turn activates the expression of genes necessary for mesodermal determination. Mutations within the catalytic domain, V1072D and R1096P, inhibit $TAF_{II}250$ E1/E2 enzymatic activity (Pham and Sauer 2000). In vivo these $TAF_{II}250$ mutants inhibit mesodermal marker

expression, indicating that dTAF$_{II}$250's E1/E2 activity is necessary for Dorsal-dependent transcriptional activation (Figure 1-9C) (Pham and Sauer 2000; Hicke 2001). TAF$_{II}$250 is unique in that it possesses both E1 and E2 enzymatic activities, and this combination enzyme may be specific for monoubiquitination. As discussed in depth below, monoubiquitination of transcription factors has been associated with transcriptional activation. It will be interesting to see whether TAF$_{II}$250 is generally responsible for monoubiquitinating transcription factors regulated in this manner, or whether TAF$_{II}$250's E1/E2 enzyme displays specificity.

TAF$_{II}$250 is a component of TFIID and is thus required for the transcription of all protein coding genes. In addition, TAF$_{II}$250 possesses three distinct enzymatic activities as a kinase, an acetyltransferase, and an E1/E2 monoubiquitinating enzyme. None of these activities have been shown to be required for the transcription of all protein-encoding genes, indicating that the different activities are likely utilized to regulate transcription from different promoters. Some mechanism(s) must exist to determine the specificity of each activity. TAF$_{II}$250 is the best studied example to date of how mediation of post-translational modifications are associated with transcriptional regulation.

Concluding remarks

Through these examples of multifunctional enzymes it is clear that an increasing number of proteins involved in transcriptional regulation are also likely to be involved in mediating/regulating post-translational modifications. As post-translational modifications are the main mechanisms by which signaling cascades regulate downstream transcription factors it is not surprising that such multifunctional transcriptional regulators exist. It will be critical to the

understanding of how transcription is regulated to elucidate and understand the exact roles of such multifunctional proteins.

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Chapter 2

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

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Tina Tootle, in majority, performed the experiments and wrote the manuscript for the published work below. However, there were specific contributions by others. In particular, Philina Lee, initiated the analysis of the NLS insertions, including generation of the constructs. Andrina Williams also assisted in the production of the transgenic fly lines.

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 cofactor 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.

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 in 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 in 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 down-regulated (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. (2001) also suggest that MAE binds to the PD of PNT-P2, and enhances PNT-P2's transcriptional activation, leading them to propose that MAE promotes PNT-P2 phosphorylation by MAPK. Thus, they speculate that by promoting phosphorylation events that simultaneously down-regulate YAN and upregulate PNT-P2, MAE facilitates downstream responses to RTK signaling.

While it is clear that MAPK phosphorylation initiates YAN downregulation, the ensuing events, with respect to both YAN and PNT-P2, remain poorly understood. Here 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 coexpressing specifically designed structural variants of YAN, we demonstrate that MAE plays a critical role in mediating YAN's nuclear export, independent of its role in promoting MAPK phosphorylation. Consistent with previous reports (Baker *et al.*, 2001), we find that overexpression of MAE decreases YAN's transcriptional repressor activity. However, whereas PNT-P2's transcriptional activity was proposed to be stimulated by MAE co-expression (Baker *et al.*, 2001), we find that overexpression of MAE inhibits PNT-P2's ability 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' ACCCCACCTAAGAAGAAGCGCAAGGTGGAGGACTCCCAG 3' and 5' GAGTCCTCC ACCTTGCGCTTCTTCTTAGGTGGGGTCTGG 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'GATCTACCCCGCCAAAGAAGA AGCGCAAGGTGGAGGACG 3' and 5'GATCCGTCCTCCACCTTGCGCTTCTTCTT TGGCGGGGTA 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' GGACTGGCAAAGTTGGGAGGCATCCAGGGGAA CCATCTGTCC 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' CTATGATAGCAGGGCCAT TGCTCGG 3'. The product was N-terminally tagged with a Myc epitope, verified by sequencing, and shuttled into both pRMHa3 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 6 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). S2 cells stainings were 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 done 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' GAATTAATACGACTCACTAT 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-TTGTTCATGCACAGGC 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 <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 2x 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 (Figure 2-1A) to the cytoplasm (Figure 2-1B) 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, 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 (Figure 2-1C). 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 detected in developing Drosophila tissues (I. Rebay, unpublished; Lai and Rubin, 1992), it was possible that the nuclear export demonstrated in S2 cultured cells (Figure 2-1A-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 amino terminus ($YAN^{N' NLS}$) or in the middle of the protein ($YAN^{Int NLS}$ and

Figure 2-1

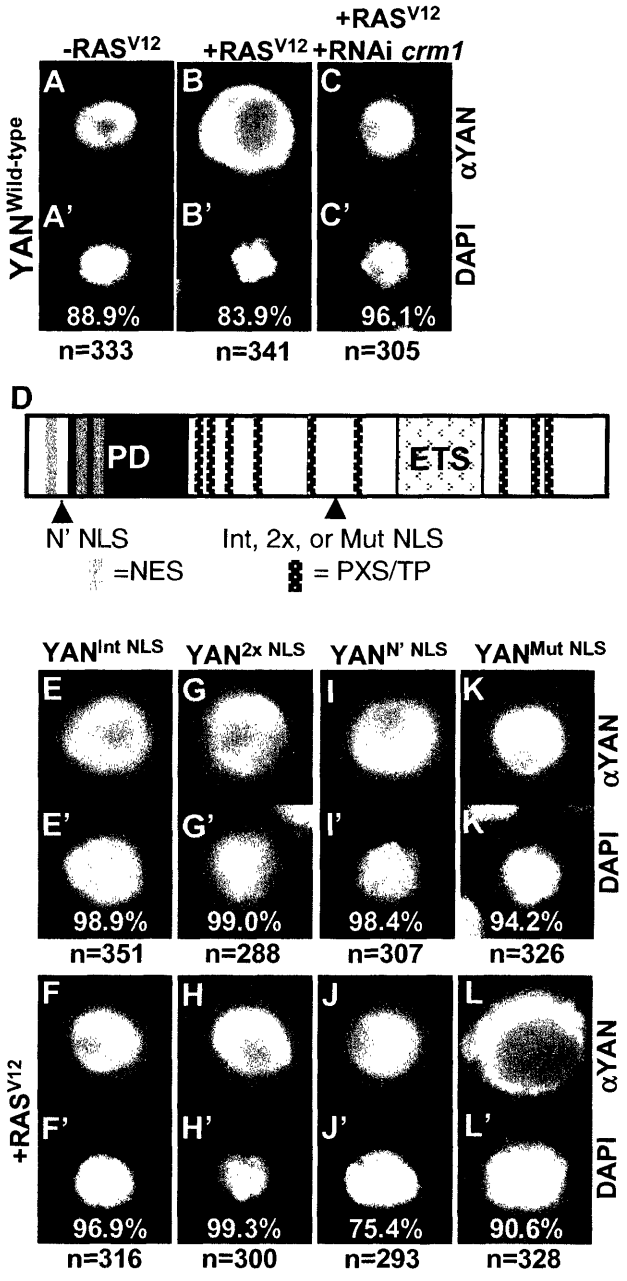

-RAS^V12
+RAS^V12
+RAS^V12 +RNAi crm1
YAN^Wild-type
αYAN
DAPI
A
A'
B
B'
C
C'
88.9%
83.9%
96.1%
n=333
n=341
n=305
D
PD
ETS
N' NLS
Int, 2x, or Mut NLS
=NES
= PXS/TP
YAN^Int NLS
YAN^2x NLS
YAN^N' NLS
YAN^Mut NLS
E
E'
G
G'
I
I'
K
K'
98.9%
99.0%
98.4%
94.2%
n=351
n=288
n=307
n=326
+RAS^V12
F
F'
H
H'
J
J'
L
L'
96.9%
99.3%
75.4%
90.6%
n=316
n=300
n=293
n=328

Figure 2-1

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 refers to the 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.

$YAN^{2x\ NLS}$) (Figure 2-1D). 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 (Figure 2-1E-H). $YAN^{N'\ NLS}$ appears less potent, presumably due to insertion in a less accessible region of the protein, and only partially restricted YAN to the nucleus (Figure 2-1I,J). The control experiment, in which $YAN^{Mut\ NLS}$ behaved indistinguishably from wild-type YAN, localizing to the nucleus in unstimulated cells (Figure 2-1K) and becoming cytoplasmic in RAS^{V12} stimulated cells (Figure 2-1L), 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 (Figure 2-2A,A') and CNS development should be inhibited as visualized by reduced expression of neuronal markers (Figure 2-2A") (Rebay and Rubin, 1995). Alternatively, if nuclear export is not required, then the NLS tagged YAN should be down-regulated 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 (Figure 2-2B,B',B").

Supporting the first model, expression of either $YAN^{Int\ NLS}$ (Figure 2-2C,C',C") or $YAN^{2x\ NLS}$ (Figure 2-2D,D',D") resulted in a YAN^{ACT} phenotype (Figure 2-2A,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 (Figure 2-2E,E') and neuronal differentiation (Figure 2-2E"). 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.

The PD is necessary for regulating the subcellular localization of YAN

Having demonstrated a requirement for nuclear export in YAN downregulation in 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) (Figure 2-1D). Two of the putative NESs reside within the Pointed Domain (PD), suggesting this motif could be involved in regulating export.

Figure 2-2

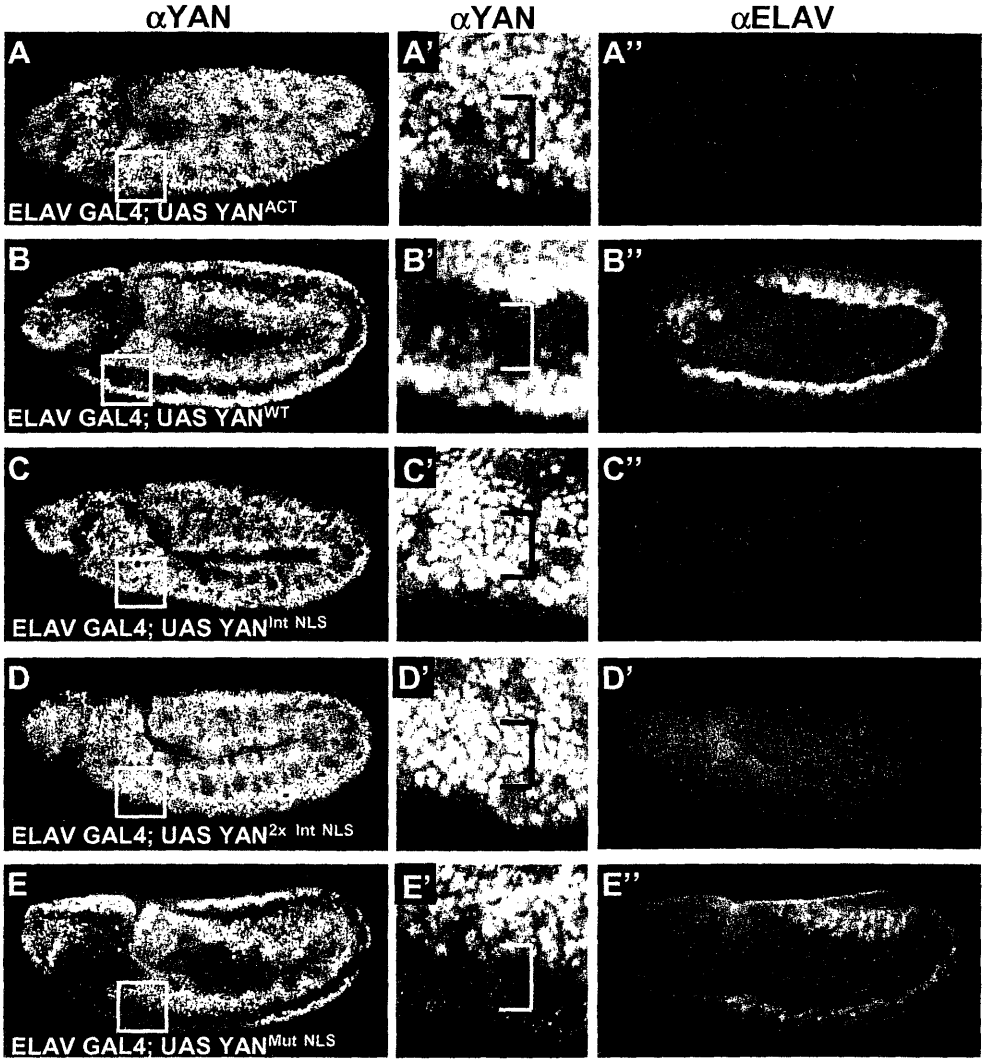

αYAN
αYAN
αELAV
A
A'
A''
ELAV GAL4; UAS YAN^ACT
B
B'
B''
ELAV GAL4; UAS YAN^WT
C
C'
C''
ELAV GAL4; UAS YAN^Int NLS
D
D'
D'
ELAV GAL4; UAS YAN^2x Int NLS
E
E'
E''
ELAV GAL4; UAS YAN^Mut NLS

Figure 2-2

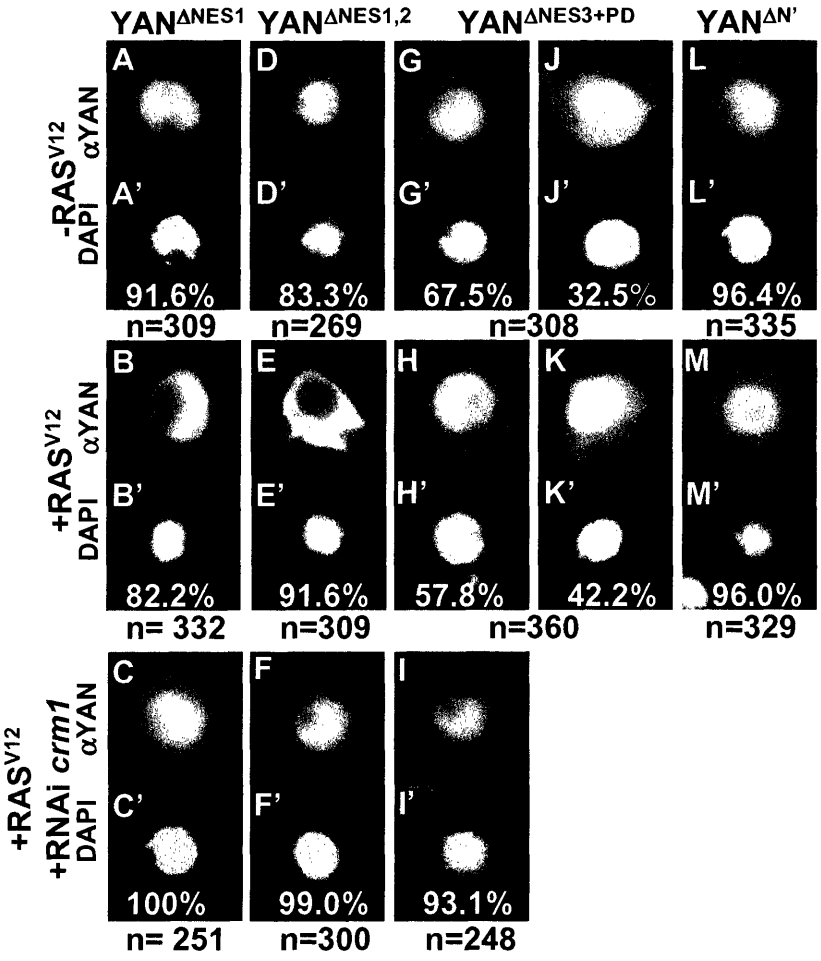
NLS insertions restrict YAN to the nucleus in vivo. (A-E") Confocal images of germband extended *Drosophila* embryos double labeled with anti-YAN (A-E, A'-E') and anti-ELAV (A"-E"). (A'-E') Higher magnification views of regions boxed in A-E with normal or failed YAN downregulation highlighted by bracket. 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 down-regulated normally in the ventral nerve cord (B, B'), allowing neuronal differentiation to proceed (B"). Just like YAN^{ACT} (A, A', A"), insertion of an NLS restricts YAN to the nucleus (C, C' and D, D'), thereby blocking CNS development(C", D").

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 (Figure 2-3A,B and D, E respectively, as compared to Figure 2-1A,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 (Figure 2-3G,J and 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 (Figure 2-3C,F,I). Finally, the deletion of the whole amino terminus ($YAN^{\Delta N'}$), including all three NESs and the PD, localized to the nucleus and remained nuclear in the presence of RAS^{V12} (Figure 2-3L,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 YAN's ability 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

Figure 2-3


YAN^ΔNES1
YAN^ΔNES1,2
YAN^ΔNES3+PD
YAN^ΔN'
-RAS^V12 αYAN DAPI
+RAS^V12 αYAN DAPI
+RAS^V12 +RNAi crm1 αYAN DAPI
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'
H
H'
I
I'
J
J'
K
K'
L
L'
M
M'
91.6%
83.3%
67.5%
32.5%
96.4%
n=309
n=269
n=308
n=335
82.2%
91.6%
57.8%
42.2%
96.0%
n= 332
n=309
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n= 251
n=300
n=248

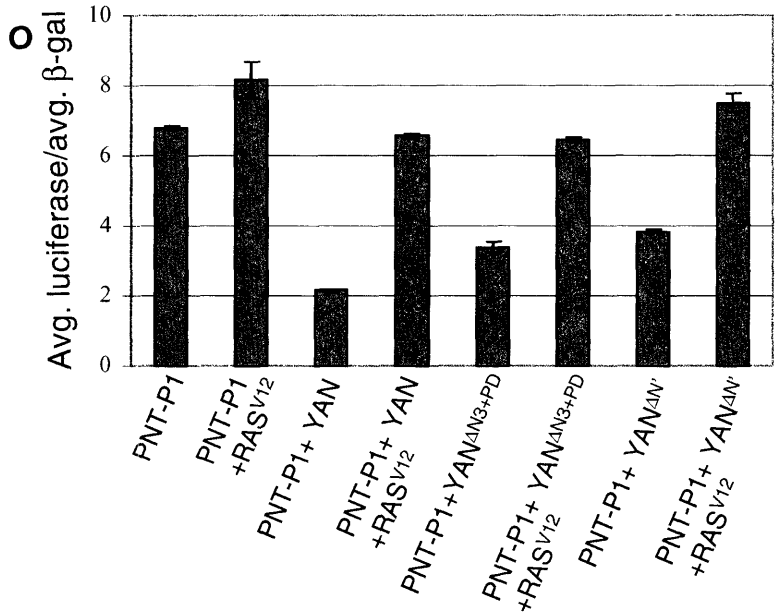
O
Avg. luciferase/avg. β-gal
10
8
6
4
2
0
PNT-P1
PNT-P1 +RAS^V12
PNT-P1+ YAN
PNT-P1+ YAN +RAS^V12
PNT-P1+YAN^ΔN3+PD
PNT-P1+ YAN^ΔN3+PD +RAS^V12
PNT-P1+ YAN^ΔN'
PNT-P1+ YAN^ΔN' +RAS^V12

Figure 2-3

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 refers to the 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 knock-down 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}.

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 (Figure 2-3O). 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 cofactors 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 curiously *mae* mutations have not been isolated in RTK pathway genetic interaction screens (for example, 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) (Figure 2-4B, compared to Figure 2-4A). 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 (Figures 2-4C,D), consistent with MAE's proposed function 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 (Figure 2-4E), Sev-RAS^{V12} exhibits 3.0 R7/ommatidium (Figure 2-4F), 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 (Figure 2-4G,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 *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 2-1), 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* (Figure 2-4I,I'), *Df(2R)PC4* (data not shown), or transheterozygotes (data not shown). YAN is not down-regulated in *mae* mutant embryos, exhibiting nuclear expression in the brain and ventral nerve cord (Figure 2-4I,I' as compared to Figure 2-4J,J'). Consistent with

Figure 2-4

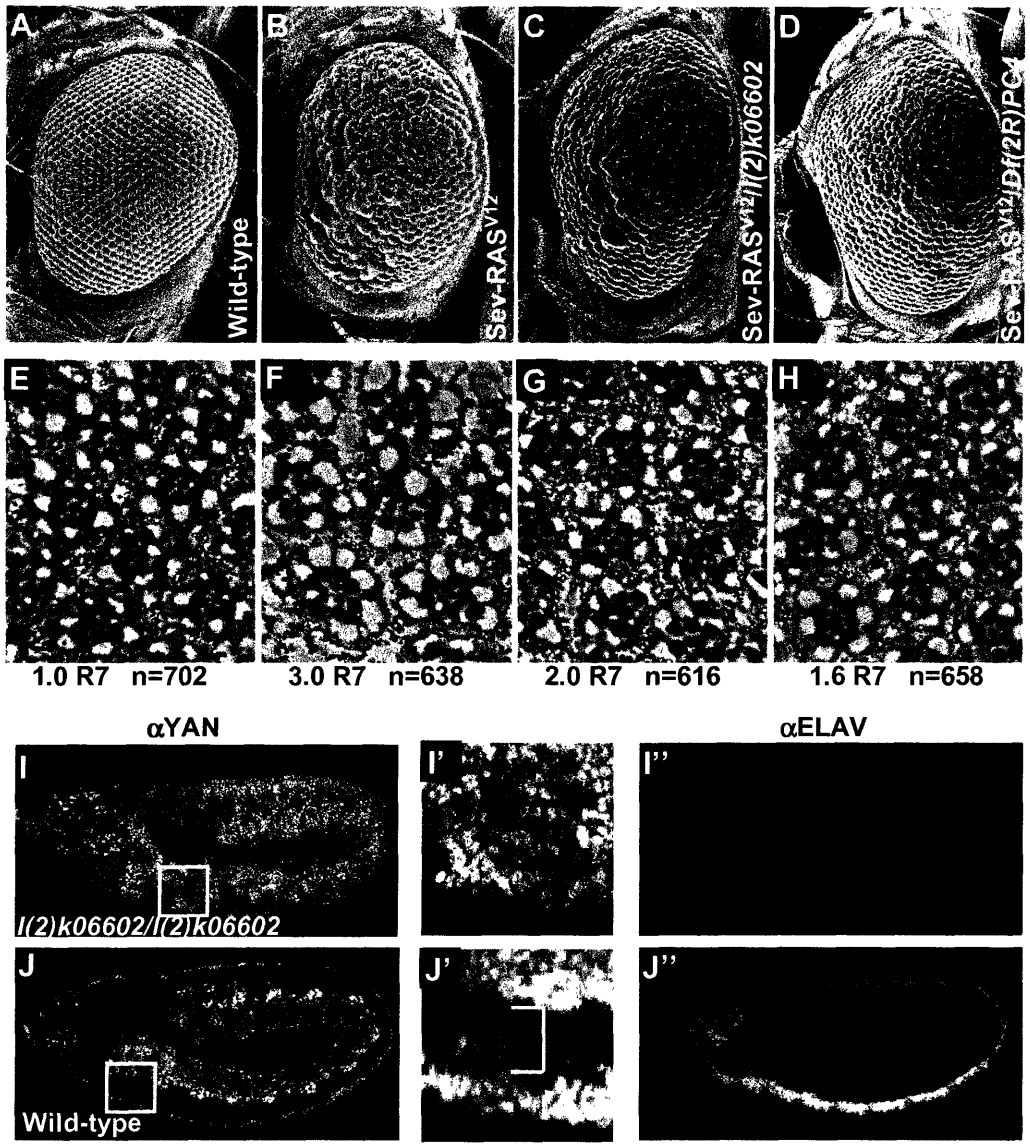
A
Wild-type
B
Sev-RAS^{V12}
C
Sev-RAS^{V12}/l(2)k06602
D
Sev-RAS^{V12}/Df(2R)PC4
E
F
G
H
1.0 R7 n=702
3.0 R7 n=638
2.0 R7 n=616
1.6 R7 n=658
αYAN
αELAV
I
I'
I''
l(2)k06602/l(2)k06602
J
J'
J''
Wild-type

Figure 2-4

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.

Table 2-1: MAE is necessary for nuclear export of YAN

YAN Localization	n=	Nuc.	Nuc. +Cyto.	Cyto.
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 (Nuc.) localization, both nuclear and cytoplasmic (Nuc.+Cyto.) localization, or exclusively cytoplasmic (Cyto.) localization. n refers to the number of cells scored in each experiment.

the presence of aberrant YAN expression in the CNS, neuronal differentiation was inhibited in *mae* mutants (Figure 2-4I" compared to J"). RNAi of *mae* performed in embryos produced identical phenotypes (data not shown). We therefore conclude that *mae* function is necessary to down-regulate 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, 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 MAE's role 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, giving us 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 2-1). Furthermore, inhibition of CRM1 mediated export resulted in localization of $YAN^{Mut\ ETS}$ to the nucleus (Table 2-1), suggesting $YAN^{Mut\ ETS}$ initially localized properly to the nucleus but due to 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).

We exploited these findings to ask whether MAE plays a role in nuclear export separate from that proposed by Baker et al. (2001) in facilitating phosphorylation. We found that RNAi of *mae* restricted $YAN^{Mut\ Ets}$ to the nucleus (Table 2-1). 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} (Figure 2-5C,D). Furthermore, inhibition of CRM1-mediated nuclear export had no effect on MAE subcellular localization (Figure 2-5E,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, MAE's localization 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 cotransfected with YAN and PNT-P2. Cotransfection of YAN with MAE alters MAE distribution. In the absence of RAS^{V12}, MAE was predominantly nuclear (Figure 2-5G) due to being bound to YAN (Figure 2-5A, lane 2) and then became both nuclear and cytoplasmic in the presence of RAS^{V12} (Figure 2-5H). This suggests that MAPK phosphorylation of YAN may result in destabilization of the YAN-MAE complex, allowing MAE to reassume uniform distribution. Coimmunoprecipitation experiments supported this interpretation, as the amount of YAN bound to MAE appeared to be significantly reduced in RAS^{V12} stimulated cells (Figure 2-5A, lane 4 as compared to lane 2; note that the total amount of YAN present is comparable +/- 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 (Figure 2-5O). These results indicate that MAE localization is

Figure 2-5

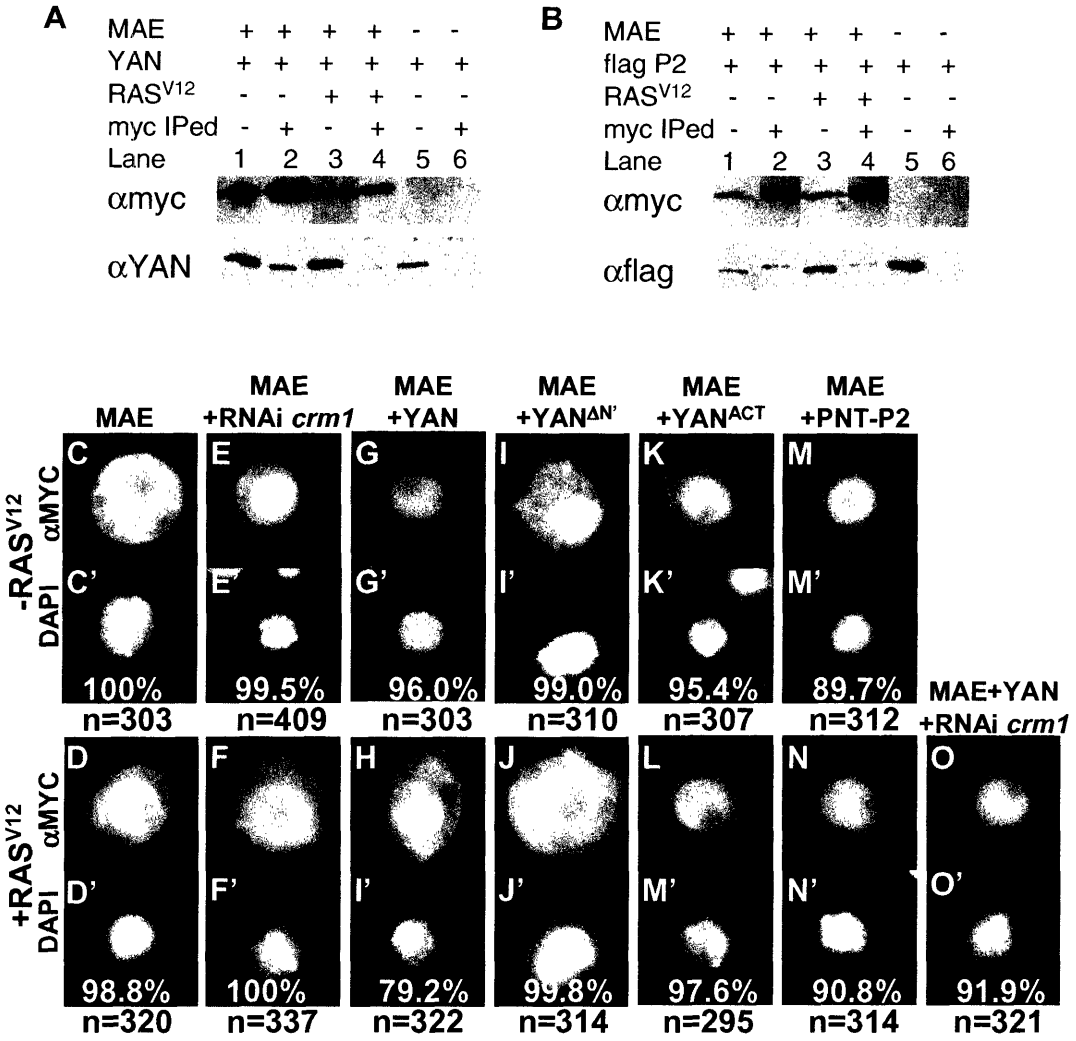
A
MAE + + + + - -
YAN + + + + + +
RASV12 - - + + - -
myc IPed - + - + - +
Lane 1 2 3 4 5 6
αmyc
αYAN
B
MAE + + + + - -
flag P2 + + + + + +
RASV12 - - + + - -
myc IPed - + - + - +
Lane 1 2 3 4 5 6
αmyc
αflag
MAE
MAE +RNAi crm1
MAE +YAN
MAE +YANΔN'
MAE +YANACT
MAE +PNT-P2
-RASV12 αMYC
DAPI
C C' 100% n=303
E E' 99.5% n=409
G G' 96.0% n=303
I I' 99.0% n=310
K K' 95.4% n=307
M M' 89.7% n=312
MAE+YAN +RNAi crm1
+RASV12 αMYC
DAPI
D D' 98.8% n=320
F F' 100% n=337
H I' 79.2% n=322
J J' 99.8% n=314
L M' 97.6% n=295
N N' 90.8% n=314
O O' 91.9% n=321

Figure 2-5

MAE localization in S2 cells depends on its binding partners' distribution. (A-C) Immuno-blots 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 refers to the 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 cotransfected. When MAE is cotransfected 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 cotransfected 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 coexpressed (I, J). Cotransfection 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).

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 cotransfected 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 (Figure 2-5I,J). Therefore, restriction of MAE to the nucleus by YAN requires the PD. We also looked at MAE localization in YAN^{ACT} expressing cells. YAN^{ACT} cannot be phosphorylated by MAPK and therefore remains restricted to the nucleus in the presence of RAS^{V12}. Cotransfection of YAN^{ACT} restricted MAE to the nucleus in the absence and presence of RAS^{V12} (Figure 2-5K,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. Cotransfection 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} (Figures 2-5M,N and 2-5B, lanes 2,4). Together these results suggest that MAE localization is not subject to direct regulation by CRM1 and RAS/MAPK signaling, but rather is determined by the presence or absence of nuclear binding partners YAN and PNT-P2 in accord with changing signaling conditions.

MAE inhibits both YAN's ability to repress and PNT-P2's ability to activate transcription

Baker at al. (2001) have proposed that overexpression of MAE inhibits YAN's ability to repress transcription and stimulates PNT-P2's ability 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 MAE's function 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. (2001). In Drosophila S2 cells, overexpression of MAE inhibited YAN mediated transcriptional repression, and slightly enhanced the RAS^{V12} mediated removal of transcriptional repression (Figure 2-6A).

However, our results disagree with Baker et al.'s (2001) conclusion that MAE stimulates PNT-P2's ability to activate transcription. We found that overexpression of MAE completely inhibited PNT-P2 mediated activation of transcription (Figure 2-6B). Therefore, MAE could have a role in downregulating, rather than stimulating, PNT-P2's ability to activate transcription.

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. Here 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.

Figure 2-6

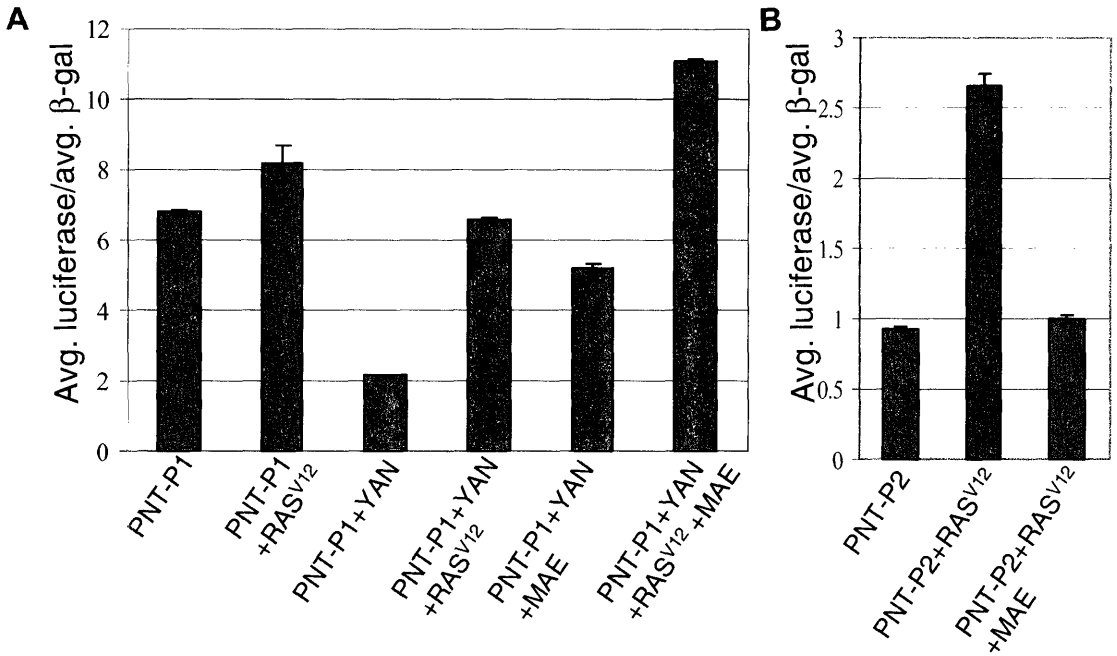

Figure 2-6

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).

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 orthologue TEL, the DNA binding domain serves as a nuclear localization sequence (NLS) (I. Rebay, 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 NES's.

Our finding that PD-mediated interactions are crucial for YAN's transcriptional repression ability agrees with similar experiments with TEL (Lopez *et al.*, 1999), but disagrees

with the results of Baker et al. (2001) who find that compromised PD function has no significant effect on YAN's transcriptional repression. 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 YAN's PD might be involved in DNA binding affinity, transcriptional repression, and maintenance of nuclear localization comes from structural studies of TEL's PD. 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, 1997 #64), and YAN has been shown to self-associate via its PD (I. Rebay, 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 published interactor with YAN's PD (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. 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.

Here 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 YAN's transcriptional repression (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, since 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. Thus we 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 cotransfected 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. (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 et al. (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-RAS^{V12}. However, in the absence of MAE, YAN cannot be down-regulated. 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, MAE's dual function 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 (Figure 2-7) in which in unstimulated cells, YAN binds with high affinity to the DNA (Figure 2-7A) and blocks PNT-P2 from contacting and activating the promoters of downstream target genes (Figure 2-7D). 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 (Figure 2-7C) and disrupts the PNT-P2-MAE complex, allowing PNT-P2 to bind to the DNA and activate transcription (Figure 2-7E). Free MAE could then interact again with PNT-P2, resulting either in its removal from the DNA,

Figure 2-7

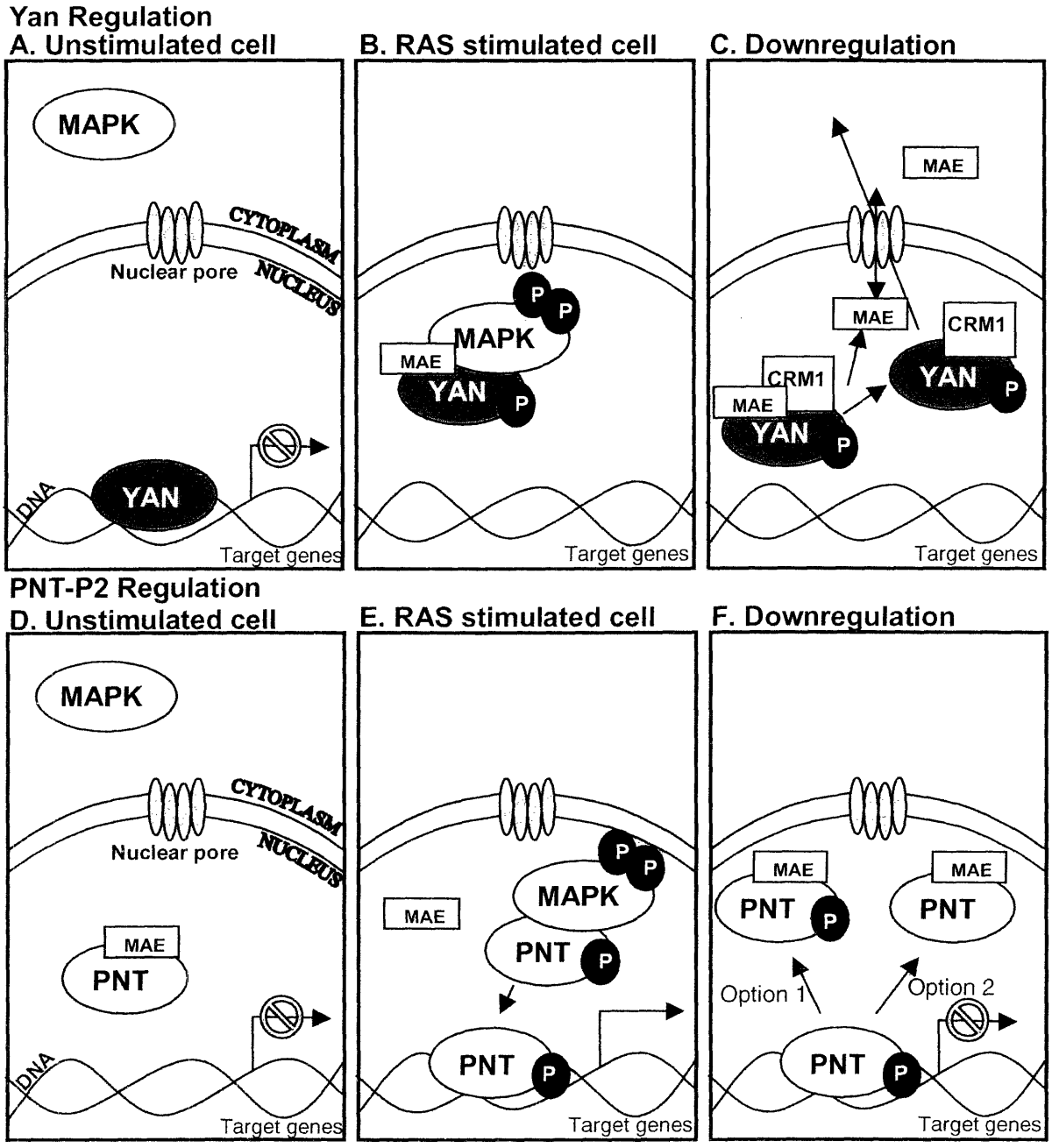
Yan Regulation
A. Unstimulated cell
B. RAS stimulated cell
C. Downregulation
MAPK
CYTOPLASM
Nuclear pore
NUCLEUS
YAN
DNA
Target genes
P
MAE
CRM1
PNT-P2 Regulation
D. Unstimulated cell
E. RAS stimulated cell
F. Downregulation
PNT
Option 1
Option 2

Figure 2-7

Model. (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 (option 1), or by MAE binding resulting in dephosphorylation of PNT-P2 (option 2), resulting in inhibition of transcriptional activation.

inhibition of transcriptional activation, or interaction with a phosphatase that returns it to an inactive state (Figure 2-7F). 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 MAE's interaction 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 cofactors that bind MAE, YAN and/or PNT-P2 will be required for fine-tuning activation and downregulation in response to changing RTK signaling conditions.

Evolutionarily conserved mechanisms of YAN downregulation

Precise regulation of RTK pathway activity appears critical 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 in 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 down-regulate 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 with a mechanism similar to what we have shown for MAE 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.

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Chapter 3

MAE, a dual regulator of the EGFR signaling pathway, is a target of the Ets transcription factors PNT and YAN

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[1]These authors contributed equally to this work

Tina Tootle performed the overexpression of MAE analysis in cultured cells and *Drosophila* eye tissue. Initial analysis of *mae* expression by in situ hybridization was performed by Tina Tootle, further analysis yielding Figure 3-2 was by Pavithra Vivekanand. Pavithra Vivekanand performed all transcriptional assays.

Abstract

Ets transcription factors play crucial roles in regulating diverse cellular processes including cell proliferation, differentiation and survival. Coordinated regulation of the *Drosophila* Ets transcription factors YAN and POINTED is required for eliciting appropriate responses to Receptor Tyrosine Kinase (RTK) signaling. YAN, a transcriptional repressor, and POINTED, a transcriptional activator, compete for regulatory regions of common target genes, with the ultimate outcome likely influenced by context-specific interactions with binding partners such as MAE. Previous work in cultured cells has led us to propose that MAE attenuates the transcriptional activity of both YAN and POINTED, although its effects on POINTED remain controversial. Here we describe a new layer of complexity to this regulatory hierarchy whereby *mae* expression is itself directly regulated by the opposing action of YAN and POINTED. In addition, we report that MAE can antagonize POINTED function during eye development, a finding that suggests MAE operates as a dual positive and negative regulator of RTK-mediated signaling in vivo. Together our results lead us to propose that a combination of protein-protein and transcriptional interactions between MAE, YAN and POINTED together establishes a complex regulatory circuit that ensures that both down-regulation and activation of the RTK pathway occur appropriately according to specific developmental context.

Introduction

Signaling through the Receptor Tyrosine Kinase (RTK) pathway leads to two primary developmental outcomes, proliferation or differentiation (Tan and Kim, 1999). Activation of the RTK is relayed to the nucleus through the evolutionarily conserved GTPase RAS and mitogen-activated protein kinase (MAPK) cascade (Marshall, 1994; Zipursky and Rubin, 1994). In the

nucleus, activated MAPK phosphorylates downstream signaling effectors, such as the two Ets family transcription factors POINTED (PNT) and YAN (O'Neill et al., 1994). *pointed* encodes two different proteins, PNTP1 and PNTP2 (Klambt, 1993); while both function as transcriptional activators that bind to ETS consensus sites, PNTP2 activity requires phosphorylation by MAPK in response to RTK/RAS signaling (O'Neill et al., 1994). YAN, a transcriptional repressor, functions as an RTK pathway antagonist by competing with PNT for access to target sequences (Flores et al., 2000; O'Neill et al., 1994; Xu et al., 2000). Under conditions of minimal RTK induction, high affinity binding of YAN to the DNA effectively outcompetes PNT and inhibits inappropriate differentiation or proliferation (O'Neill et al., 1994; Rebay and Rubin, 1995). Upon RAS/MAPK stimulation, MAPK-mediated phosphorylation of YAN results in abrogation of YAN repressor activity, allowing PNT to activate transcription of formerly repressed genes (O'Neill et al., 1994).

Multiple regulatory mechanisms are employed to fine-tune not only the competition between YAN and PNT for access to the promoter regions of target genes but also their transcriptional activities. One key component involved in these processes is MAE, a gene product structurally related to YAN and PNT by virtue of a shared protein-protein interaction motif called the pointed domain (PD) (Baker et al., 2001; Tootle et al., 2003). MAE has been shown to interact with both YAN and PNT via heterotypic PD-PD associations (Baker et al., 2001; Tootle et al., 2003; Yamada et al., 2003). While the mechanistic consequences of YAN-MAE and PNT-MAE complexes are not fully understood, MAE appears to contribute critical regulation that modulates the balance between YAN-mediated repression and PNT-mediated activation of downstream target genes.

We and others have recently shown that MAE plays multiple independent roles in modulating YAN activity. Specifically, MAE facilitates both MAPK phosphorylation (Baker et al., 2001) and nuclear export (Tootle et al., 2003) of YAN in response to RTK pathway activation, and antagonizes YAN's repressor activity in the absence of pathway activation (Tootle et al., 2003). Although MAE has been proposed to positively influence PNT activity (Baker et al., 2001), transcriptional activity studies in cultured cells have led to a competing hypothesis whereby MAE participates in a negative feedback loop that downregulates PNT (Tootle et al., 2003; Yamada et al., 2003). Based on this capacity to inhibit the transcriptional properties of both YAN and PNT, MAE could potentially play dual positive and negative roles in modulating transcriptional responses downstream of the RTK signaling pathway.

Here we report that superimposed on the complex web of protein level interactions whereby MAE modulates YAN and PNT function, is a transcriptional regulatory network in which YAN and PNT directly regulate *mae* transcription. These results reveal an important new mechanism for precisely modulating MAE levels in order to ensure appropriate transcriptional responses to RTK/Ras/MAPK pathway activation during development and highlights the extraordinary complexity of the meshwork of interactions and feedback loops that fine tunes and coordinates the activities of YAN, PNT and MAE in vivo. To explore these regulatory circuitries further, we have investigated the mechanisms whereby MAE antagonizes the transcriptional output of YAN and PNT by examining the consequences of overexpressing MAE in both cultured cells and in vivo. We find that while increased MAE expression abrogates YAN-mediated repression, it does not do so by facilitating nuclear export and down-regulation of YAN, suggesting instead a direct interference with transcription. In the eye imaginal disc, we find that elevated MAE expression antagonizes PNT function, arguing that one function of MAE

in vivo could be to limit the transcriptional output of PNT, a model consistent with our previous work in cultured cells. Together our results suggest that multiple layers of regulatory feedback loops involving the nuclear effectors MAE, YAN and PNT ensure finely-tuned and context-appropriate RTK signaling levels.

Results

YAN and PNT regulate mae expression

Because *mae* expression in wild-type embryos is reminiscent of the expression patterns of genes such as *argos* (*aos*) and *orthodenticle* (*otd*) that have been shown to be regulated by Epidermal Growth Factor Receptor (EGFR) signaling (Gabay et al., 1996), we decided to investigate whether *mae* expression might be similarly regulated by the downstream EGFR pathway effectors, YAN and PNT. Analysis of the genomic region around *mae* reveals two clusters of ETS DNA binding consensus sites (EBS; defined as GGAA/T; Nye et al., 1992), one upstream of the transcription start site (MaeEBS1) and the other in the intron of *mae* (MaeEBS2) (Figure 3-1A), further suggesting that YAN and PNT might regulate *mae* expression. To explore this possibility, in situ hybridization experiments were performed to determine whether *mae* expression was affected by altering the dosage of YAN and PNT. As predicted based on the presence of EBS clusters in the *mae* genomic region, *mae* expression is significantly increased in *yan* mutant embryos (Figure 3-2B compared to 3-2A), while it is lost in *pnt* mutant embryos (Figure 3-2C). Conversely, ubiquitous overexpression of YAN^{ACT} or PNT results in down- (Figure 3-2E) and up- regulation (Figure 3-2F) of *mae* expression respectively (compare to Figure 3-2D). Taken together, these results suggest that *mae* expression is regulated by the Ets transcription factors PNT and YAN.

Figure 3-1

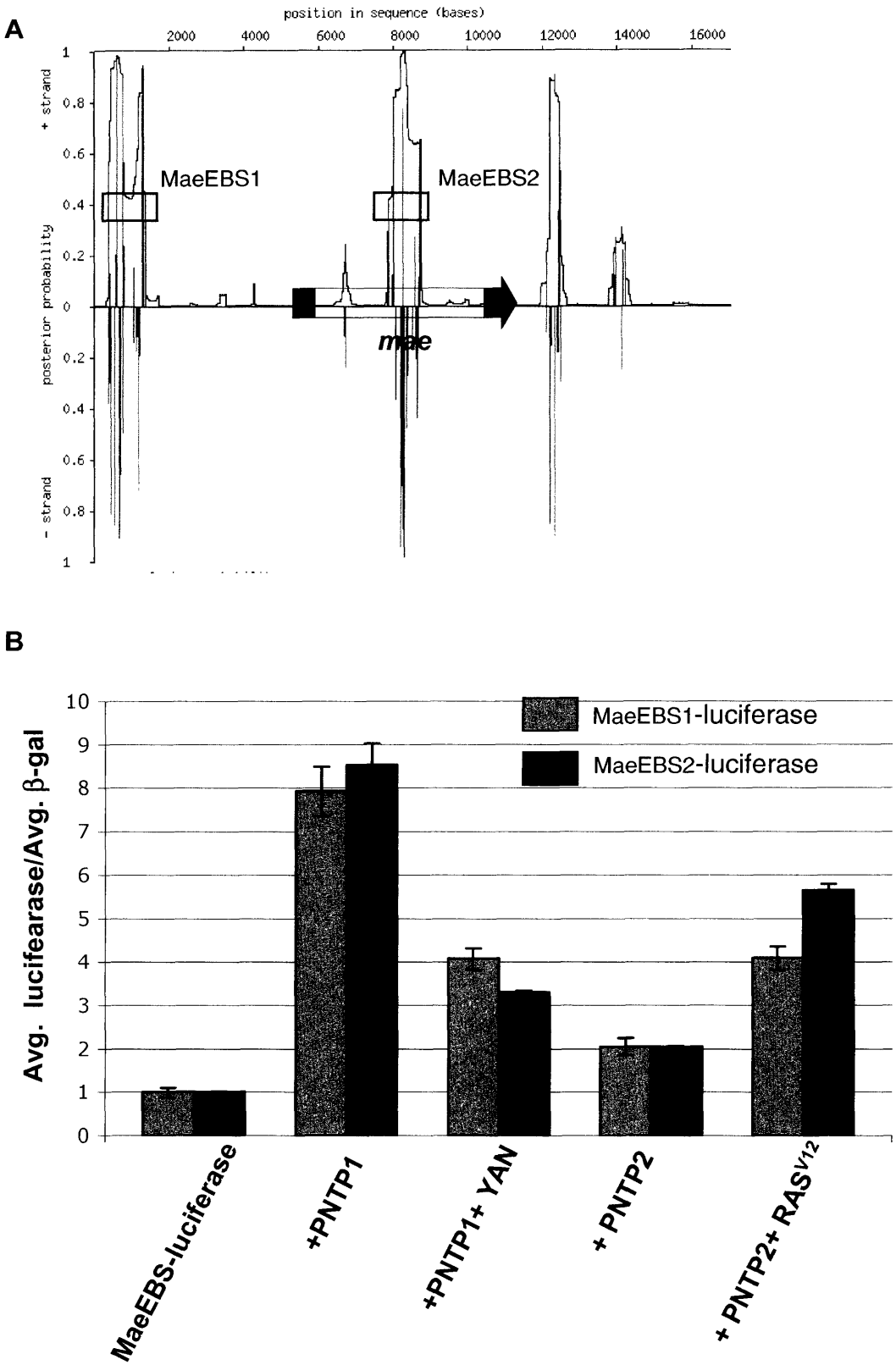
A
position in sequence (bases)
2000
4000
6000
8000
10000
12000
14000
16000
+ strand
posterior probability
- strand
MaeEBS1
MaeEBS2
mae
B
Avg. lucifearase/Avg. β-gal
MaeEBS1-luciferase
MaeEBS2-luciferase
MaeEBS-luciferase
+PNTP1
+PNTP1+ YAN
+ PNTP2
+ PNTP2+ RASV12

Figure 3-1

PNT and YAN directly regulate *mae* transcription. A) Two EBS clusters, one upstream of the *mae* transcription start site and the other in the intron of *mae* are predicted by Cister (Cis-element cluster finder). (B) Transcription assays with *MaeEBS-luciferase* show that PNTP1, YAN and PNTP2 regulate *mae*. PNTP1 and PNTP2+RAS^{V12} activate transcription while addition of YAN results in transcriptional repression.

To determine whether PNT and YAN regulate *mae* levels directly, the EBS clusters were cloned upstream of a minimal promoter and luciferase cDNA to generate two different MaeEBS-luciferase reporters, MaeEBS1-luciferase (upstream cluster, Figure 3-1A) and MaeEBS2-luciferase (intronic cluster, Figure 3-1A). This enabled us to assess the effects of PNT and YAN on these putative regulatory elements by performing transcription assays in *Drosophila* S2 cells. If PNT and YAN directly regulate *mae* transcription, then the prediction would be that PNT and YAN would bind to the EBSs and activate and repress transcription of the reporter, respectively.

Both the upstream and the intronic EBS clusters behaved similarly in these luciferase reporter assays. Addition of the constitutively activated form of PNT, PNTP1, resulted in activation of the reporter, while co-transfection of YAN with PNTP1 resulted in two to three fold repression in transcription (Figure 3-1B). Similarly, co-transfection of PNTP2 and RAS^{V12} resulted in transcriptional activation of the reporter (Figure 3-1B). The transcriptional modulation of the MaeEBS-luciferase reporters by PNT and YAN supports our hypothesis that *mae* expression is directly regulated by PNT and YAN in vivo.

MAE-mediated antagonism of YAN repression activity does not involve nuclear export of YAN

Based on our previous finding that overexpression of MAE inhibits YAN's ability to repress transcription (Tootle et al., 2003), we decided to investigate further the underlying mechanisms. One possibility was that MAE overexpression might disrupt YAN's nuclear localization. In *Drosophila* S2 cultured cells YAN localizes to the nucleus (Figure 3-3A), and upon activation of the RAS/MAPK cascade is exported to the cytoplasm (Figure 3-3B) (Rebay and Rubin, 1995). Previous analyses revealed that MAE is necessary for nuclear export of YAN, as YAN remains restricted to the nucleus in the absence of *mae* both in cultured cells and in vivo

Figure 3-2

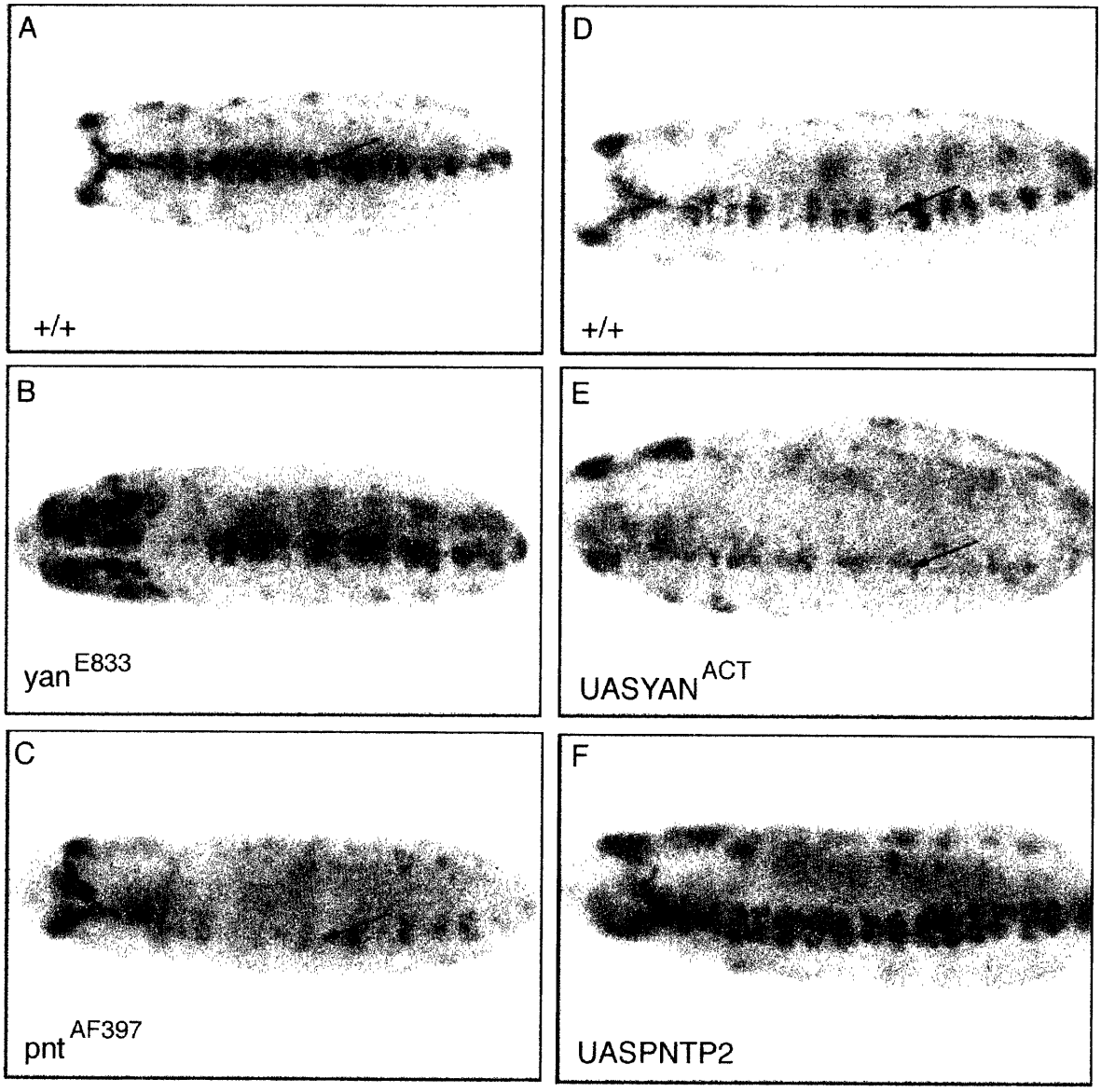

Figure 3-2

mae expression is modulated by PNT and YAN. (A-F) in situ hybridizations with *mae* DNA probe in (A, D) wild-type; (B) *yan* mutant; (C) *pnt* mutant; (E) Ub-GAL4; UAS-YAN^{ACT}; (F) Ub-GAL4; UAS-PNTP2 embryos. *mae* expression is upregulated in *yan* mutant (B) and in embryos over-expressing PNTP2 (F) while its expression is down-regulated in *pnt* mutant (C) and in embryos over-expressing YAN^{ACT} (E) when compared to wild-type embryos.

(Tootle et al., 2003). Therefore we asked whether the abrogation of YAN-mediated repression that results from MAE overexpression (Tootle et al., 2003) could be the result of premature nuclear export of YAN. However, no effect on nuclear YAN localization was observed (Figure 3-3C). In order to rule out the possibility that the MYC epitope tag on MAE was interfering with normal MAE function to produce a misleading result, we confirmed the finding using a non-tagged MAE construct (Figure 3-3D). Thus the mechanism whereby MAE antagonizes YAN-mediated repression appears to involve a more direct interference with transcriptional activity, rather than simply inducing inappropriate nuclear export.

Genetic interactions between mae and yan suggest MAE is both a positive and a negative regulator of RTK signaling in the eye

The *Drosophila* eye provides a powerful and sensitive system in which to unravel the molecular circuitries underlying RTK-mediated signaling events. For example, the rough eye phenotype that results from expression of a constitutively active allele of *yan* (referred to as sev-YAN^{ACT} (Rebay and Rubin, 1995); Figure 3-4A) has been used successfully as a dose-sensitive background to demonstrate genetic interactions with novel components of the EGFR/Ras/MAPK pathway (Rebay et al., 2000). Using this sensitized system, we recently demonstrated that heterozygosity for *mae* mildly enhances the severity of the YAN^{ACT} rough eye phenotype, consistent with its role in downregulating YAN activity and positioning it as a positive regulator of RTK-mediated signaling events (Figure 3-4B; Tootle et al., 2003).

If, as predicted by Baker et al. 2001, MAE functions exclusively as a positive pathway component, then excess MAE should suppress the sev-YAN^{ACT} phenotype. However if MAE serves dual positive and negative functions, as suggested by its ability to inhibit transcriptional output of both YAN and PNT, then depending on which role prevails in this context, either

Figure 3-3

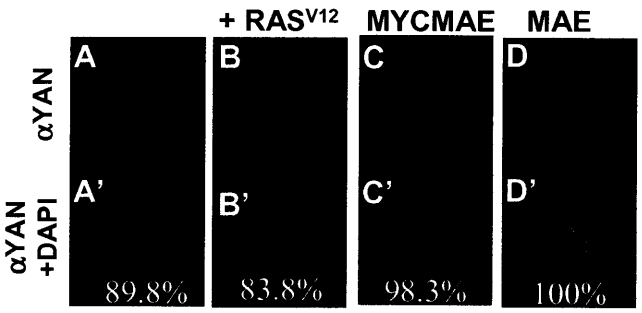

Figure 3-3

MAE-YAN complexes in cultured cells are refractory to normal YAN downregulation. (A-D) Transient transfections of Drosophila S2 cultured cells expressing YAN and the indicated constructs stained with anti-YAN. (A'-D') DAPI staining overlaying YAN antibody staining of the same cells. The percentage of transfected cells exhibiting nuclear localization (A, C-D) or exclusively cytoplasmic localization (B) is indicated, $n \geq 250$. (A, C-D) YAN localization in the absence of RAS^{V12}. (B) YAN localization in the presence of RAS^{V12}. (C, D) Coexpression of MYCMAE or MAE has no effect on the nuclear localization of YAN.

Figure 3-4

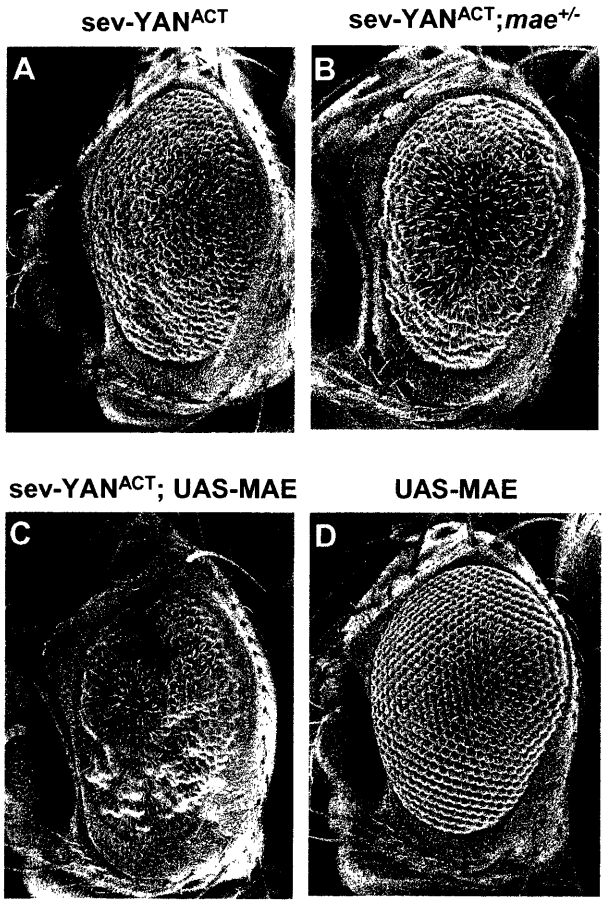

Figure 3-4

MAE is a dual positive and negative regulator of EGFR signaling in the eye. (A-D) Scanning electron micrographs of adult *Drosophila* eyes. (A) sev-YAN^{ACT}; sev-GAL4; (B) sev-YAN^{ACT}; *l(2)k06602/+*; (C) sev-YAN^{ACT};UAS-MYCMAE/ sev-GAL4; (D) UAS-MYCMAE/ sev-GAL4. Both loss of function and overexpression of *mae* enhance the rough eye phenotype of activated YAN, although the system appears more sensitive to increased MAE levels.

suppression or enhancement might be observed. Intriguingly, we found that sev-GAL4 driven MAE strongly enhanced the severity of the sev-YAN^{ACT} rough eye phenotype (Figure 3-4C, compared to A). Confirming that the enhancement was not due to an additive effect, overexpression of MAE alone using sev-GAL4 exhibited a wild-type eye (Figure 3-4D). This result argues that MAE is a dual regulator of RTK signaling, and that in this context, its function as a pathway antagonist appears to prevail.

MAE antagonizes POINTED function in the developing eye

If MAE has a role as an RTK pathway antagonist during eye development, given its close functional association with YAN and PNT (Tootle et al., 2003), three possible mechanisms of action can be considered: MAE could directly antagonize PNT function, a model consistent with cell culture transcription assays and with the enhancement of sev-YAN^{ACT} (Tootle et al., 2003; Yamada et al., 2003; Rebay et al., 2000); MAE could potentiate YAN activity, a mechanism that would not be predicted by our previous work (Tootle et al., 2003); or MAE could simultaneously impair activity of both PNT and YAN in such a way that the overall effect is to reduce pathway output. Because loss of *mae* results in a failure to down-regulate YAN (Tootle et al., 2003), the presence of a stable repressor completely damps down signaling output and makes it impossible to assess whether PNT activity is directly compromised in this context. Therefore to address the possibility that MAE might antagonize PNT activity in vivo, we examined in further detail the consequences of overexpressing MAE in the developing eye. Although MAE expression driven by a weak sev-Gal4 driver has no phenotypic consequences (Figure 3-4D), using the stronger GMR-Gal4 driver we found that overexpression of MAE results in gross disorganization of the external morphology of the adult eye (Figure 3-5B, compared to GMR-Gal4 alone in 3-5A).

To determine the developmental cause(s) of the phenotype, eye imaginal discs from GMR-GAL4; UAS-MAE larvae were examined. We first looked at expression of the neuronal marker ELAV, which is expressed in the developing photoreceptors (Figure 3-5D), and found a substantial reduction in the number recruited (Figure 3-5E), a phenotype consistent with a reduction in RTK/RAS pathway function. ELAV expression in the first three recruited photoreceptors, R8, R2 and R5 appeared least affected while the later specified photoreceptors, R3, R4, R1, R6 and R7, were most frequently lost. Although loss of these photoreceptors would be predicted to result in a rough eye, the severity of the adult phenotype (Figure 3-5B) suggests that many more cell types are affected. Therefore, we also examined whether cone cell recruitment was compromised and found that overexpression of MAE significantly reduces the number of cone cells (Figure 3-5H compared to 3-5G). As the cone cells are essential for recruiting all subsequent cell types, the severity of the adult rough eye likely reflects the loss of the non-neuronal support cells.

Another factor that could contribute to the decreased number of photoreceptors that is brought about by MAE overexpression is cell death, another characteristic of reduced RTK signaling output. In wild-type eye imaginal discs there is a small amount of cell death immediately anterior to the furrow (Figure 3-5J). Overexpression of MAE results in increased cell death posterior to the furrow (Figure 3-5K). Therefore, both the loss of differentiated cell types and ectopic cell death during eye development contribute to the severe rough eye seen in the adult.

We also asked whether elevated MAE levels altered YAN expression in the eye disc. YAN is normally expressed strongly in the morphogenetic furrow and at slightly lower levels in the basally localized nuclei of undifferentiated cells posterior to the furrow (Figure 3-5M).

Interestingly, we found that YAN expression at the furrow was slightly elevated, while basal YAN expression posterior to the furrow was distinctly reduced (Figure 3-5N).

To determine whether MAE exerts independent effects on YAN and PNT in this context, we asked whether we could rescue some or all of the MAE overexpression phenotypes by coexpressing PNTP2. We were unable to perform the converse test, because overexpression of YAN results in lethality, even with eye specific drivers (T.T. and I.R., unpublished). Our results suggest that the phenotypic consequences of overexpressing MAE occur primarily due to loss of *pnt-p2* function.

Specifically we generated recombinant UAS-PNTP2, UAS-MAE flies and expressed the transgenes using GMR-GAL4. The control experiment indicated that overexpression of PNTP2 alone using the GMR-GAL4 driver results in normal photoreceptor recruitment, extra cone cells, wild-type levels of cell death, and increased basal YAN expression compared to wild-type (data not shown). Overexpression of PNTP2 completely rescues the severe rough eye of GMR-GAL4; UAS-MAE (Figure 3-5C) and at the cellular level restores normal recruitment of photoreceptors and cone cells (Figure 3-5F, I), reduces cell death to wild-type levels (Figure 3-5L), and returns YAN expression to normal levels (Figure 3-5O). Therefore, the phenotypes associated with overexpression of MAE in the eye appears to be due to loss of PNTP2 function, strongly suggesting that MAE can antagonize RTK pathway output by attenuating PNTP2 activity.

Discussion

A precise balance between the opposing activities of the repressor YAN and the activator POINTED is essential for achieving appropriate transcriptional response both in the presence and in the absence of RTK signaling (Rebay and Rubin, 1995). MAE, a small PD containing protein

Figure 3-5

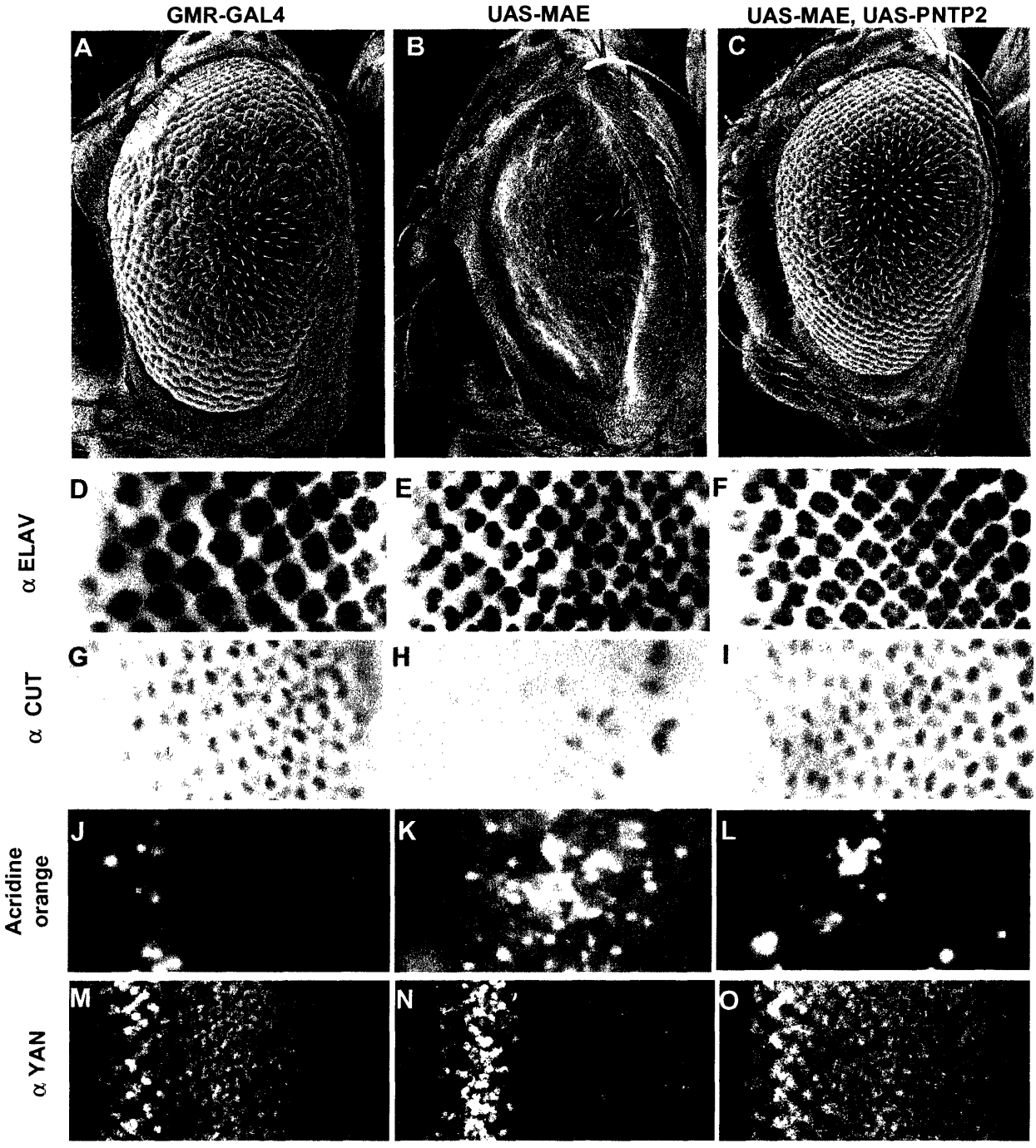
GMR-GAL4
UAS-MAE
UAS-MAE, UAS-PNTP2
A
B
C
D
E
F
α ELAV
G
H
I
α CUT
J
K
L
Acridine orange
M
N
O
α YAN

Figure 3-5

MAE inhibits PNTP2 function in the eye. (A-C) Scanning electron micrographs of adult *Drosophila* eyes. (D-O) Various stainings of third instar larval eye imaginal discs. (A, D, G, J, M) GMR-GAL4; (B, E, H, K, M) GMR-GAL4; UAS-MYCMAE; (C, F, I, L, O) GMR-GAL4; UAS-MYCMAE, UAS-PNTP2. (D-F) Anti-ELAV staining. (G-I) Anti-cut staining. (J-L) Acridine orange staining. (M-O) Basal anti-YAN staining. Overexpression of MAE results in a complete loss of eye tissue in the adult fly due to loss of photoreceptor and support cell recruitment and increased apoptosis in the developing eye. All of these phenotype are rescued by overexpression of PNTP2, suggesting that overexpression of MAE inhibits PNTP2 function.

that has been shown to bind directly to both transcription factors, plays a pivotal role in modulating their activities (Baker et al., 2001; Tootle et al., 2003). For example, our previous work showed that MAE contributes positively to RTK signaling output by facilitating nuclear export and down-regulation of YAN in response to pathway activation (Tootle et al., 2003). In this study we demonstrate that MAE also antagonizes PNT function, putting it in the unique position of being a dual positive and negative regulator of EGFR-mediated signals. Intriguingly, we find that *mae* expression is itself regulated by PNT and YAN, suggesting a whole new layer of feedback loops that fine-tune and down-regulate signaling.

MAE negatively regulates both YAN and PNTP2

We have shown that while overexpression of MAE blocks YAN's repression capability (Tootle et al., 2003), this occurs without altering YAN nuclear localization. Thus increased MAE expression appears to interfere directly with YAN-mediated transcriptional repression. An intriguing model to explain this finding originates from the observation that homotypic interactions mediated by the PD (pointed domain) of TEL, the mammalian ortholog of YAN, result in the formation of TEL polymer that may facilitate transcriptional repression by wrapping around the target DNA (Kim et al., 2001). YAN is similarly capable of self-association and the residues that are required for TEL polymerization have been conserved, suggesting YAN-YAN polymerization might similarly be critical for repression (Jousset et al., 1997). In this context, perhaps clusters of EBSs, similar to those we have described in *mae*, by recruiting multiple YAN molecules to a common target site may provide a scaffold for nucleating and promoting YAN polymerization.

Such a model requires a mechanism to limit the extent of polymer formation, such that the cell can achieve efficient but reversible repression of target genes. Considering its

multifaceted role in down-regulating YAN activity and its ability to bind the PD of YAN, MAE is a prime candidate to fill such a role. Consistent with this prediction, recent studies have found PD-mediated polymerization of YAN is required for transcriptional repression and that MAE effectively "caps" YAN oligomerization by occluding the residues required for polymerization (Jim Bowie, personal communication). Thus it is tempting to speculate that MAE's ability to abrogate YAN-mediated repression may reflect a role in "depolymerizing" YAN at the DNA, an intriguing model that remains to be validated in vivo.

Assuming the model holds true, then our combined results suggest that MAE antagonizes YAN activity both in the absence and as a consequence of RTK pathway stimulation. Under lower levels of pathway activation, MAE would play a key role in mitigating the strength of YAN-mediated repression, essentially ensuring that the threshold barrier that blocks inappropriate cellular responses to RTK signaling is not set too high. In response to pathway activation, MAE would then contribute at multiple levels to the phosphorylation and nuclear export mechanisms that ensure timely and efficient down-regulation of YAN.

In addition to antagonizing YAN activity, our work suggests that MAE also negatively regulates PNTP2 function, thus positioning it uniquely within the RTK pathway as both a positive and negative regulator. For example, the phenotypes associated with misexpression of MAE in the *Drosophila* visual system are completely suppressed by co-expression of PNTP2, arguing strongly that MAE can antagonize EGFR signaling in the eye by interfering with the activity of PNTP2. While the photoreceptor loss and increased apoptosis phenotypes associated with MAE overexpression resemble the consequences of blocking YAN nuclear export and down-regulation (Rebay and Rubin, 1995), the reduced YAN expression observed in MAE-expressing eye disc argues against such an explanation. Furthermore, if MAE were inducing

premature downregulation of YAN in these cells, one would expect to observe ectopic photoreceptors, rather than the neuronal loss that actually occurs. Thus, although we cannot rule out a direct effect on YAN, we favor the interpretation that the primary consequence of MAE overexpression is reduction in activity of PNTP2, and that the loss of YAN expression is a secondary outcome. It is important to note that both our cell culture and in vivo experiments employ overexpression strategies that are subject to the caveats inherent to such analyses. Thus we view these experiments as an opportunity to reveal new mechanistic hypothesis that will provide an important foundation for future studies designed to unravel the complex regulatory circuitries that exist between MAE, YAN and PNT in vivo.

YAN and PNTP2 regulate mae expression

Induction of both positive and negative feedback loops by signal transduction pathways plays an important role in regulating the response to pathway activation (Freeman, 2000; Rebay, 2002). Activation of PNTP2 by EGFR/RAS/MAPK results in the transcription of target genes including *Argos* and *Kekkon1*, which have been shown to negatively regulate the pathway (Ghiglione et al., 1999; Golembo et al., 1996). We have identified another target of the Ets transcription factors PNT and YAN, *mae*, which performs the dual role of promoting and inhibiting signaling by the EGFR/RAS/MAPK pathway. Based on the effects on *mae* expression pattern observed in *pnt* and *yan* mutants and in embryos overexpressing PNT and YAN, we propose that PNT activates while YAN represses *mae* transcription (Figure 3-6).

Based on MAE's ability to antagonize EGFR signaling output, activation of *mae* transcription by PNTP2 provides a negative feedback loop that would prevent runaway pathway activation (Figure 3-6). While Kekkon-1 and the secreted antagonist Argos act at the level of the receptor to down-regulate signaling (Jin et al., 2000), the induction of *mae* transcription would ensure the

down-regulation of the pathway by inhibiting the function of the effector PNTP2. This would result in cell autonomous inhibition of the EGFR/RAS/MAPK pathway at the level of the transcription factor. Moreover, while the previously identified inhibitors Argos, Sprouty (Casci et al., 1999) and Kekkon1 function solely as antagonists of RTK signaling, as discussed above, MAE is unusual in that it acts both as a positive and negative regulator of the pathway by inhibiting both YAN and PNTP2 function. Because MAE negatively regulates both YAN and PNTP2 function, imposing constraints on MAE protein levels becomes critical. This appears to be achieved by regulating *mae* expression levels directly by YAN and PNT. For example, and as discussed above, because excess MAE could potentially break up YAN-YAN polymer to such an extent that YAN would no longer able to repress appropriate target genes, the negative regulation of *mae* expression by YAN sets up a situation whereby excessive levels of MAE do not accumulate. Thus in the absence of RTK signaling, repression of *mae* by YAN would ensure that only low MAE levels are present in the nucleus, allowing YAN to repress transcription. Emphasizing the importance of fine-tuning the expression levels of these three nuclear RTK pathway regulators and further complicating the circuitry, it has been suggested that YAN and PNT may also directly regulate each others transcription, setting up additional positive and/or negative regulatory loops (Rohrbaugh et al., 2002). For example, our finding that overexpression of PNTP2 leads to upregulation in YAN in the eye disc is consistent with a feedback loop whereby the activity of PNTP2, a positive pathway effector, attenuates its own activity by increasing expression of the YAN repressor. A great deal of future work will be needed to unravel the precise in vivo contexts in which these complex transcriptional regulatory networks operate.

Figure 3-6

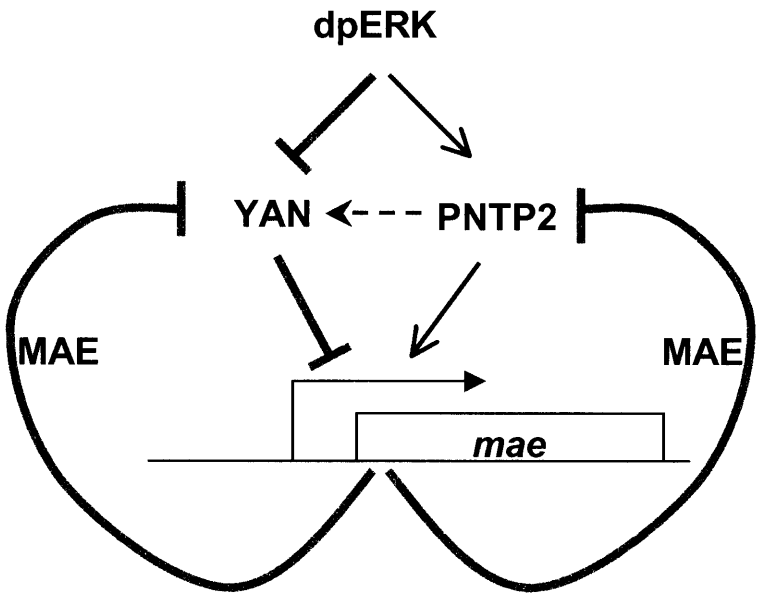

Figure 3-6

Model for regulation of PNT, YAN and *mae*. EGFR/RAS/MAPK signaling leads to MAE-mediated phosphorylation and down-regulation of YAN, resulting in activation of targets by PNTP2. As *mae* itself is a target of PNT and YAN and negatively regulates their function, activation of PNTP2 would result in induction of *mae* and subsequent down-regulation of the pathway by inhibition of PNTP2 to limit the duration of the signal (see text).

Assuming developmental contexts in which YAN and MAE are coexpressed, something that at present is difficult to ascertain given the lack of antibody reagents specific to MAE, why does endogenous MAE not inhibit YAN function in the absence of RTK signaling? One possible answer stems from the finding by Yamada et al. (2003) that MAE binds PNTP2 with much higher efficiency than YAN. Therefore, under conditions of moderate MAE expression, binding to PNTP2, or some currently unidentified other binding partner, would result in MAE being sequestered away from YAN. In this scenario, activation of the RTK/RAS/MAPK pathway and subsequent phosphorylation of PNTP2 might result in dissociation of the PNT-MAE complex. Free MAE could then facilitate phosphorylation and nuclear export of YAN, thereby permitting subsequent activation of target genes by PNTP2. As *mae* is itself one such target gene and as it inhibits PNTP2 function, the resulting increased MAE expression would establish a negative feedback loop resulting in attenuation of RTK signaling. Alternatively, MAE might require a co-factor whose expression or function is regulated by EGFR signaling to down-regulate YAN activity. In the absence of signaling, the co-factor would be inactive/absent and MAE would be unable to inhibit YAN function.

In conclusion, MAE joins the panoply of regulators of EGFR signaling that have been shown to play an important role in modulating and restricting the strength, range and duration of signaling events. By establishing negative feedback loops that act at multiple levels within a signal transduction cascade, a robust checkpoint is established to attenuate as well as prevent constitutive signaling by the RTK pathway.

Materials and Methods

Molecular biology

The upstream EBS cluster (MaeEBS1) was generated by PCR amplification using forward EBS1 5' TTGGGATCCTTTCCGCTTCCTGTGGCCCAGATTA3' and reverse EBS1 5' TTAAGATCTTTGAGCCTAGACAATTGCATTTCCT3' and ligated with BamHI/BglII digested pBluescript-Sk+; the intronic cluster (MaeEBS2) by PCR amplification using forward EBS2 5' TTGCTCGAGGCCAAATGACAGGAAACGC-GTCAT3' and reverse EBS2 5' TTGGTCGACCTGCATTCACTTCCGCCCACGTTA-GAA3' and ligated with XhoI/SalI digested pBluescript-Sk+. These constructs were subcloned into KpnI/PstI digested pBSSK-*luciferase* (Silver et al., 2003) to obtain *MaeEBS1-luciferase* and *MaeEBS2-luciferase.*

Transfections

Drosophila S2 cells were transfected with various combinations of plasmids (Pascal and Tjian, 1991) and transcription assays were done as described previously (Tootle et al., 2003).

Immunohistochemistry and in situ hybridization

Fixation and staining of S2 cells and embryos were performed as previously described (Fehon et al., 1991; Fehon et al., 1990; Tootle et al., 2003). Antibodies used were mouse anti-YAN MAb 8B12, mouse anti-myc (a gift from R. Fehon), rat anti-ELAV MAb 7E8A10 and mouse anti-CUT MAb2B10. 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). Eye imaginal discs were dissected from third instar larvae in Schneiders S2 media and fixed in 4% paraformaldehyde in PBS for 10 minutes. Discs were washed in PBS+ 0.1% TritonX-100 (PT) for 15 minutes on ice and then incubated in antibody, in PBS+5% normal goat serum+ 0.1% TritonX-100 (PNT) overnight. Discs were washed three

times in PNT and then incubated in secondary antibody for 2 hours at 4°. Discs were washed three times with PNT. If HRP secondary was used then discs were incubated in diaminobenzidine (0.5 mg/ml diaminobenzidine, 0.1% saponin, 0.003% H_2O_2 in PBS) until pattern was visible and then washed three times in PT. Discs were mounted in 50% PT and 50% glycerol. Acridine orange staining was performed by incubating dissected discs in 1:500 1mM acridine orange in ethanol at room temperature for 10 minutes, washing in media and mounting in PBS.

In situ hybridizations were performed as described previously (Tautz and Pfeifle, 1989) using a 500bp *mae* probe generated by PCR amplification (5' CCCAAGTGGAAT-CGAGCTATACC 3'; 5' CTATGATAGCAGGGCCATTGCTCGG 3').

Histology

Preparation of tissue for scanning electron microscopy was performed as previously described (Tootle et al., 2003). Fixation and tangential sections of adult eyes was performed as previously described (Tomlinson et al., 1987).

Acknowledgements

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 and the Developmental Studies Hybridoma Bank for providing hybridoma lines. We also thank Serena Silver for initially identifying the EBS sites in *mae* and David Doroquez for critical reading of the manuscript. T.L.T was supported by the Ludwig Foundation. This work was supported in part by American Cancer Society Grant RPG-00-308-01-DDC.

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Chapter 4

The transcription factor Eyes absent is a protein tyrosine phosphatase.

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This *Letter to Nature* work was a collaborative effort. Initial efforts to isolate substantial amount of Eya protein from a variety of sources and determine whether Eya possesses phosphatase activity were performed by Erin Davies, Victoria Newman, and myself. Successful purification of Eya from bacteria and subsequent kinetic analysis was a joint effort by myself and Serena Silver. The observation that Eya is tyrosine phosphorylated in Drosophila insect cultured cells by Beth Parlikar led me to analyze the levels of tyrosine phosphorylation of wild-type versus catalytic mutants. I found that catalytic mutants exhibit higher tyrosine phosphorylation than wild-type, and went on to show in an in vitro assay that bacterially purified Eya can dephosphorylate tyrosine phosphorylated Eya from cultured cells.

Summary

Post-translational modifications provide sensitive and flexible mechanisms to dynamically modulate protein function in response to specific signaling inputs(Hunter 2000). In the case of transcription factors, changes in phosphorylation state can influence protein stability, conformation, subcellular localization, interactions with cofactors, transactivation potential and transcriptional output(Hunter 2000). Here we show that the evolutionarily conserved transcription factor Eyes absent(Treisman 1999; Wawersik and Maas 2000) belongs to the phosphatase subgroup of the haloacid dehalogenase superfamily(Collet et al. 1998; Thaller et al. 1998) and propose a novel function for it as a non-thiol based protein tyrosine phosphatase. In vitro assays demonstrate that Eyes absent has intrinsic phosphatase activity that is blocked by mutations altering the active site. Experiments performed in Drosophila cultured cells and in vitro indicate that Eyes absent has protein tyrosine phosphatase capability and may act autocatalytically to dephosphorylate itself. Confirming the biological significance of this function, mutations that disrupt the phosphatase active site severely compromise Eyes absent's ability to promote eye specification and development in Drosophila. Given the functional importance of phosphorylation-dependent modulation of transcription factor activity, our evidence of a nuclear transcriptional coactivator with intrinsic phosphatase activity suggests exciting new mechanisms for fine-tuning transcriptional regulation.

Results and Discussion

The transcriptional coactivator Eyes absent (EYA) is a member of an evolutionarily conserved set of nuclear transcription factors and cofactors collectively termed the retinal determination (RD) gene network(Bonini et al. 1997; Treisman 1999; Wawersik and Maas 2000). While RD network members are perhaps best known for their roles in eye specification,

redeployment of these genes, either individually or as a network, contributes to a diverse array of essential developmental processes in all metazoans(Treisman 1999; Wawersik and Maas 2000). EYA family members are defined by a conserved ~275 amino acid motif, referred to as the EYA domain (ED), that has been shown to bind two other RD members, Sine oculis (SO) and Dachshund (DAC)(Bonini et al. 1997; Chen et al. 1997; Pignoni et al. 1997). Together, EYA and SO form a potent transcriptional activator(Silver et al. 2003), while the mechanistic implications of EYA-DAC interactions are less clear(Ikeda et al. 2002; Kim et al. 2002). Emphasizing the functional conservation among EYA homologs, mammalian EYA transgenes can rescue the "eyeless" phenotype of Drosophila *eya* mutations(Bonini et al. 1997; Bui et al. 2000).

We have explored a new function for EYA's C-terminal ED that is suggested by protein motif searches and structural modeling studies. These investigations place EYA within the phosphatase subgroup of the Haloacid dehalogenase (HAD) superfamily (Figure 4-1a and Supplementary Figure 4-S1a). HAD family members constitute a diverse collection of enzymes found in all organisms ranging from bacteria and archaea to humans, that includes dehalogenases, ATPases, phosphonatases, phosphomutases, epoxy hydrolases and a growing number of magnesium-dependent phosphatases(Collet et al. 1998; Thaller et al. 1998; Collet et al. 1999). Understanding of the in vivo function of HAD family phosphatases remains extremely limited, particularly in eukaryotic systems.

X-ray crystallography combined with mutagenesis studies of several HAD family proteins has revealed a conserved α/β-hydrolase fold that unites three non-contiguous sequence motifs to form the catalytic core of the enzyme(Aravind et al. 1998; Collet et al. 1999; Selengut 2001). Structural modeling studies predict that the ED will form a HAD α/β-hydrolase-like fold

(Figure 4-1b). Five conserved residues brought together by this tripartite configuration surround the active site and are essential for catalysis(Aravind et al. 1998; Collet et al. 1999; Selengut 2001). These residues are strikingly conserved in the ED of all EYA proteins (Figure 4-1a and Supplementary Figure 4-S1b). In Motif 1 (DXDX (T/V)) the invariant first aspartic acid serves as the nucleophile in all HAD family proteins and likely forms a phospho-aspartate intermediate(Ridder and Dijkstra 1999; Cho et al. 2001). The second aspartic acid distinguishes the phosphatase/phosphohydrolase subgroup from other branches of the HAD superfamily (Collet et al. 1998; Thaller et al. 1998; Selengut 2001) and is strictly conserved in all EYA homologs. Motif 2 contains an essential Serine/Threonine at the end of the β-strand and Motif 3 contributes at least three required residues, a lysine and two aspartic acids, the second of which has undergone a conservative substitution to glutamic acid in EYA proteins. Requirement for the two acidic residues within Motif 3 appears strictest within the phosphatase/phosphohydrolase branch of the HAD superfamily (Thaller et al. 1998). The high degree of conservation of this catalytic quintet (D, S/T, K, D, E) in invertebrate, vertebrate and plant EYA homologs suggests that EYA belongs to the phosphatase subgroup of the HAD superfamily.

To investigate whether EYA has intrinsic phosphatase activity, we tested the ability of recombinant GST-tagged ED fusions to dephosphorylate the synthetic substrate para-nitrophenyl phosphate (pNPP). Using a murine homolog, we demonstrate that EYA can function as a phosphatase (Figure 4-2). Mutations altering the presumptive HAD active site residues severely compromise activity (Figure 4-2a; see Supplementary Information for details). Sensitivity to the tyrosine phosphatase inhibitor vanadate, but not to inhibitors of serine/threonine phosphatases (Figures 4-2a, b), and a requirement for Mg^{++} are consistent with EYA being a HAD family phosphatase(Selengut and Levine 2000). We also tested recombinant Drosophila EYA in these

Figure 4-1

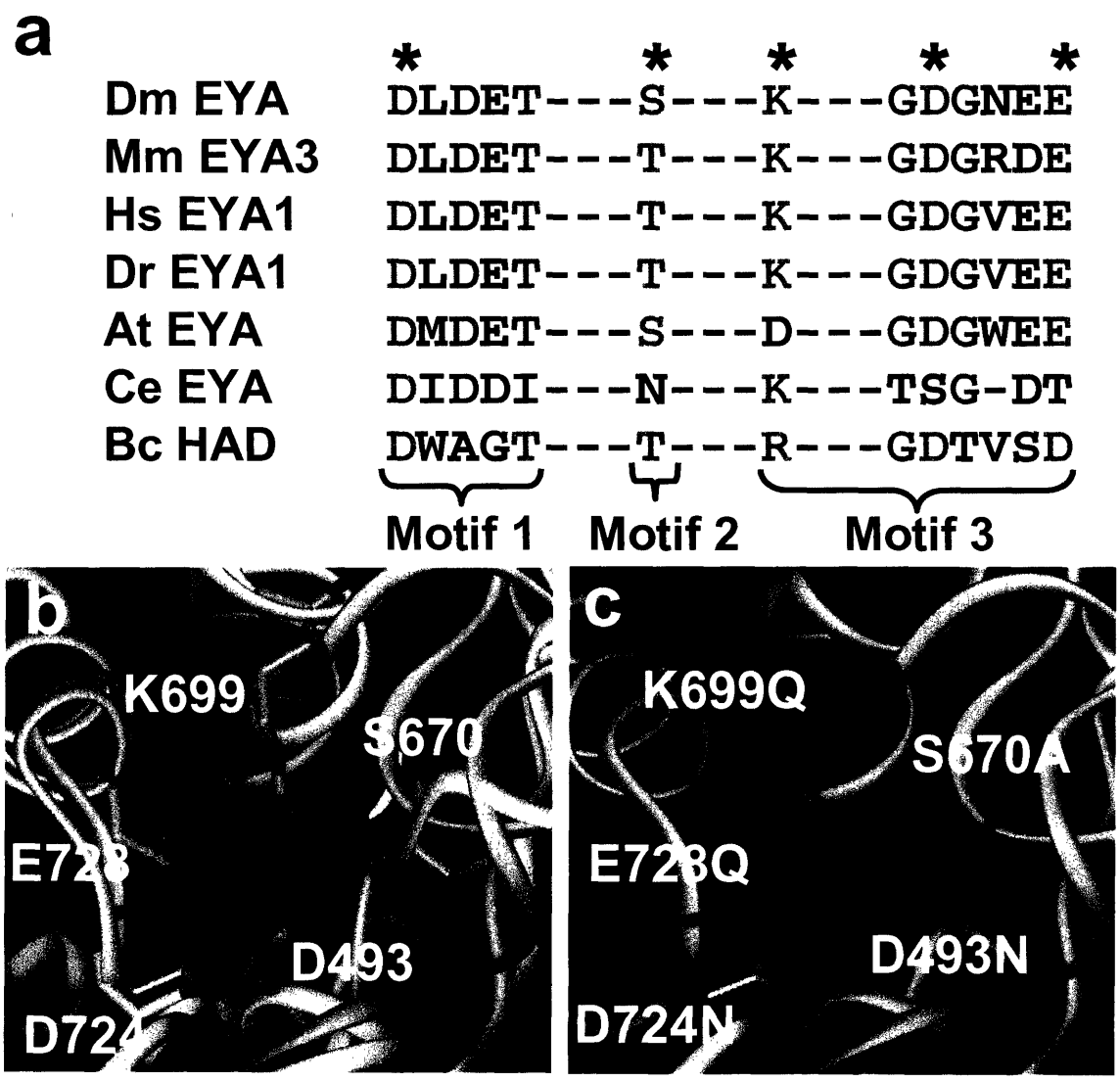

a
Dm EYA DLDET---S---K---GDGNEE
Mm EYA3 DLDET---T---K---GDGRDE
Hs EYA1 DLDET---T---K---GDGVEE
Dr EYA1 DLDET---T---K---GDGVEE
At EYA DMDET---S---D---GDGWEE
Ce EYA DIDDI---N---K---TSG-DT
Bc HAD DWAGT---T---R---GDTVSD
Motif 1
Motif 2
Motif 3
b
K699
S670
E728
D493
D724
c
K699Q
S670A
E728Q
D493N
D724N

Figure 4-1

EYA is a member of the phosphatase subgroup of the HAD superfamily.

a, The non-contiguous sequences comprising the HAD Motifs 1, 2, and 3. Pink residues define the HAD motif, with those mutated in this study marked with a "*". Blue residues are most strongly conserved among the phosphatase subgroup of the HAD superfamily. Green residues are highly conserved in both ATPases and phosphatases(Aravind and Koonin 1998; Collet et al. 1999).

b, Structural modeling studies predict a similar active site configuration for Drosophila EYA and other HAD proteins. The HAD template backbone is identified with a white ribbon and the EYA model backbone is rendered with a cyan ribbon. Key active site residues are highlighted as sticks, either white for the HAD or yellow for EYA.

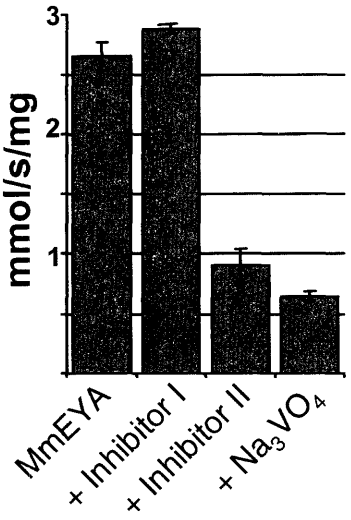
c, Superimposition of mutant DmEYA^{HAD} residues on the DmEYA model. Alignment of the substitutions (in magenta) and their wild type counterparts (in yellow) is shown.

Figure 4-2

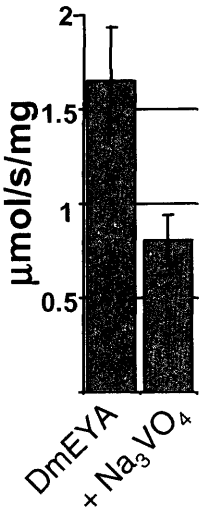
a

GST-MmEYA	Km (mM)	kcat (min^{-1})	kcat/Km ($M^{-1}min^{-1}$)
Mm EYA	93	0.017	0.18
+ Na_3VO_4	190	0.011	0.058
D246N	680	0.0012	0.0018
T420A	290	0.010	0.034
K449Q	590	0.021	0.035
D474N	66	0.019	0.29
E478Q	>>>	<<<	<<<
D246N + D474N	>>>	<<<	<<<

b



c



d

GST-MmEYA	Km (µM)	kcat (min^{-1})	kcat/Km (M^{-1} min^{-1})
Mm EYA	99	2.7×10^{-4}	2.71
D246N	560	9.0×10^{-5}	0.16

Figure 4-2

EYA exhibits phosphatase activity in vitro.

a, Kinetics for mouse EYA3 GST-ED fusion proteins (GST-MmEYA). D246N, T420A, K449Q, D474N and E478Q are mutations analogous to the D493N, S670A, K699Q, D724N and E728Q described for Drosophila EYA. For those mutant enzymes whose activity was too low to be measured, >>> indicates a Km significantly higher and an efficiency (kcat/Km) significantly lower than that measured for D246N.

b, Like the HAD family phosphatase MDP1(Selengut and Levine 2000), phosphatase activity of MmEYA is blocked by tyrosine phosphatase inhibitors (Inhibitor II and Na_3VO_4) but not serine/threonine phosphatase inhibitors (Inhibitor I).

c, Phosphatase activity of Drosophila EYA (DmEYA), although significantly weaker than that obtained with MmEYA, is also blocked by addition of the tyrosine phosphatase inhibitor Na_3VO_4.

d, Kinetics for mouse EYA3 GST-ED fusion proteins (GST-MmEYA) with respect to the tyrosyl phosphorylated peptide substrate $_{1(pY)GEF}$.

assays, and although its activity is significantly lower, it remains Mg^{++}-dependent and vanadate sensitive (Figure 4-2c). While the most likely explanation for the weak in vitro activity of the fly ED is that we have not identified appropriate conditions for purifying properly folded and active protein, we cannot rule out the possibility that Drosophila EYA, although it retains all the conserved residues comprising the HAD motif (Figure 4-1), may have only limited ability to function as a phosphatase. However, the fact that the mouse EYA isoform used in our in vitro assays is able to substitute for Drosophila EYA in vivo(Bui et al. 2000), when considered together with the results of the in vivo experiments described below, leads us to propose that EYA proteins possess a conserved phosphatase function.

To investigate whether EYA might have protein phosphatase capability, something that has not been definitively demonstrated for any other HAD family protein(Selengut 2001), several phosphotyrosine or phosphothreonine containing synthetic peptides were tested as substrates. We find that EYA exhibits robust activity toward one of the tyrosyl phosphorylated peptides, with a Km significantly lower than that measured using pNPP as a substrate (Figure 4-2d). No measurable activity was detected with the phosphothreonine or other phosphotyrosine containing peptides (data not shown; see Methods for details). These results demonstrate that EYA has protein tyrosine phosphatase (PTP) capability, although they do not rule the possibility that EYA could dephosphorylate other substrates as well. The fact that not all tyrosyl phosphorylated peptides were hydrolyzed suggests EYA has specific sequence preferences with respect to its putative protein substrates. Because HAD family phosphatases employ a catalytic aspartate(Ridder and Dijkstra 1999; Cho et al. 2001) as the nucleophile rather than the cysteine residue used by standard PTPs(Andersen et al. 2001), these results suggest EYA is the founding member of a new class of non-thiol-based PTPs.

We have used the genetically tractable Drosophila system to investigate the physiological relevance of EYA's putative PTP activity. For these experiments, site-directed mutagenesis was used to target the five HAD active site residues in Drosophila EYA (Figure 4-1c). Five single and four double mutant combinations were generated and will be referred to collectively as the EYA^{HAD} mutants. These EYA^{HAD} mutants were first tested in transfected Drosophila S2 cultured cells where immunostaining and western blotting analyses revealed no apparent changes in subcellular localization (data not shown) or expression levels (Supplementary Figure 4-S2a) relative to EYA^{WT}.

EYA, like most other RD genes, induces formation of eye tissue outside the normal eye field when ectopically expressed(Bonini et al. 1997; Treisman 1999; Wawersik and Maas 2000; Hsiao et al. 2001). Scoring the percentage of flies exhibiting ectopic eye formation provides a sensitive measurement of EYA activity(Hsiao et al. 2001). To determine whether the HAD active site mutants compromise EYA's ectopic eye induction potential, we generated transgenic lines carrying full-length EYA^{HAD} mutant expression constructs. All EYA^{HAD} mutants exhibit strikingly reduced ectopic eye induction relative to EYA^{WT} (Figure 4-3a). Protein expression levels from the EYA^{HAD} transgenes were comparable to those from EYA^{WT} lines (Supplementary Figure 4-S2b), indicating that the reduction in ectopic eye inducing potential reflects a change in protein activity rather than reduced expression. Comparable reductions in EYA activity were also observed with EYA^{HAD} transgenes in which two of the five HAD active site residues were mutated simultaneously (data not shown).

Because the HAD motif active site mutants compromise EYA's ability to induce ectopic eye formation, we asked whether an intact HAD motif is required for normal EYA function during eye development. We compared the ability of EYA^{WT} versus EYA^{HAD} transgenes to

Figure 4-3

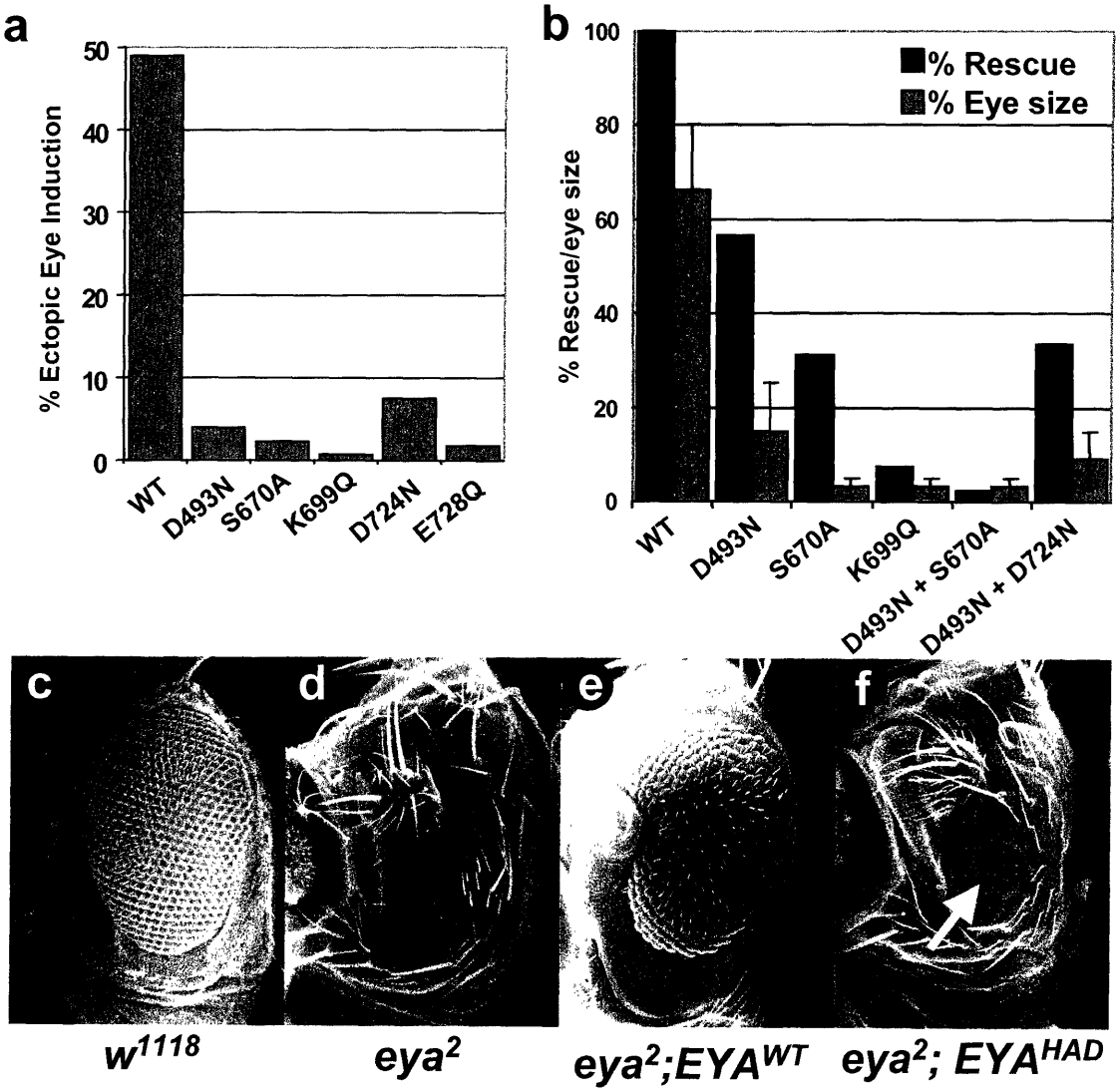

a
% Ectopic Eye Induction
50
40
30
20
10
0
WT
D493N
S670A
K699Q
D724N
E728Q
b
% Rescue/eye size
100
80
60
40
20
0
% Rescue
% Eye size
WT
D493N
S670A
K699Q
D493N + S670A
D493N + D724N
c
d
e
f
w1118
eya2
eya2;EYAWT
eya2; EYAHAD

Figure 4-3

EYA^{HAD} mutants exhibit severely reduced activity relative to EYA^{WT} in ectopic eye induction and genetic rescue assays.

a, The frequency of ectopic eye induction associated with expression of EYA transgenes was calculated from multiple independent transgenic lines: EYA^{WT}, 2465 flies from 8 lines(Hsiao et al. 2001); EYA^{D493N}, 1502 flies from 5 lines; EYA^{S670A}, 955 flies from 3 lines; EYA^{K699Q}, 953 flies from 3 lines; EYA^{D724N}, 265 flies from a single line; EYA^{E728Q} - 1239 flies from 4 lines.

b, The percentage of eyes from flies of the genotype *eya^2;UAS-EYA/dpp-GAL4* exhibiting rescue of the *eya^2* "eyeless" phenotype (black bars) and average size of the rescued tissue relative to a wild type eye (grey bars) is plotted. Data derives from the following lines: EYA^{WT}, 155 flies from two independent lines; EYA^{D493N}, 124 flies from a single line; EYA^{S670A}, 281 flies from a single line; EYA^{K699Q}, 176 flies from a single line; $EYA^{D493N+S670A}$, 209 flies from two independent lines; $EYA^{D493N+D724N}$, 151 flies from two independent lines.

c-f, Scanning electron micrographs of adult eyes. c, *w^{1118}*. d, *eya^2*. e, *eya^2;UAS-EYA^{WT}/dpp-GAL4*. f, *eya^2;UAS-EYA^{HAD}/dpp-GAL4*, arrow points to a small patch of rescued eye tissue.

complement the eye-specific loss-of-function *eya*^{2} allele. *eya*^{2} homozygous mutant flies exhibit a completely penetrant "eyeless" phenotype, in which the entire eye is missing (Figure 4-3c, d). For these experiments we define "rescue" as the ability of a given transgene to produce recognizable eye tissue within the normal eye field of an adult fly. We also estimate the size of the rescued eye tissue relative to that of a wild type eye in order to compare the extent of rescue.

Expression of EYA^{WT} transgenes rescues the *eya*^{2} "eyeless" phenotype with complete penetrance (Figure 4-3b, e) in both eyes of each individual fly (data not shown). In striking contrast, all EYA^{HAD} mutant transgenes exhibit a significantly reduced frequency and extent of rescue, with rescue usually occurring in only one of the two eye fields of an individual (Figure 4-3b, f). For all EYA^{HAD} transgenes tested, even in cases where rescue efficiency is only two to three fold lower than that of EYA^{WT}, the size of the rescued eye tissue is always significantly (5-10 fold) reduced relative to that obtained with EYA^{WT} lines (Figure 4-3b, e, f). Western blot analyses of eye imaginal discs again ruled out the possibility that reduced protein expression might be responsible for this result (Supplementary Figure 4-S2c). In combination with the ectopic eye induction assay data, the results of these rescue experiments argue strongly that EYA's activity as a putative HAD family phosphatase is required to promote normal eye development in Drosophila.

Because the region of the ED that binds to the RD gene network protein SO(Pignoni et al. 1997; Bui et al. 2000) partially overlaps with Motif 1 of the HAD domain (Supplementary Figure 4-S1b), we checked whether the EYA^{HAD} missense mutations compromise EYA's ability to interact productively with SO. EYA and SO interact to form a potent transcriptional activator required for eye specification, in which SO contributes the DNA binding domain and EYA provides the transactivation potential (Pignoni et al. 1997; Ikeda et al. 2002; Silver et al. 2003).

Using a transcription assay in Drosophila S2 cultured cells(Silver et al. 2003), we find that the ability of EYA^{HAD} mutant proteins to synergize with SO to activate transcription of a reporter gene is comparable to that of EYA^{WT} (Figure 4-4 and Supplementary Figure 4-S3). Although we cannot rule out the formal possibility that in vivo the EYA^{HAD} mutations disrupt interactions with other proteins rather than blocking phosphatase activity, the finding that mutational disruption of the HAD motif active site does not abrogate EYA's ability to function as a transcriptional coactivator in conjunction with SO leads us to propose that EYA proteins have two essential functions: a previously described role as a transcription factor and a novel role as a protein tyrosine phosphatase.

To investigate further EYA's intrinsic PTP capability with respect to physiologically relevant substrate candidates, we exploited our finding that EYA can be tyrosine phosphorylated in Drosophila S2 cells (Figure 4-5a; see Supplementary Information for discussion) by affinity purifying full-length EYA from these cells and using it as a protein substrate in an in vitro phosphatase reaction. Because the phosphotyrosine signal associated with the EYA^{HAD} mutant proteins was consistently elevated relative to EYA^{WT} (Figure 4-5a; see Supplementary Information for discussion), the EYA^{HAD} protein was used as the substrate. We find that incubation of EYA^{HAD} protein with recombinant murine GST-ED fusion protein strongly reduces the phosphotyrosine signal (Figure 4-5b). HAD active site mutants that exhibit impaired activity both in vitro and in vivo (Figures 4-2 and 4-3) also have severely reduced activity in this assay (Figure 4-5b). These results demonstrate that EYA has PTP capability with respect to a full-length endogenous protein substrate and that such activity depends on an intact HAD motif. Although we do not yet understand the physiological relevance of tyrosine phosphorylation and dephosphorylation of EYA, the results of these experiments (Figure 4-5), together with our

previous demonstration that EYA is able to self-associate(Silver et al. 2003), suggest that EYA may act autocatalytically to dephosphorylate itself.

In conclusion, we propose that EYA is both the founding member of a novel class of non-thiol based PTPs and to our knowledge, the first example of a transcription factor with intrinsic phosphatase activity. Further work will be required to understand how tyrosine phosphorylation and dephosphorylation regulates EYA function in vivo, and what substrates, potentially including EYA itself, may be regulated by its PTP activity. Elucidation of the biochemical regulatory mechanisms that coordinate EYA's dual functions as transactivator and phosphatase during eye specification will provide new insights into the function of the RD gene network, and more generally a new paradigm for transcriptional regulatory strategies. Although preliminary analyses have not identified other HAD-motif containing proteins that are annotated as transcriptional regulators (R. R. L. and I. R., unpublished observation), it seems likely that dual function mechanisms analogous to that we propose for EYA may prove to be a general strategy for fine-tuning transcriptional output, particularly in highly regulated developmental systems.

Figure 4-4

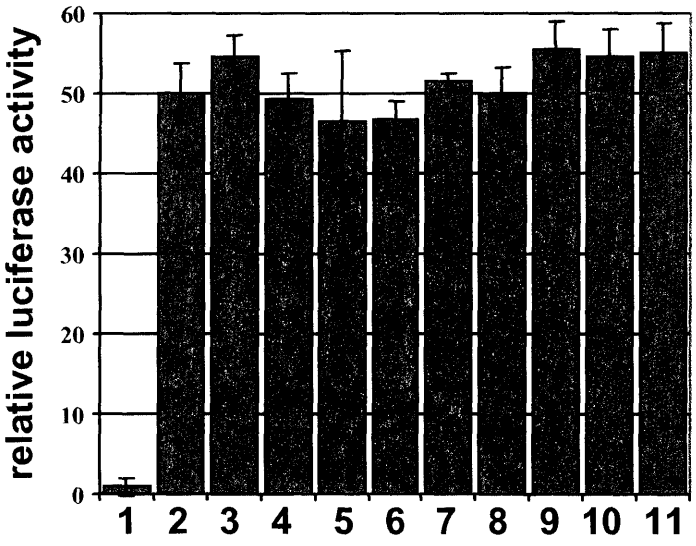

relative luciferase activity
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11

Figure 4-4

EYA^{HAD} mutations do not disrupt EYA's role as a transcriptional coactivator in conjunction with Sine oculis. The Drosophila cell culture based transcription assays were performed as recently described(Silver et al. 2003). Lanes: 1, Are-Luciferase; 2, WT; 3, D493N; 3, S670A; 4, K699Q; 5, D724N; 5, E728Q; 6, D493N + S670A; 7, D493N + K699Q; D493N + D724N; D493N + E728Q. Are-Luciferase is a multimer of SIX family binding sites. See Supplementary Figure S3 for further details.

Figure 4-5

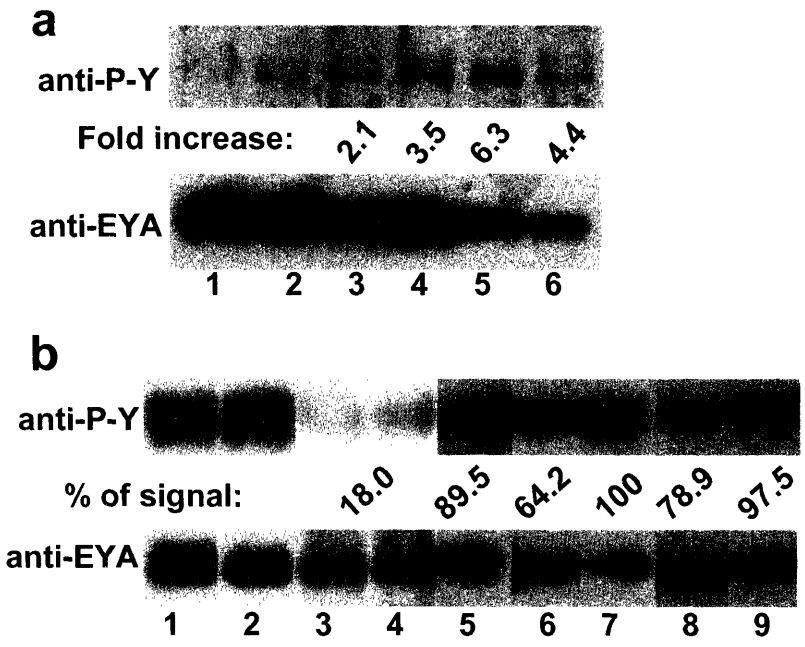

a
anti-P-Y
Fold increase:
2.1
3.5
6.3
4.4
anti-EYA
1 2 3 4 5 6
b
anti-P-Y
% of signal:
18.0
89.5
64.2
100
78.9
97.5
anti-EYA
1 2 3 4 5 6 7 8 9

Figure 4-5

EYA has protein tyrosine phosphatase capability.

Top panels show immunoblots probed with anti-phosphotyrosine (anti-P-Y); bottom panels show immunoblots of the same samples probed with anti-Flag to detect EYA (anti-EYA).

a, Lanes: 1-2, independent transfections of EYA^{WT}; 3, $EYA^{D493N + S670A}$; 4, $EYA^{D493N + K699Q}$; 5, $EYA^{D493N + D724N}$; 6, $EYA^{D493N + E728Q}$. Fold increase in P-Y levels for the EYA^{HAD} mutants relative to an average of the P-Y signal in the two EYA^{WT} lanes, and corrected relative to the strength of the anti-EYA signal, is indicated underneath the anti-P-Y blot.

b, Dephosphorylation of Drosophila EYA by recombinant GST-ED. Full length tyrosine phosphorylated Drosophila $EYA^{D493N + D724N}$ (all lanes) was immunoprecipitated and incubated with recombinant mouse GST-ED, either wild type (WT) or HAD mutant variants (Lanes: 1-2, control; 3-4, WT; 5, D246N, 6, T420A; 7, K449Q; 8, D474N; 9, E478Q). The percentage of anti-P-Y signal on $EYA^{D493N + D724N}$ relative to controls and corrected for relative protein levels is indicated. Numbers shown are an average from two independent experiments for each GST-ED tested; results from only one of the two experiments are shown for the GST-ED HAD mutants. Samples were run on the same gel to allow quantitative comparisons.

Methods

Bioinformatics

For a description of the computational analyses, see Supplementary Information.

Phosphatase assays

Phosphatase assays were performed using GST-ED fusion proteins (purification protocol described in Supplementary Information). For enzyme kinetics with the synthetic substrate p-Nitrophenyl Phosphate (pNPP, Sigma), assays were done in triplicate with six substrate concentrations over six timepoints. 80ul reactions performed in microfuge tubes at 30°C in 200mM PIPES pH 7.0, 5mM EDTA and 10mM $MgCl_2$ were quenched by addition of 40ul of 10M NaOH. PNP anion was detected at 405 nm (extinction coefficient $\varepsilon_M = 1.78 \times 10^4$ /cm M) using a Tecan GENios plate reader. Reactions were normalized to buffer alone controls and the results analyzed by Lineweaver-Burk plot using Microsoft Excel. Synthetic peptide substrates tested were: I (pY)GEF and TSTGPE (pY)EPGENL (Calbiochem); END (pY)INASL, DADE (pY)LIPQQG and RRA (pT)VA (Promega). 50ul reactions were performed at 25°C in 200mM HEPES pH7.0, 10mM $MgCl_2$, 5mM EDTA and quenched with 50µl of Molybdate Dye Solution (Promega). Malachite Green/Ammonium Molybdate-phosphate complex was detected at 595 nm and converted to moles of free phosphate using a phosphate standard curve. Assays with I (pY)GEF were carried out for five substrate concentrations over five timepoints and the results were analyzed as described for pNPP.

Phosphatase Inhibitor Cocktail Sets I and Set II (Calbiochem; see Supplementary Information for details) were used at 1:50 in pNPP phosphatase assays. Sodium Orthovanadate was used at 4mM final concentration in pNPP phosphatase assays.

Amino terminally flag epitope tagged EYA constructs were subcloned into the copper inducible metallothionein promoter vector. 5 μg of DNA for each construct was transfected into S2 cells as previously described(Tootle et al. 2003). Following published protocols(Imbert et al. 1994; Cohen et al. 1997; Huyer et al. 1997; Ruff et al. 1997; Scanga et al. 2000), cells were treated with 100μM $NaVO_3$, 200μM H_2O_2 for 15 minutes prior to lysis in 100mM NaCl, 50mM Tris, pH 7.5, 2mM EDTA, 2mM EGTA, 1% NP-40, 1mM Na_3VO_4, and one mini-complete protease inhibitor tablet (Roche) per 10 ml. All subsequent solutions include 1mM Na_3VO_4. Clarified lysates were incubated with 25μl of anti-flag M2 agarose affinity gel (Sigma) for 1.5 hours at 4°C. Beads were washed twice in lysis buffer and twice in 10mM Tris, pH 7.5, 150mM NaCl, resuspended in 30μl of 2x SDS sample buffer, boiled and 10μl were loaded per lane. Westerns were performed as previously described(O'Neill et al. 1994) except that blocking and antibody incubations were performed in 1% Casein According to Hammarsten (EM Science). Antibodies: guinea pig anti-Eya 1:16,000, rabbit anti-phosphotyrosine 1:400 (.21 mg/ml, Upstate); HRP-conjugated goat anti-guinea pig and anti-rabbit 1:5000 (Jackson ImmunoResearch). Determination of fold increase in phospho-tyrosine signal relative to EYA protein amounts was performed using NIH Image software; samples analyzed in this way were always run together on the same gel.

To obtain sufficient tyrosine phosphorylated Drosophila EYA to use as a substrate in the in vitro phosphatase assay, a stable cell line expressing flag-tagged $Eya^{D493N + D724N}$ was generated. 500μl of cells were immunoprecipitated for each reaction as described above, except the 1mM Na_3VO_4 was omitted from the wash buffer. The washed immunoprecipitates were incubated in phosphatase assay reaction buffer (as described above but without pNPP), either

with GST agarose or with 100μg GST-ED proteins for 1 hour at 30°C, processed for western blotting and analyzed as described above.

Molecular Biology and Genetics

Site-directed mutagenesis, subcloning, generation of transgenics, crosses, ectopic eye scoring, calculation of % ectopic eye induction and scanning electron microscopy were performed as previously described(Hsiao et al. 2001; Tootle et al. 2003). Fly crosses were at 25°C with the exception of the genetic rescue assays which were performed at 20°C.

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Supplementary Information

Discussion

We have found that EYA is a novel protein tyrosine phosphatase member of the HAD superfamily. Specific point mutations shown previously to compromise the HAD active site in other superfamily members severely reduce EYA's phosphatase activity. The in vitro assays, one using pNPP or a tyrosyl phosphorylated peptide as artificial substrates (Figure4-2) and the other using tyrosine phosphorylated Drosophila EYA protein as a physiologically relevant substrate (Figure 4-5), and the in vivo assays, ectopic eye induction and genetic rescue (Figure 4-3), consistently reveal this trend. However there are subtle activity differences between the various mutants tested, as well as slight variations between the same mutants tested in different assays.

The most notable example results from our analyses of the D493N mutant (D246N in murine EYA3). This mutation alters the aspartic acid residue that serves as nucleophile and forms a phospho-aspartate intermediate in the reaction(Ridder and Dijkstra 1999; Cho et al. 2001). Given its critical role in catalysis, the prediction is that a mutation in this residue should severely compromise activity. In fact, in all the in vitro assays (Figure 4-2 and Figure 4-5b) and in the ectopic eye induction assays (Figure 4-3a), the activity of D246N is greatly compromised. However in the genetic rescue experiment, although activity is significantly reduced relative to EYA^{WT}, the residual activity is greater than that measured for any of the other HAD mutants (Figure 4-3b). While the reason for this is unclear, it presumably reflects the complexity of assaying function in the context of normal eye development, where both functions of EYA as a phosphatase and as a transcriptional cofactor are required. Elucidation of the mechanisms whereby EYA's dual functions as transcriptional coactivator and phosphatase are coordinated

will be required to fully understand such subtle distinctions in activity between the different HAD active site mutants in vivo and how these correlate with their relative activities in vitro.

A second example is the D724N mutant (D474N in murine EYA3), which unlike all the other mutants tested and consistent with its previously characterized role in Mg^{++} binding rather than substrate binding(Cho et al. 2001), does not increase the Km measured in the pNPP assay (Figure 4-2). Activity in both the PTP and ectopic eye induction assays is comparable, although perhaps slightly elevated, to that measured for the other HAD active site mutants. In general, interpreting modest differences in activities between different mutants in the various assays must be performed with caution because we do not yet understand the biochemical mechanisms that coordinate and regulate EYA's dual functions as transcription factor and phosphatase.

The Drosophila EYA^{HAD} protein used in the PTP assay (Figure 4-5b) was purified from a stably transfected S2 cell line that had been artificially stimulated with pervanadate. Confirming that EYA is tyrosine phosphorylated in the absence of pervanadate, we find that EYA^{HAD} is tyrosine phosphorylated in unstimulated cells, although the signal is reduced relative to that observed in stimulated cells (Figure 4-S4a). We have been unable to immunoprecipitate sufficient EYA^{WT} protein from transient transfections to detect a signal in the absence of pervanadate and efforts to generate a stable cell line have not yet been successful (Clark et al. 2002). Therefore, to facilitate detection of PTP activity in our assay (Figure 4-5b), we elected to purify EYA^{HAD} protein from pervanadate stimulated cells in order to increase the pool of tyrosine phosphorylated protein substrate.

The most likely explanation as to why we require the use of pervanadate to detect tyrosine phosphorylation of EYA in transiently transfected cells (Figure 4-5a) is that EYA has autocatalytic activity and actively dephosphorylates itself. Drosophila S2 cells express

significant levels of endogenous wild-type EYA. We have recently shown that EYA has the ability to self-associate (Silver et al. 2003), leading us to postulate that endogenous EYA may associate with the transfected EYA and dephosphorylate it. Addition of pervanadate presumably greatly impairs, but does not totally knock out phosphatase activity resulting in a weak but detectable signal for EYA^{WT} (Figure 4-5a, Lanes 1 and 2). This interpretation is consistent with our finding that addition of vanadate in the pNPP assay doubles the apparent Km, but does not completely inactivate the enzyme (Figure 4-2a). EYA^{HAD} mutants exhibit increased phosphotyrosine signal in this assay (Figure 4-5a), likely reflecting their reduced activity as a phosphatase (Figure 4-2). In the case of the stable cell lines expressing the catalytically inactive EYA^{HAD} mutants, in the absence of pervanadate, the endogenous EYA is sufficiently active to dephosphorylate a significant portion, but not all, of the overexpressed EYA^{HAD} mutant protein (Figure 4-S4a). However, because these experiments were carried out in Drosophila cells rather than in vitro, it is possible that rather than reflecting impairment of intrinsic PTP activity in the EYA^{HAD} mutants, the increased phosphotyrosine signal resulted from a second coprecipitating PTP that interacts more strongly with EYA^{WT} than with EYA^{HAD}, or from the EYA^{HAD} proteins serving as better substrates for the relevant tyrosine kinase. Arguing against this interpretation, we find that incubation with recombinant EYA^{WT} fusion protein, but not EYA^{HAD} fusion protein, strongly reduces the phosphotyrosine signal associated with Drosophila EYA^{HAD} (Figure 4-5b). This suggests that EYA may serve as its own substrate, likely acting in trans to dephosphorylate itself. Based on our genetic analyses indicating that EYA's phosphatase activity is required for eye specification and development (Figure 4-3), we propose that EYA may autoactivate by dephosphorylating itself on specific tyrosine residues.

Methods

Bioinformatics

The phylogenetic tree of a number of different hydrolase family members (PFAM 00702.6) was generated by the neighbor-joining method using ClustalX(Thompson et al. 1997). EYA protein sequences from Drosophila (Dm, gi:17737399), mouse (Mm, gi:6753794), human (Hs, gi:3183005), zebrafish (Dr, gi:18858653), arabidopsis (At, gi:21593200) and nematode (Ce, gi:3875091) were aligned with the Bacillus cereus phosphonoacetaldehyde hydrolase (Bc, gi:10835405) HAD protein using ClustalX (Thompson et al. 1997). For the structural modeling analyses, Prospect Pro (Xu and Xu 2000) was used to thread the Drosophila EYA ED sequence (DmEYA) against structures in the protein data bank. From these comparisons, Phosphonoacetaldehyde hydrolase (PDB:1FEZ) was selected as a suitable modeling template. DmEYA and the template initially were aligned according to threading results and then modified by anchoring several phosphonatase active site residues to their synonymous positions within DmEYA. The structural model of DmEYA was created with Modeler (Sali and Blundell 1993) employing the alignment and using the coordinates of the Phosphonoacetaldehyde hydrolase structure. The original template, 1FEZ, and the DmEYA model were aligned using CCP4 (Otwinowski 1993). Key active site positions within the DmEYA model were replaced with the variants described in this study using the Builder module within InsightII (Accelrys ™, 2001).

Molecular Biology and Genetics

The cell culture based transcription assays were performed as recently described(Silver et al. 2003). For western blot analyses of protein expression levels, equivalent samples of either S2 cells, embryos or dissected eye-imaginal discs were lysed in SDS-sample buffer, separated by SDS-PAGE and immunoblotted with GP anti-EYA antiserum diluted 1:10,000.

Protein Purification

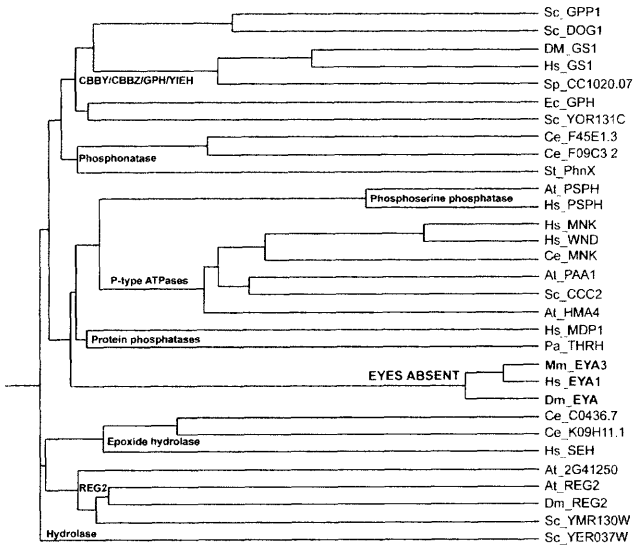
GST-MmEYA3 ED (aa 237-510 of mouse EYA3) or GST-DmEYA ED (aa 438-760 of Drosophila EYA) fusion proteins were purified from BL21 E. coli cells grown to an OD_{600} of 1.0 and then induced with IPTG for 2.5 hours at 18°C. Cells were harvested by centrifugation, resuspended in 50mM Tris buffer pH 8.0, 1mM EDTA and 100mM NaCl plus protease inhibitor cocktail (1 tablet per 50 mls, Roche) and lysed by three passes through a French Press at 1000 psi. Clarified lysates were rocked overnight at 4°C with glutathione-agarose beads. Beads were washed five times with 50mM HEPES, 300mM NaCl, and left in a final 1:1 slurry. Protein concentrations were estimated by Coomassie Blue staining of an SDS-PAGE gel run with a dilution series of GST-ED and a BSA standard curve. Depending on the protein concentration, appropriate amounts of beads were diluted for the phosphatase assays. To determine Km and enzyme efficiency, GST-ED was eluted by rocking for 20 minutes at 4°C with 300mM reduced glutathione in 50mM Tris pH 7.0, 150mM NaCl. For peptide assays, 10mM reduced glutathione was used in the elutions. Protein concentrations were determined by Bradford Assay.

Phosphatase Inhibitor Cocktail Ingredients

Inhibitor Cocktail Set I (Calbiochem) contains p-Bromotetramisole (inhibits alkaline phosphatase), Oxalate 2.5mM, Cantharidin 500uM (inhibits serine/threonine protein phosphatase), Microcystin LR 500nM (inhibits serine/threonine protein phosphatase). Inhibitor Cocktail Set II (Calbiochem) contains 200mM Imidazole (inhibits alkaline phosphatase), 100mM Sodium Fluoride (inhibits acid phosphatase), 115 mM Sodium Molybdate (inhibits acid phosphatase), 100mM Sodium Orthovanadate (inhibits protein tyrosine phosphatase and alkaline phosphatase), and 400mM Sodium Tartrate Dihydrate (inhibits acid phosphatase).

Figure 4-S1

a



b

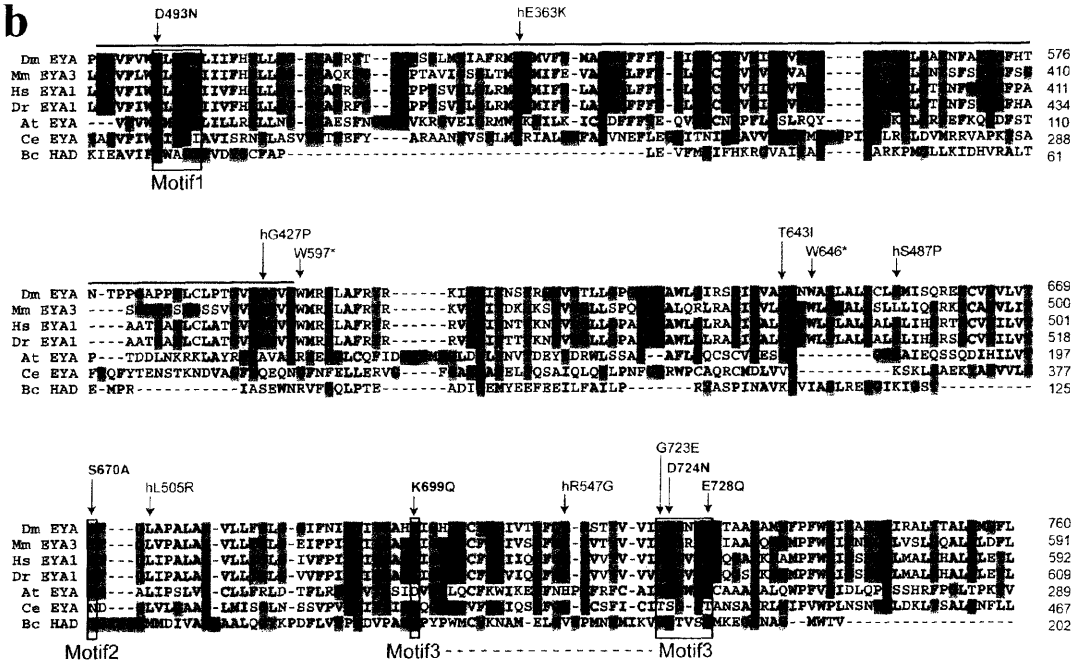


Figure 4-S1

EYA is a member of the phosphatase subgroup of the HAD superfamily.

a, A HAD superfamily phylogenetic tree suggests EYA proteins are related to protein phosphatases. Branches are labeled with the two letter species abbreviation and common protein name. The EYA branch is highlighted in red.

b, Multiple Sequence Alignment reveals that the conserved EYA domain shares similarity with the HAD hydrolase domain, particularly in those regions implicated in forming the active site (PFAM 00702.6). Residues comprising Motifs 1-3 of the HAD domain are boxed. Labeled arrows designate the positions of variant residues used in this study or identified in Drosophila and human EYA mutations. Site-directed mutations generated in this study are shown in red. Additional variants associated with specific Drosophila *eya* alleles(Bui et al. 2000) or derived from human patients suffering from the EYA1-specific branchio-oto-renal syndrome (Azuma et al. 2000) are shown in black, with the human mutations prefaced with the letter "h". While none of these variants overlap with the five residues focused on in this study, one, the Drosophila G723E mutation, affects a conserved glycine residue in Motif 3 of the HAD domain. The putative Sine oculis binding site(Pignoni et al. 1997; Bui et al. 2000) is denoted with a solid black line.

Figure 4-S2

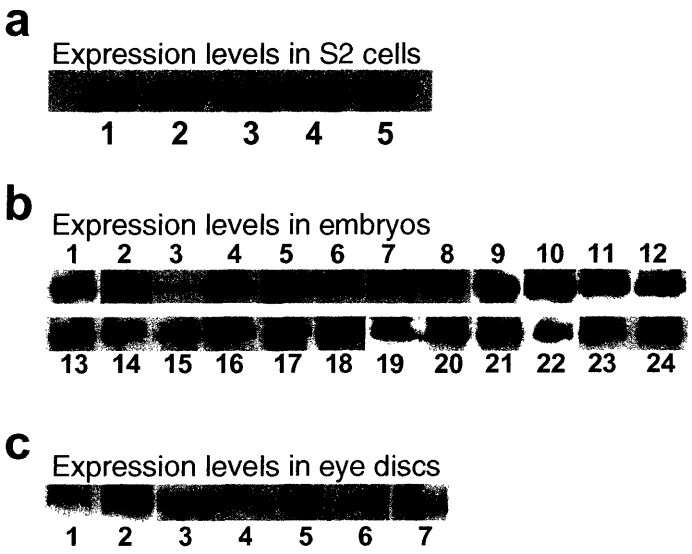

a
Expression levels in S2 cells
1 2 3 4 5
b
Expression levels in embryos
1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16 17 18 19 20 21 22 23 24
c
Expression levels in eye discs
1 2 3 4 5 6 7

Figure 4-S2

HAD active site mutations do not result in appreciable changes in protein levels relative to wild type EYA.

a, Western blot of transfected S2 cells showing comparable expression of EYA^{HAD} mutants relative to EYA^{WT}. Equivalent samples from pools of stably transfected S2 cell lines expressing EYA^{WT} and four different EYA^{HAD} mutants. Lanes 1-5: EYA^{WT}; $EYA^{D493N + S670A}$; $EYA^{D493N + K699Q}$; $EYA^{D493N + D724N}$; $EYA^{D493N + E728Q}$.

b, Western blots of equivalent samples of embryos in which the EYA transgenes have been expressed using a *ubiquitin*-GAL4 driver line reveal comparable expression levels in EYA^{HAD} lines relative to EYA^{WT}. Each lane represents an independent transgenic line. Lanes: 1-4, EYA^{WT}; 5-9, EYA^{D493N}; 10-13, EYA^{S670A}; 14-17, EYA^{K699Q}; 18, EYA^{D724N}; 19-22, EYA^{E728Q}; 23-24, $EYA^{D493N + D724N}$.

c, Western blots of equivalent samples of eye imaginal discs in which the EYA transgenes have been expressed using a *dpp*-GAL4 driver line reveal comparable expression levels in EYA^{HAD} lines relative to EYA^{WT}. Each lane represents an independent transgenic line used in the rescue assay. EYA sometimes runs as a doublet (for example, lanes 1 and 2 and lanes 16-20 of panel 3c). Lanes: 1, EYA^{WT}; 2, EYA^{D493N}; 3, EYA^{S670A}; 4, EYA^{K699Q}; 5&6, $EYA^{D493N + S670A}$; 7, $EYA^{D493N + D724N}$.

Figure 4-S3

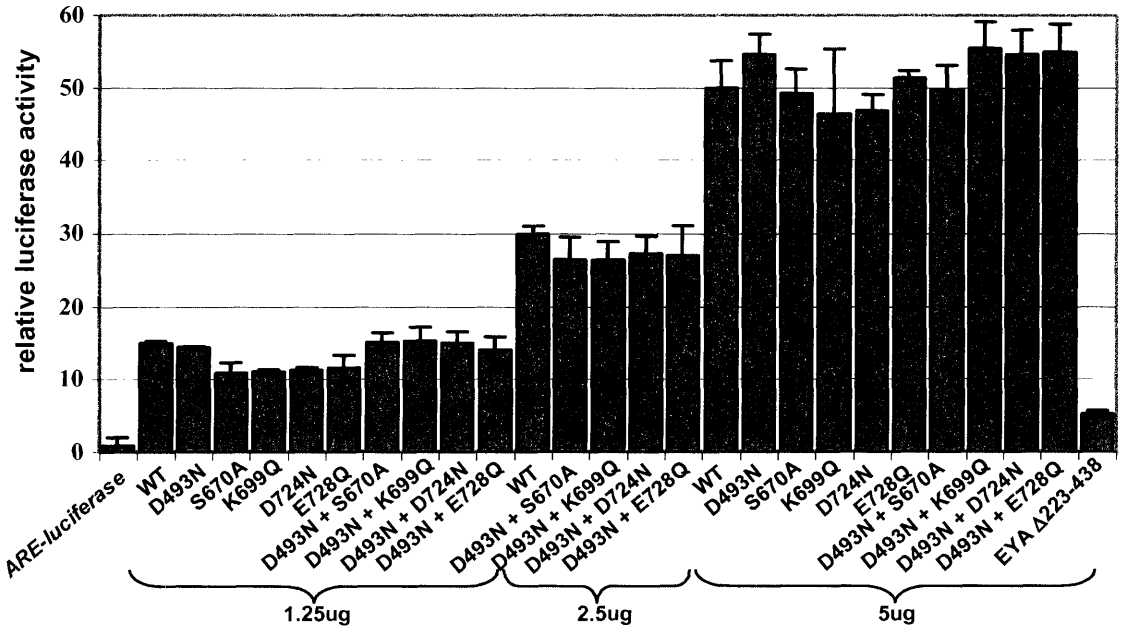

relative luciferase activity
60
50
40
30
20
10
0
ARE-luciferase
WT
D493N
S670A
K699Q
D724N
E728Q
D493N + S670A
D493N + K699Q
D493N + D724N
D493N + E728Q
WT
D493N + S670A
D493N + K699Q
D493N + D724N
D493N + E728Q
WT
D493N
S670A
K699Q
D724N
E728Q
D493N + S670A
D493N + K699Q
D493N + D724N
D493N + E728Q
EYA Δ223-438
1.25ug
2.5ug
5ug

Figure 4-S3

EYA^{HAD} mutations do not disrupt EYA's role as a transcriptional coactivator in conjunction with Sine oculis. EYA^{WT} versus EYA^{HAD} activity was tested at three different concentrations of EYA DNA (1.25 ug, 2.5 ug and 5 ug) to confirm the linearity of response. A mutation (D223-438; last sample in graph) that deletes the transactivation domain of EYA, but can still bind SO4, demonstrates that it is possible to block activity in this assay system. The reporter baseline level in the absence of EYA and SO is shown (ARE-luciferase; first sample in graph).

Figure 4-S4

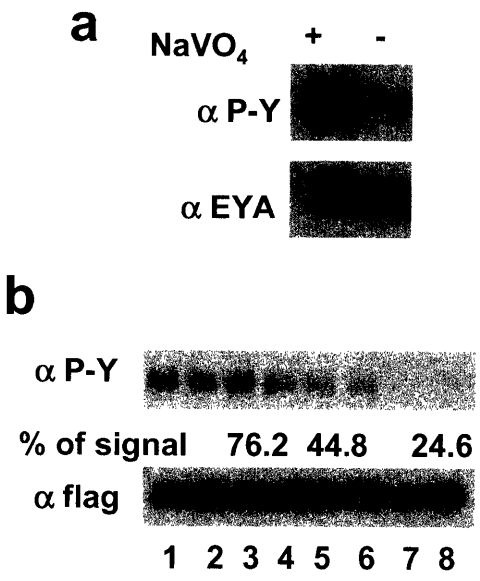


Figure 4-S4

Using tyrosine phosphorylated EYAHAD as a substrate in an in vitro phosphatase reaction.

a, Tyrosine phosphorylation of EYAHAD in the absence of pervanadate is reduced relative to levels achieved in the presence of pervanadate. Quantitation was not performed because the exposure time necessary to obtain a sufficiently strong signal in the (-) pervanadate lane placed the signal in the (+) pervanadate range outside of the linear range of detection.

b, A titration curve of amounts of GST-ED necessary to achieve maximal activity in the PTP assay was performed. Duplicate experiments are shown. Lanes: 1-2, 1ug, set at 100%; 3-4, 5ug; 5,6, 10ug; 7,8, 50ug. EYAD493N+D724N was used as the substrate as in Figure 4b.

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Chapter 5

Efforts to determine where and by whom EYA is tyrosine phosphorylated.

Tina Tootle and Ilaria Rebay

Abstract

Eyes absent, EYA, functions as both a transcriptional coactivator in the Retinal Determination Network, and as a protein tyrosine phosphatase. EYA is tyrosine phosphorylated and can dephosphorylate itself. It is unclear how tyrosine phosphorylation regulates EYA's activities. We find that EYA is likely to be phosphorylated within the EYA domain (ED), and a putative site of phosphorylation is tyrosine 719. Attempts to identify the kinase(s) responsible for this phosphorylation have thus far been unsuccessful. This work, along with future efforts discussed in Chapter 6, should clarify where EYA is tyrosine phosphorylated and the effects of such phosphorylation.

Introduction

Eyes absent, EYA, is an evolutionarily conserved transcriptional coactivator in the Retinal Determination (RD) Network, which encompasses a signaling cascade of transcriptional regulators best known for their necessity during *Drosophila* eye development. Eye specific mutations in RD network members exhibit eyeless phenotypes, while overexpression either alone or in conjunction with other RD network members results in ectopic eye tissue. While one outcome of this signaling cascade is eye formation, known null mutations within this network exhibit lethality, indicating further roles during development.

The RD Network functions in a hierarchical manner in that Twin on Eyeless (TOY) turns on the expression of Eyeless (EY), which in turn leads to expression of EYA and Sine oculis (SO), who turn on Dachshund (DAC) (Chen et al. 1997; Pignoni et al. 1997; Czerny et al. 1999; Hauck et al. 1999). TOY and EY are Pax6 homologs and contain both Paired and Homeo-Domain DNA binding domains (Quiring et al. 1994). SO is a homeodomain transcription factor (Cheyette et al. 1994; Serikaku and O'Tousa 1994). EYA and DAC are more novel nuclear

factors. It has recently been suggested that DAC possesses intrinsic DNA binding ability (Kim et al. 2002), while EYA is known to function as a transcriptional coactivator with SO (Ohto et al. 1999; Silver et al. 2003).

Structure-function analysis has yielded insight into how the various domains within EYA function in relation to its transcriptional activity (Figure 5-1). There are two evolutionarily conserved domains in EYA, EYA Domain 2 (ED2) and EYA Domain (ED) (Xu et al. 1997; Zimmerman et al. 1997). The ED2 is a tyrosine rich domain contained within a larger proline, serine, threonine (PST) rich region. The PST region is necessary for EYA's transcriptional activity with SO (Silver et al. 2003). There are two MAPK phosphorylation sites, shown to positively regulate EYA, at the carboxy-terminus of the PST region (Hsiao et al. 2001). The ED has been shown to bind to SO and DAC (Pignoni et al. 1997); in addition the ED possesses homology to a broad family of enzymes called haloacid dehalogenases (HADs) (Li et al. 2003; Rayapureddi et al. 2003; Tootle et al. 2003).

Homology between the ED and HADs suggested that EYA, in addition to acting as a transcriptional coactivator, could be functioning as a phosphatase. It has been shown, Chapter 4, that EYA is a protein tyrosine phosphatase (Rayapureddi et al. 2003; Tootle et al. 2003), while others have observed that EYA is a dual specific phosphatase (Li et al. 2003). Phosphatase activity is necessary for EYA function, as phosphatase mutants cannot rescue *eya* mutant phenotypes or induce ectopic Eyes at a comparable level to wild-type EYA (Tootle et al. 2003). In addition EYA is tyrosine phosphorylated by an unknown kinase(s) in *Drosophila* insect cultured cells (see Figure 4-5) (Tootle et al. 2003). In vitro assays have shown that the bacterially purified EYA protein tyrosine phosphatase (PTP) domain can dephosphorylate EYA

Figure 5-1

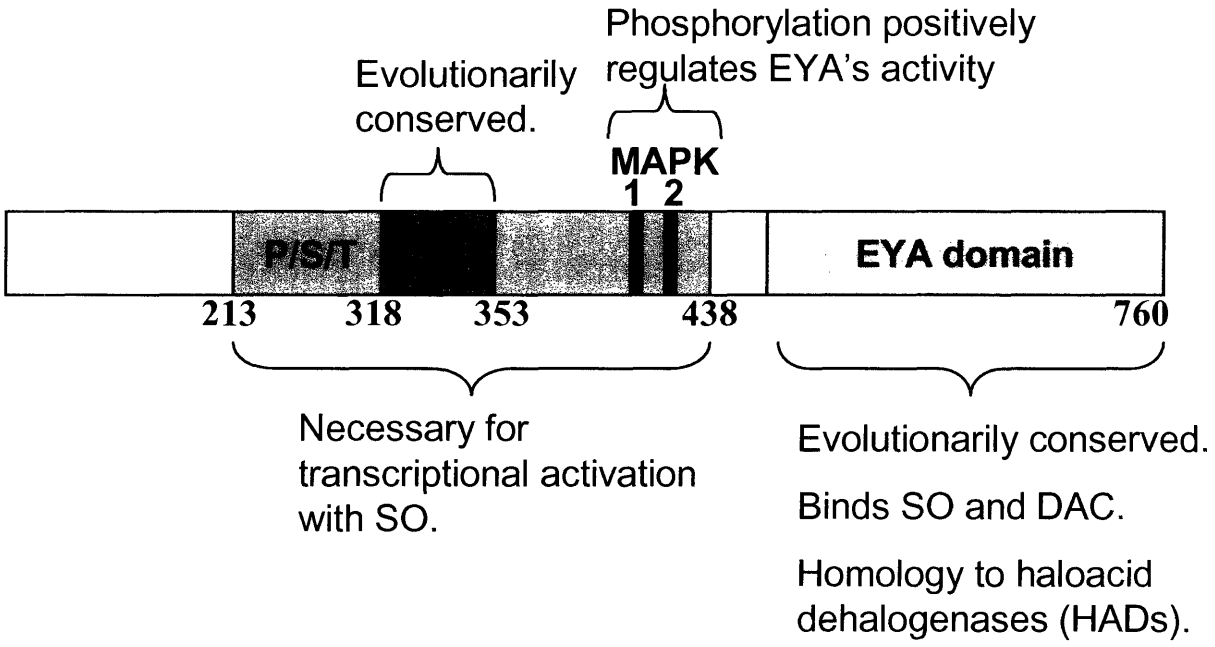


Figure 5-1

Schematic of the EYA protein. The known domains of EYA are demarcated, and the corresponding functions are marked.

(Tootle et al. 2003), suggesting that EYA may regulate itself, and its two activities, by dephosphorylation of tyrosine residues.

Tyrosine phosphorylation of nuclear factors is a poorly studied area (Chapter 1). Therefore, it is of interest both to understand the general mechanisms of regulating nuclear proteins by tyrosine phosphorylation and to specifically understand how EYA and its two functions are regulated by tyrosine phosphorylation. To begin to understand how tyrosine phosphorylation regulates EYA, I have attempted to determine where within the protein EYA is tyrosine phosphorylated, and which kinase/s is/are responsible for this phosphorylation.

Materials and Methods

Subcloning

The deletion construct $EYA^{D/D+\Delta ED2}$ and $EYA^{D/D+\Delta PST}$ were made exactly as described previously, except $EYA^{D/D}$ was used as the starting construct instead of EYA^{WT}.

ED D/D construct was made by PCR amplifying from pRMHa3 $Eya^{D/D}$, using the forward primer, EYA 1309-Sma (5' TGACCCGGGGTGGGTACCGCCGGCTCTGGG 3'), which contains a SmaI site at its 5' end and the reverse primer, EYA A2676Stop-Sal (5' TAATGTCGACTCATAAGAAGCCCATGTCGAGGGC 3'), which contains a SalI site at its 5' end. The PCR product was first digested with SmaI and then with SalI. The digested product was ligated into pBS flag that was SmaI/SalI digested. The resulting pBS flag-ED D/D was digested with SacI and SalI, and subsequently ligated into pRMHa3 similarly digested.

$EYA^{\Delta ED}$ construct was made by ligating the SmaI and SalI digested pRMHa3 Gal4BDB EYA N term and the similarly digested pRMHa3 flag-EYA^{WT}.

Protein biochemistry

Transient transfections, 5 μg/construct, were performed as previously described in Chapter 2 [Tootle, 2003 #622]. Immunoprecipitation and western blot analyses were performed as described in Chapter 4 [Tootle, 2003 #883], using 3ml of transfected cells/immunoprecipitation. Upstate rabbit anti-phosphotyrosine was used 1:400, Sigma rabbit anti-flag was used 1:5000, and Jackson Laboratories HRP conjugated goat anti-rabbit was used 1:5000.

Peptide analysis

Peptides were synthesized by Tuft University Core Facility, with an acetyl group at it amino-terminus and a $-NH_2$ group at its carboxy-terminus. Stock solutions of peptides were made with phosphate free water. Initial phosphatase assays were performed in duplicate at two substrate concentrations (50 and 100 μM) for one time point (10 min). Free phosphate was measured as previously described (Chapter 4) [Tootle, 2003 #883] except the dye, BIOMOL Green, was from Biomol Research Laboratories Inc. and used according to the product specifications. Kinetic analysis and calculations were performed as previously describe in Chapter 4 [Tootle, 2003 #883], using five substrate concentrations and five timepoints.

RNAi screen

RNAi primers were designed as previously described in Chapter 2 [Tootle, 2003 #622]. See primer list at end of chapter for RNAi primers used.

Approximately 1 x 10^6 cells of a stable S2 cell line expressing $EYA^{D/D}$ under the control of the metallothionine promoter was suspended in 1 ml of serum free Gibco Schneider's Insect Media, and 40 μg of dsRNA per kinase for 1.5 hrs. 2 ml of media +12.5% serum were then added, and $EYA^{D/D}$ expression was induced on Day 4, approximately 76 hours after the dsRNA

was added. Assays were performed on Day 5, approximately 94 hours after the dsRNA was added.

1 ml of cells was used for phosphotyrosine western blot analysis, see above. The remaining 2 ml of cells were used for RNA isolation, following the RNA STAT-60 protocol (TEL-TEST "B" Inc.). The RNA pellet was resuspended in 30 µl of DEPC H_2O. 15 µl was DNase treated (15 µl DEPC H_2O, 4 µl DNase, 5 µl DNase Buffer) for 2 hrs at 37°c, and then 1 µl 25 mM EDTA was added and it was heat inactivated for 10 minutes at 70°C. 2 µg of RNA were used per reverse transcriptase (RT) reaction. The RNA, dNTPs, primers and DEPC H_2O were incubated for 5 minutes at 70°C (concentrations according to kit used, either Ambion RetroScript or Promega Reverse Transcription System). The mixture was cooled on ice for 5 minutes, and then the following were added: RT PCR buffer, RNase Inhibitor, and reverse transcriptase. The total reaction volume was 20 µl. The reaction was performed according to the kit directions, except that reactions were allowed to proceed for 1 hour. Control reactions without the reverse transcriptase were performed to verify that the DNase treatment was complete.

Specific PCR reactions using 3 µl of the RT reaction were performed using the RNAi primers for the kinase, and control primers for Rps17, a ribosomal protein (Forward- 5' CGAACCAAGAC GGTGAAGAAG 3', reverse- 5' CCTGCAACTTG ATGGAGATACC). Conditions vary per primer set. PCR reactions were analyzed by running products on 3% gels.

Knock-down of multiple kinases at once was performed in the same manner except that 3 ml of cells were used for western analysis and 3 ml were used for RT analysis.

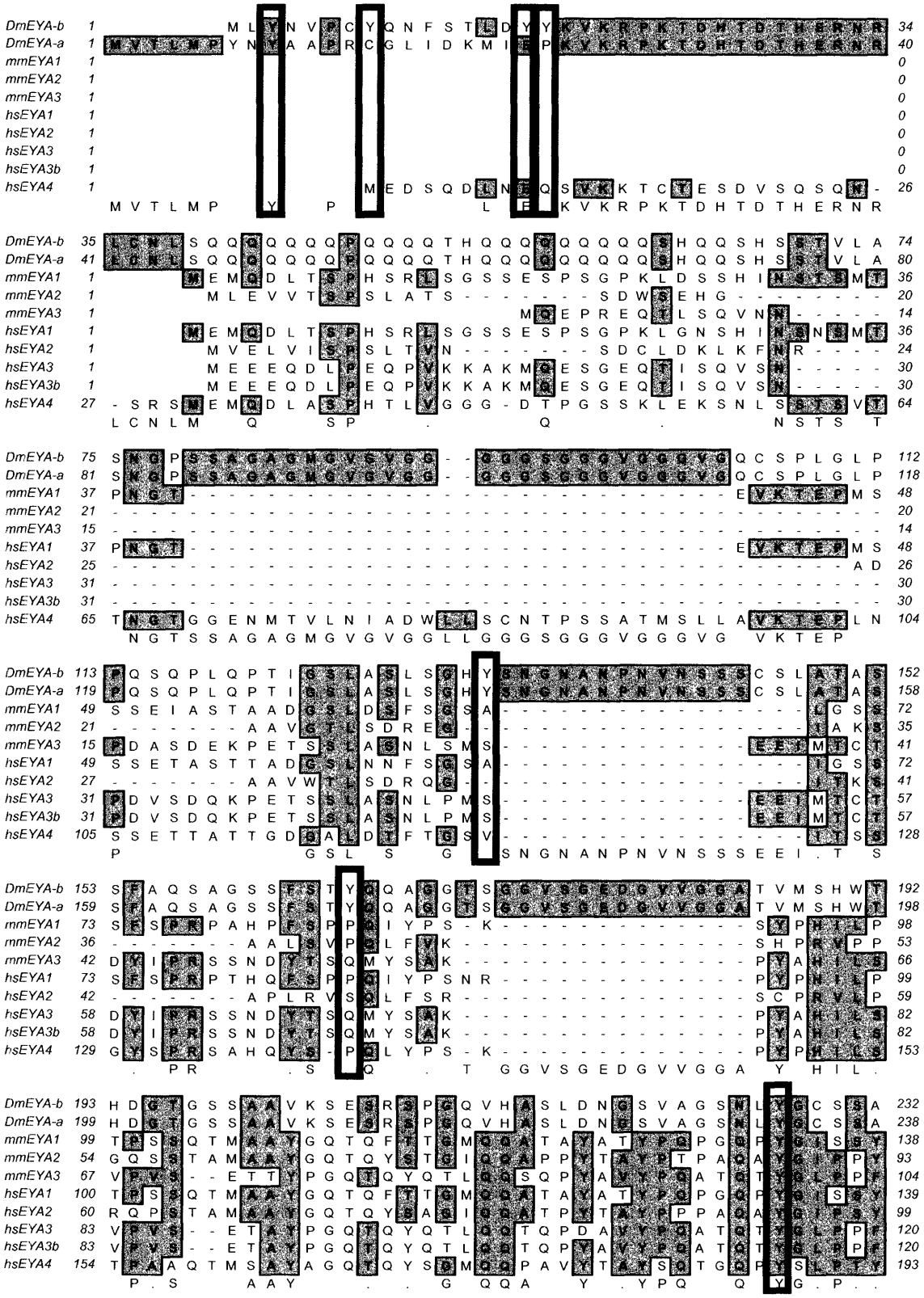
Results

The ED of EYA is likely to by tyrosine phosphorylated

It has been shown that EYA is tyrosine phosphorylated in *Drosophila* insect cultured cells. There are thirty-nine tyrosines within the EYA protein, eleven of these are conserved among all mouse and human homologs, while an additional seven are conserved among some members, three of which are tyrosine to phenylalanine substitutions in the non-conserved members (Figure 5-2). All of the conserved tyrosine residues are within domains of EYA that have been shown to play roles in both transcription and protein-protein interactions (Figure 5-1). The two most conserved domains of EYA, the ED and the ED2 contain eight and five conserved tyrosines respectively. The ED has been shown to have roles in protein-protein interactions, as it mediates binding to SO and DAC, in addition to possessing protein phosphatase activity. The ED2 resides within the PST region that has been shown to be required for transcriptional activation.

Deletion analysis was performed to determine which region of the EYA protein is tyrosine phosphorylated. Previous studies had shown that protein tyrosine phosphatase catalytic mutants exhibit a higher level of tyrosine phosphorylation than wild-type, presumably due to their significantly reduced phosphatase activity (Figure 4-5A). Therefore the analysis was performed using the mutant exhibiting the highest level of tyrosine phosphorylation, $EYA^{D/D}$. The various deletion constructs (Figure 5-3A) were transiently expressed in *Drosophila* insect culture cells, immunoprecipitated, and the level of tyrosine phosphorylation was analyzed by anti-phosphotyrosine western blots. The results definitively show that $EYA^{D/D+\Delta ED2}$ is tyrosine phosphorylated (Figure 5-3B lane 2, and 5C lanes 5-6), while $EYA^{\Delta ED}$ and ED only+D/D are not phosphorylated (Figure 5-3C lanes 9-10 and 7-8, respectively). A background band of the same size complicates the analysis of $EYA^{D/D+\Delta PST}$. Initial experiments clearly indicate that

Figure 5-2


DmEYA-b
DmEYA-a
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mmEYA2
mmEYA3
hsEYA1
hsEYA2
hsEYA3
hsEYA3b
hsEYA4

Figure 5-2

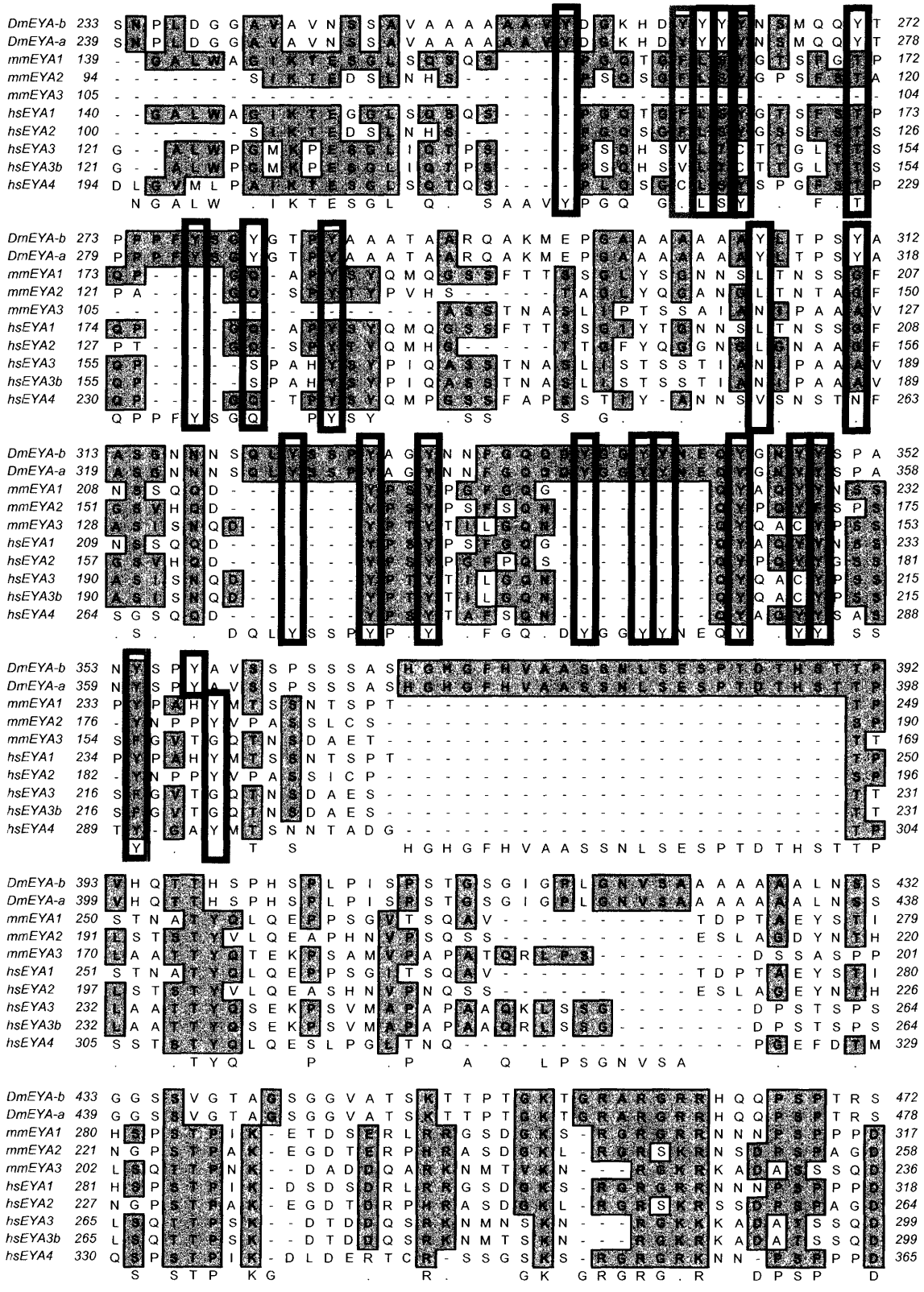
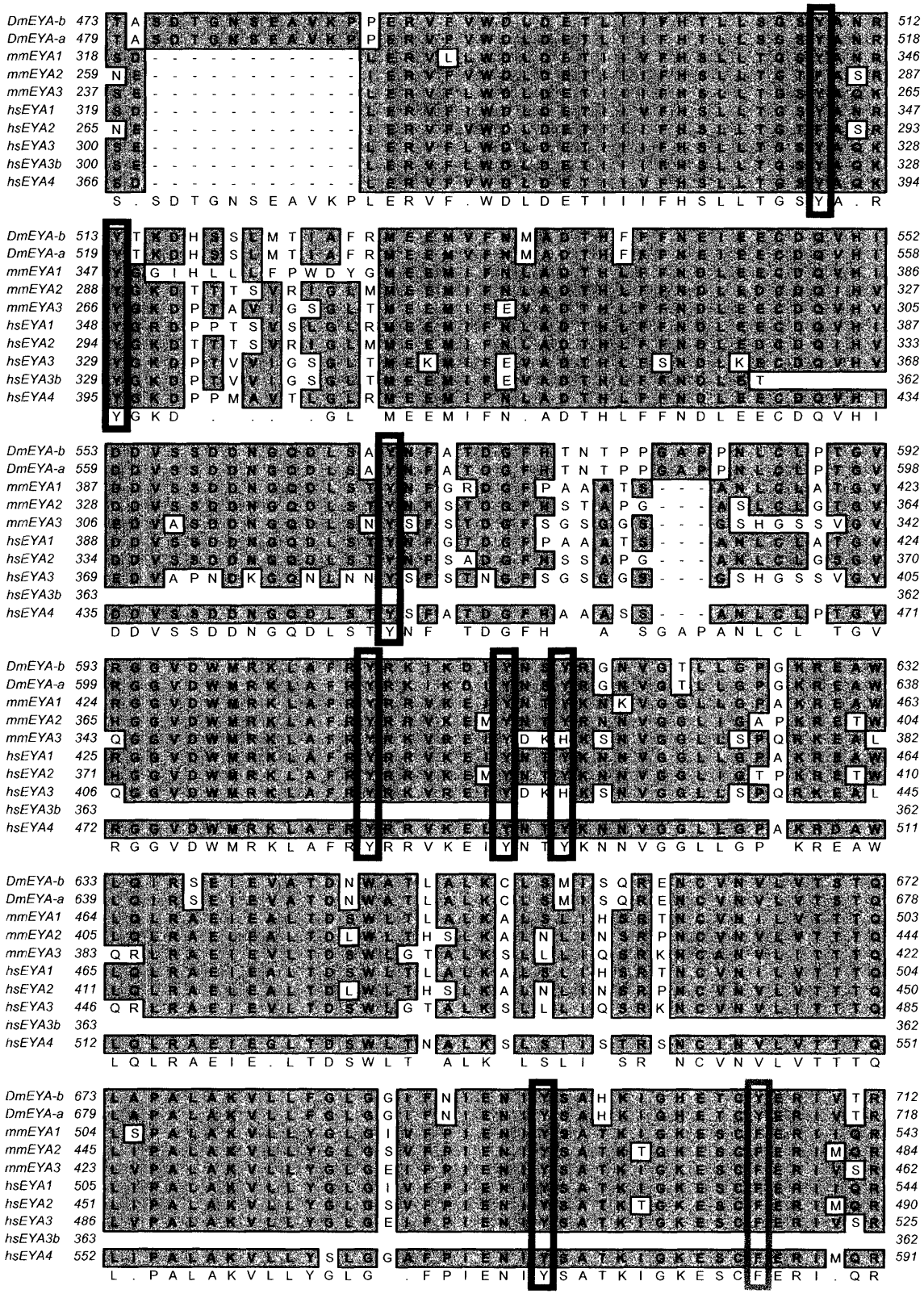

DmEYA-b
DmEYA-a
mmEYA1
mmEYA2
mmEYA3
hsEYA1
hsEYA2
hsEYA3
hsEYA3b
hsEYA4

Figure 5-2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DmEYA-a 479 518
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mmEYA2 259 287
mmEYA3 237 265
hsEYA1 319 347
hsEYA2 265 293
hsEYA3 300 328
hsEYA3b 300 328
hsEYA4 366 394
S . S D T G N S E A V K P L E R V F . W D L D E T I I F H S L L T G S Y A . R
DmEYA-b 513 552
DmEYA-a 519 558
mmEYA1 347 386
mmEYA2 288 327
mmEYA3 266 305
hsEYA1 348 387
hsEYA2 294 333
hsEYA3 329 368
hsEYA3b 329 362
hsEYA4 395 434
Y G K D . . . G L M E E M I F N . A D T H L F F N D L E E C D Q V H I
DmEYA-b 553 592
DmEYA-a 559 598
mmEYA1 387 423
mmEYA2 328 364
mmEYA3 306 342
hsEYA1 388 424
hsEYA2 334 370
hsEYA3 369 405
hsEYA3b 363 362
hsEYA4 435 471
D D V S S D D N G Q D L S T Y N F T D G F H A A S G A P A N L C L T G V
DmEYA-b 593 632
DmEYA-a 599 638
mmEYA1 424 463
mmEYA2 365 404
mmEYA3 343 382
hsEYA1 425 464
hsEYA2 371 410
hsEYA3 406 445
hsEYA3b 363 362
hsEYA4 472 511
R G G V D W M R K L A F R Y R R V K E I Y N T Y K N N V G G L L G P A K R E A W
DmEYA-b 633 672
DmEYA-a 639 678
mmEYA1 464 503
mmEYA2 405 444
mmEYA3 383 422
hsEYA1 465 504
hsEYA2 411 450
hsEYA3 446 485
hsEYA3b 363 362
hsEYA4 512 551
L Q L R A E I E . L T D S W L T A L K L S L I S R N C V N V L V T T T Q
DmEYA-b 673 712
DmEYA-a 679 718
mmEYA1 504 543
mmEYA2 445 484
mmEYA3 423 462
hsEYA1 505 544
hsEYA2 451 490
hsEYA3 486 525
hsEYA3b 363 362
hsEYA4 552 591
L . P A L A K V L L Y G L G . F P I E N I Y S A T K I G K E S C F E R I . Q R

Figure 5-2

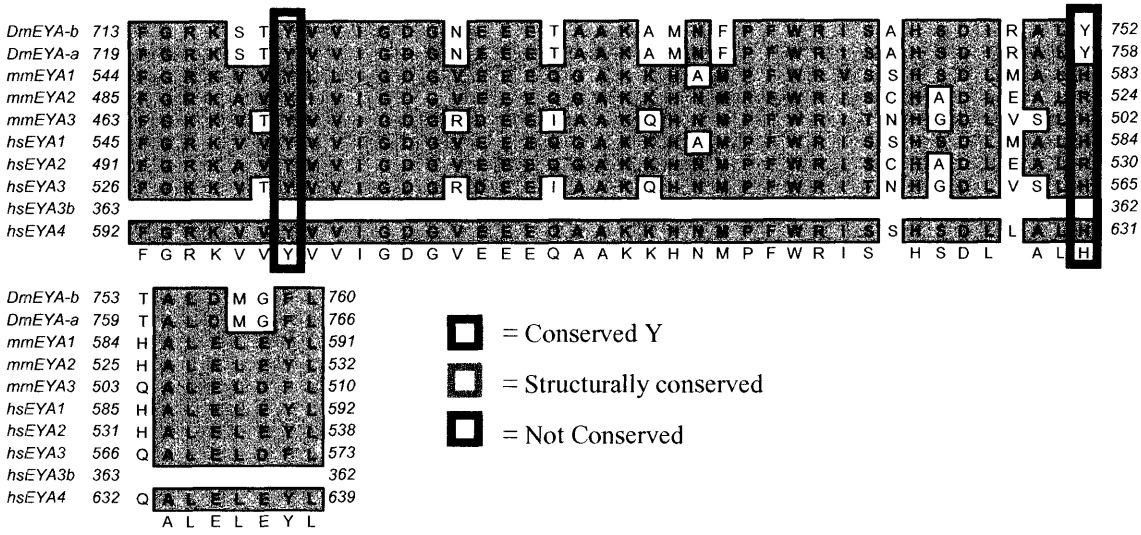


Figure 5-2

ClustalX alignment of *Drosophila,* mouse, and human EYA proteins. Conserved tyrosines are boxed in red. Structurally conserved, meaning Y/F, are boxed in green, while non-conserved tyrosines are boxed in blue.

Figure 5-3

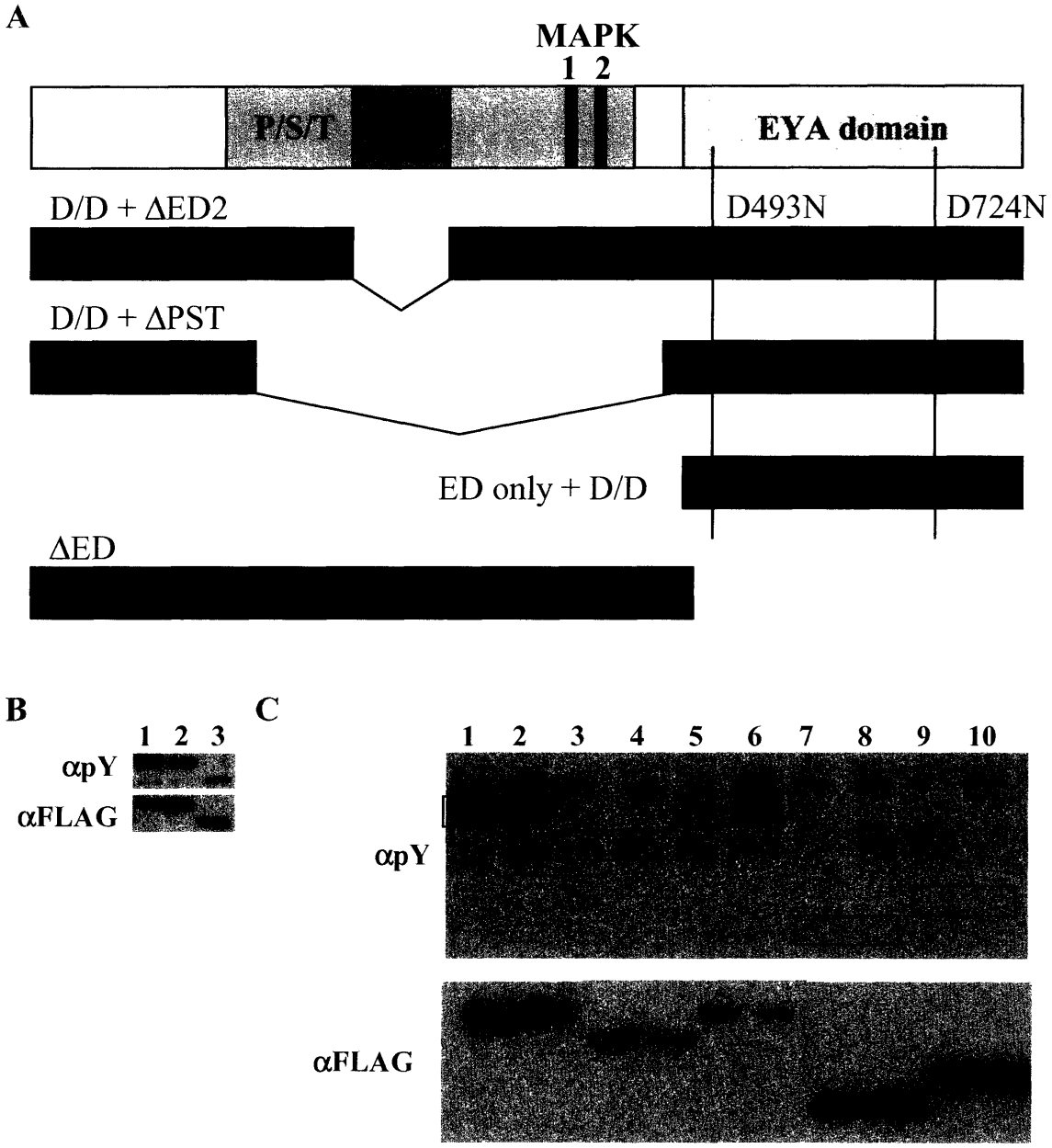

A
MAPK
1 2
P/S/T
EYA domain
D/D + ΔED2
D493N
D724N
D/D + ΔPST
ED only + D/D
ΔED
B
1 2 3
αpY
αFLAG
C
1 2 3 4 5 6 7 8 9 10
αpY
αFLAG

Figure 5-3

EYA is tyrosine phosphorylated within its ED. A) Schematic of the deletion constructs used to determine where EYA is tyrosine phosphorylated. B and C Western blot analysis, with the top row showing the level of tyrosine phosphorylation (Upstate Rabbit anti-phosphotyrosine 1:400) and the bottom showing the protein expression level (Sigma Rabbit anti-flag 1:5000). B) Lane 1 is $EYA^{D/D}$ Lane 2 is $EYA^{D/D+\Delta ED2}$, Lane 3 is $EYA^{D/D+\Delta PST}$. All three EYA constructs are tyrosine phosphorylated. C) Lane 1-2 $EYA^{D/D}$, Lane 3-4 $EYA^{D/D+\Delta PST}$, Lane 5-6 $EYA^{D/D+\Delta ED2}$, Lane 7-8 ED D/D, Lane 9-10 $EYA^{\Delta ED}$. As before, B, $EYA^{D/D}$, $EYA^{D/D+\Delta ED2}$, and $EYA^{D/D+\Delta PST}$ are tyrosine phosphorylated, although the level of tyrosine phosphorylation of $EYA^{D/D+\Delta PST}$ appears reduced (see text for discussion). Neither $EYA^{\Delta ED}$ or the ED D/D are tyrosine phosphorylated.

$EYA^{D/D+\Delta PST}$ is tyrosine phosphorylated (Figure 5-3B lane 3), although possibly to a lesser extent, while in later experiments it is difficult to determine (Figure 5-3C lanes 3-4). The PST domain contains two completely and three partially conserved tyrosines outside of the ED2. All constructs tested expressed appropriately sized proteins (Figure 5-3B,C, anti-flag).

These data suggest that EYA is likely to be tyrosine phosphorylated with the ED. While the lack of phosphorylation of ED only+D/D appears to disagree with this, it could be due to severe alterations in protein structure resulting in destabilization of the protein, as double the amount of expression plasmid transfected was required to obtain similar levels of protein expression (data not shown) and in vivo analysis of ED alone constructs reveal they are non-functional (I. Rebay, personal communication).

One other caveat to the above deletion analysis is that the subcellular localization of these constructs could affect tyrosine phosphorylation even if the normally phosphorylated tyrosine residues are retained. Analysis of the localization of the deletion constructs revealed that like $EYA^{D/D}$, $EYA^{D/D+\Delta ED2}$ is strictly nuclear (Figure 5-4A,B), while $EYA^{D/D+\Delta PST}$, $EYA^{\Delta ED}$, and ED only+D/D are both nuclear and cytoplasmic (Figure 5-4C-E). Thus, all of the constructs exhibiting no or reduced tyrosine phosphorylation have altered subcellular localizations. However, a significant portion of all the deletion constructs localize properly to the nucleus, suggesting that some tyrosine phosphorylation should be able to occur. This altered subcellular localization might be the reason for reduced phosphotyrosine signal of $EYA^{D/D+\Delta PST}$, while it remains possible that this reduced signal is due to deletion of some of the tyrosines which are phosphorylated. As $EYA^{\Delta ED}$ localizes to the nucleus but it is not tyrosine phosphorylated, it seems likely that EYA is indeed tyrosine phosphorylated within the ED.

Figure 5-4

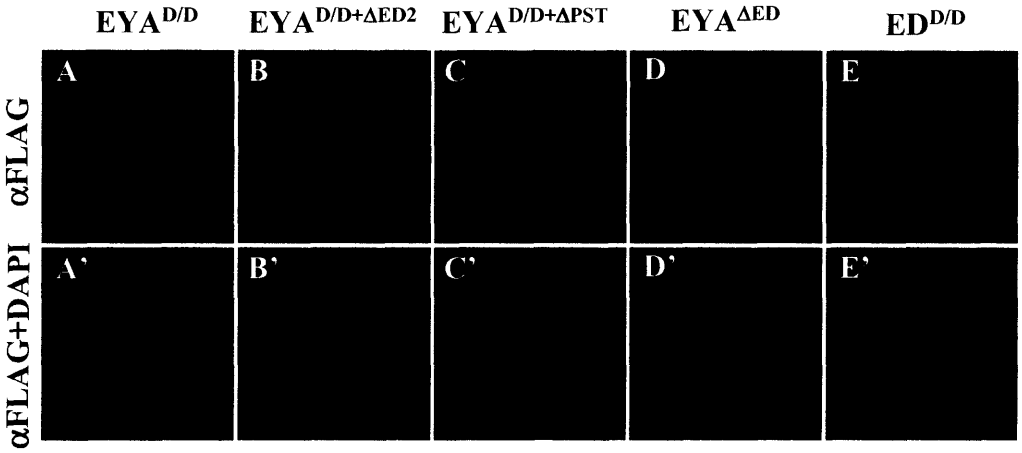


Figure 5-4

Like $EYA^{D/D}$, $EYA^{D/D+\Delta ED2}$ is strictly nuclear, while the other deletion constructs are nuclear and cytoplasmic. The top row is EYA localization by anti-flag (1:10000; CY3 secondary 1:2000), and bottom row is DAPI (1:10000) overlayed on top of the anti-flag signal. (A, A') $EYA^{D/D}$. (B, B') $EYA^{D/D+\Delta ED2}$. (C, C') $EYA^{D/D+\Delta PST}$. (D, D') $EYA^{\Delta ED}$. (E, E') ED D/D. (A-B') $EYA^{D/D}$ and $EYA^{D/D+\Delta ED2}$ are strictly nuclear. (C-E') $EYA^{D/D+\Delta PST}$, $EYA^{\Delta ED}$, and ED D/D are nuclear and cytoplasmic.

MmEYA3 is tyrosine phosphorylated

There are eighteen tyrosines that are at least partially conserved among the mouse and human homologs, specifically eight out of ten tyrosines within the ED of EYA are conserved. To verify that the phosphorylated tyrosine(s) is/are likely to be conserved, Murine EYA3 (MmEYA3) was expressed in *Drosophila* S2 insect cultured cells and analyzed by phosphotyrosine western blot analysis. Indeed MmEYA3 is tyrosine phosphorylated (Figure 5-5A) and thus it is likely that a conserved tyrosine(s) is phosphorylated. Tyrosine phosphorylation and dephosphorylation could be a conserved mechanism of regulating the functions of EYA.

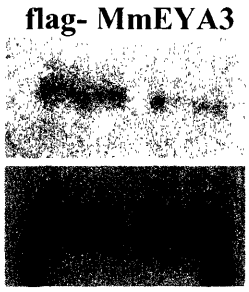
Determining which tyrosines are phosphorylated: Initial Phosphopeptide analysis

The sites of tyrosine phosphorylation within EYA are also likely to be sites of dephosphorylation by EYA, as EYA can dephosphorylate itself (Chapter 4) (Tootle et al. 2003). Therefore, we reasoned that a quick way to assess which tyrosine residues are likely to be phosphorylated was to synthesize phosphotyrosine peptides and analyze which are the best substrates for EYA by in vitro phosphatase assays.

We initially focused on tyrosines within the ED, as the above deletion analysis suggests EYA is tyrosine phosphorylated within the ED. Three phosphopeptides were synthesized and tested. Peptides 1 and 3 are conserved tyrosines, while Peptide 2 is a nonconserved tyrosine whose sequence resembles the known EYA substrate Src Substrate II (Figure 5-5B). Structural modeling predicts that these three tyrosines are surface exposed, and thus could be targets of phosphorylation (R. Latek, personal communication). An initial in vitro phosphatase trial assay was performed using one substrate concentration. From this analysis it was apparent that Peptide 3 could serve as a substrate for GST-MmEYA3. Kinetics were then performed with Peptide 3,

Figure 5-5

A



B

	Peptide 1	Peptide 2	Peptide 3
DmEYA	IENIYSAHK	HETCYERIV	RKSTYVVIG
MmEYA1	IENIYSATK	KESCFERII	RKVVYLLIG
MmEYA2	IENIYSATK	KESCFERIM	RKAVYIVIG
MmEYA3	IENIYSATK	KESCFERIV	KKVTYVVIG
HsEYA1	IENIYSATK	KESCFERII	RKVVYVVIG
HsEYA2	IENIYSATK	KESCFERIM	RKAVYVVIG
HsEYA3	IENIYSATK	KESCFERIV	KKVTYVVIG
HsEYA4	IENIYSATK	KESCFERIM	RKVVYVVIG

C

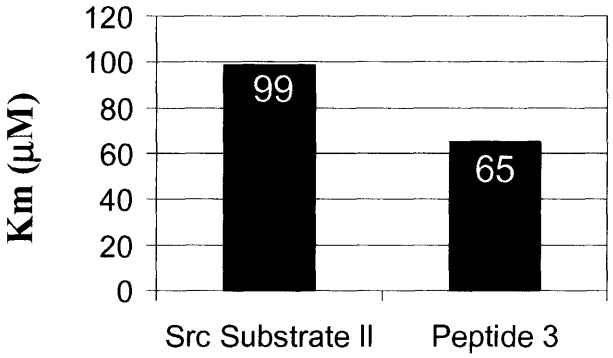


Figure 5-5

Mouse EYA3 is tyrosine phosphorylated and Peptide 3 (Y719) is an excellent substrate for GST-mEYA3. (A) Western blot analysis, with the top row showing the level of tyrosine phosphorylation (Upstate Rabbit anti-phosphotyrosine 1:400) and the bottom showing the protein expression level (Sigma Rabbit anti-flag 1:5000). MmEYA3 is tyrosine phosphorylated in *Drosophila* cultured cells. (B) Alignment of the three *Drosophila* EYA phosphopeptides synthesized with the corresponding mouse and human homolog sequences. (C) Graph of the Km of Peptide 3 in comparison to the known Km with Src Substrate II phosphopeptide. Peptide 3 with a Km of 65 μM is the best substrate for EYA identified thus far.

revealing the Km=65μM (Figure 5-5C). The Km with Src Substrate II was 99 μM (Figure 4-2D) (Tootle et al. 2003). Therefore, Peptide 3, a conserved tyrosine from the EYA Domain, is the best substrate for EYA identified thus far.

Y719, the phosphotyrosine in Peptide 3, is an excellent candidate for a site of phosphorylation within EYA. For discussion on how further peptide analysis, and subsequent experiments can be utilized to determine where EYA is tyrosine phosphorylated see Chapter 6.

RNAi screen to determine the tyrosine kinase which phosphorylates EYA

EYA is tyrosine phosphorylated in *Drosophila* insect cultured cells. There are forty-one tyrosine kinases in *Drosophila*, eighteen of which are non-membrane bound kinases. To elucidate which kinase or kinases are responsible for tyrosine phosphorylating EYA an RNAi screen was initiated utilizing a stable cell line expressing $EYA^{D/D}$, a catalytically inactive mutant exhibiting a high level of tyrosine phosphorylation. These cells are soaked in dsRNA to a particular kinase, and then assayed by RT-PCR to verify knock-down of the kinase and anti-phosphotyrosine western blot analysis to determine whether loss of the kinase reduces the level of tyrosine phosphorylation of EYA (Figure 5-6).

During the first attempt at the screen cells were soaked in dsRNA to one kinase at a time, four days were allowed for knock-down and then cells were assayed. No repeatable difference in the level of tyrosine phosphorylation of EYA was seen (data not shown). However, RT-PCR technical difficulties occurred early in the screening process, and it is likely that expression of the majority of the kinases screened was not knocked-down. It is also possible that loss of one kinase alone is not sufficient to reduce the tyrosine phosphorylation of EYA enough to visualize the difference.

Another caveat to the screen is that the cells were treated with pervanadate to increase the level of tyrosine phosphorylation and there is some thought that such a treatment also stimulates kinases, possibly leading to aberrant phosphorylation. By utilizing more cells per immunoprecipitation it is possible to visualize a phosphotyrosine signal for EYA in the absence of pervanadate (Figure 5-7).

The plan for the second attempt at the screen was to knock down multiple kinases at once and to not treat the cells with pervanadate. To narrow the number of kinases to test, I first determined which tyrosine kinases are expressed in *Drosophila* insect cultured cells via RT-PCR. I found that only twenty-two of the forty-one kinases are definitely expressed in the cells, while an additional five may be expressed (Table 5-1). At attempt was made to knock-down the expression of four kinases at once in the absence of pervanadate treatment, while extending the incubation time. However, the RNAi was not successful as all the kinases were still expressed. The experiment was useful in that it showed that the phosphotyrosine signal of EYA in the absence of pervanadate is not robust enough to perform the screen with (data not shown). Although this experimental approach has not been successful to date I believe that once the technical difficulties are overcome it should elucidate the kinase(s) responsible. The steps that should be taken to get the system working are as follows: 1) the stable cell line should be maintained at a particular density, $2\text{-}3x10^6$; 2) particular care should be taken to perform RNAi on $1x10^6$ cells suspended in 1 ml of serum free media for 1.5 hrs; 3) the experiment should be done as a time course, with the cells being grown for 3, 4, 5, and 6 days after addition of dsRNA before analysis. Additionally, it is probably best to perform that screen by treating the cells with pervanadate and knocking down the expression of one kinase at a time. By using a time course one can make sure enough time is allowed for knock-down of a specific kinase, while lethality

Figure 5-6

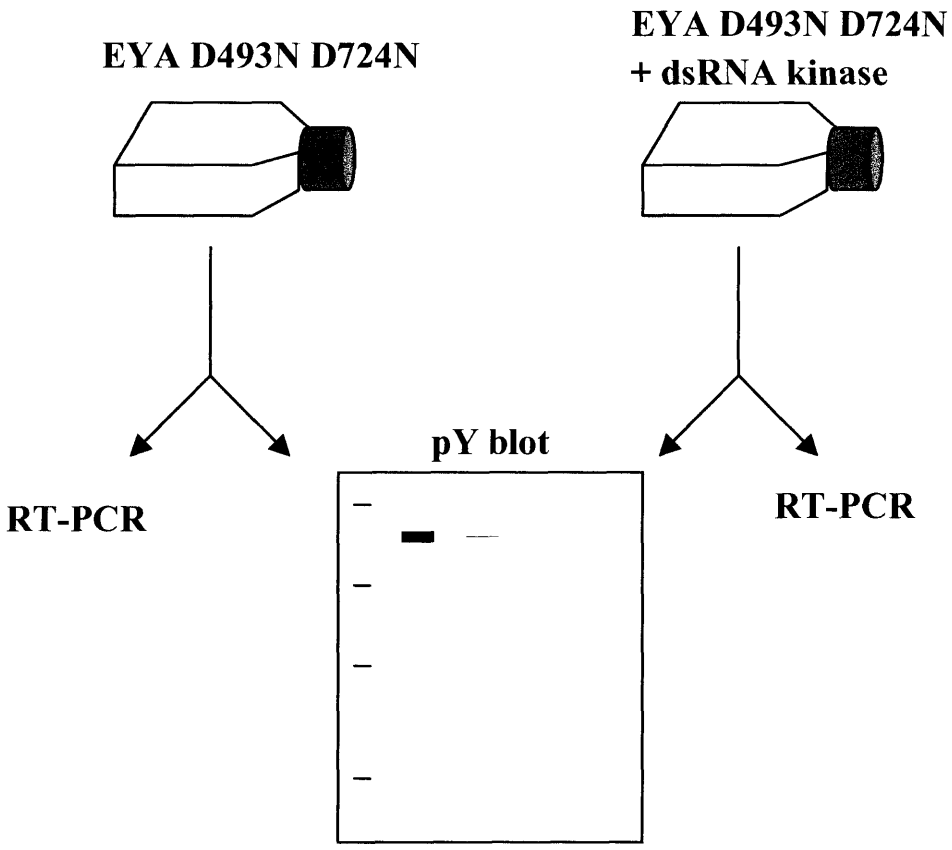


Figure 5-6

Schematic of the cultured cell based RNAi screen to identify the kinase(s) responsible for the phosphorylation of EYA.

Figure 5-7

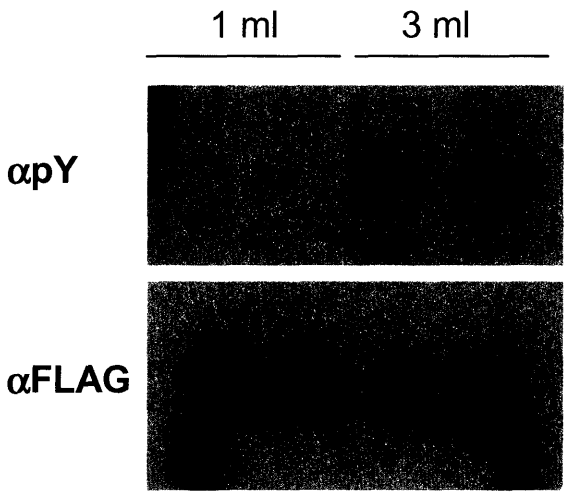


Figure 5-7

EYA is tyrosine phosphorylated in *Drosophila* insect cultured cells in the absence of pervanadate treatment. Western blot analysis, with the top row showing the level of tyrosine phosphorylation (Upstate Rabbit anti-phosphotyrosine 1:400) and the bottom showing the protein expression level (Sigma Rabbit anti-flag 1:5000).

Table 5-1

Non-membrane	Expressed	Membrane associated	Expressed
Abl	Yes	Alk	No
Ack	Yes	Btk29A	No
CG10673	Yes	Btl	Yes
CG3277	No	Cad96Ca	Maybe
Csk	Yes	CG10743	No
Doa	Yes	CG31640	Maybe
Fak56D	Yes	Ddr	Maybe
Fps85D	Maybe	Dnt	Yes
Hop	Maybe	Drl	No
Mnb	No	Drl-2	No
Myt1	Yes	Egfr	Yes
Pr2	Yes	Eph	Yes
Shark	Yes	Htl	No
Slpr	No	InR	Yes
Src42A	No	Nrk	Yes
Src64B	Maybe	Otk	Yes
Twf	Yes	Pvr	Yes
Wee	Yes	Ret	No
		Ror	Maybe
		Sev	No
		Stam	Yes
		Tie	Maybe
		Tor	No

Tyrosine kinases in *Drosophila.* Table shows whether kinases are expressed in *Drosophila* S2 cultured cells.

due to knock-down can be avoided. Performing the screen in this manner should determine which kinase(s) tyrosine phosphorylates/phosphorylate EYA.

Concluding remarks

The above experiments suggest that EYA is likely to be tyrosine phosphorylated within the ED, and a putative site of phosphorylation is Y719. Further work in this area, as discussed in Chapter 6, should definitively determine the tyrosine residues within EYA that are tyrosine phosphorylated. From there we can begin to study how phosphorylation regulates the two functions of EYA, as a transcriptional activator and as a protein tyrosine phosphatase.

Successful completion of the RNAi screen we have begun will determine which kinase or kinases tyrosine phosphorylate EYA. This too, will be a jumping off point from which one can begin to understand how tyrosine phosphorylation regulates EYA.

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RNAi primers for kinase screen
Gene:CG3277
CG3277 F
5' GAATTAATACGACTCACTATAGGGAGAagtgctggcttgaggagccc 3'
CG3277 REV
5' GAATTAATACGACTCACTATAGGGAGACGTTTAAATCTCACAGCACA 3'

Gene:Abl tyrosine kinase
Abl F
5' GAATTAATACGACTCACTATAGGGAGAGTGATAGCCGCTCCGGTCAC 3'
Abl Rev
5' GAATTAATACGACTCACTATAGGGAGAGAGTTGCTCCTGATCATTTG 3'

Gene: C-ter Src kinase (CSK)
Csk F
5' GAATTAATACGACTCACTATAGGGAGAATGAACAGCCACGCGACTGC 3'
Csk Rev
5' GAATTAATACGACTCACTATAGGGAGATGCTGGTTCGTCGTCTGTTG 3'

Gene: Hopscotch
Hop F
5' GAATTAATACGACTCACTATAGGGAGACTGTTGACAACTGAATGAAA 3'
Hop Rev
5' GAATTAATACGACTCACTATAGGGAGAGTTCAGTGTTTATGCGTGGT 3'

Gene: Minibrain
Mnb F
5' GAATTAATACGACTCACTATAGGGAGATGTACTCCTTGAATCCCCAC 3'
Mnb Rev
5' GAATTAATACGACTCACTATAGGGAGAGTCTTGGGGTCAAAGTCGAG 3'

Gene:Wee
Wee F
5' GAATTAATACGACTCACTATAGGGAGAGTGGTCAGCTGGACAGCTACA 3'
Wee Rev
5' GAATTAATACGACTCACTATAGGGAGAGTGTCCTGGGTGGGCAGTTT 3'

Gene: Src42a
Src42a F
5' GAATTAATACGACTCACTATAGGGAGAATGGGTAACTGCCTCACCAC 3'
Src42a Rev
5' GAATTAATACGACTCACTATAGGGAGACGTGTCATTCAGTATCTCCA 3'

Gene: FAK-like tyrosine kinase/PR2
Pr2 F

5' GAATTAATACGACTCACTATAGGGAGAGGTGAAATCTATGACCAACT 3'
Pr2 Rev
5' GAATTAATACGACTCACTATAGGGAGAGTCTGAAGAGAGTCAGGACT 3'

Gene: Shark
Shark F
5' GAATTAATACGACTCACTATAGGGAGACGGTTCCGCCACAGCCCAAG
Shark Rev
5' GAATTAATACGACTCACTATAGGGAGATGTTCCTGGGCACGTTGTAC

Gene: Fak56d
Fak56d F
5' GAATTAATACGACTCACTATAGGGAGATGCTCGGTGTAAAGCCTTTCC 3'
Fak56d Rev
5' GAATTAATACGACTCACTATAGGGAGACCGCGGGATTTTGTGCAATA 3'

Gene: Ack
Ack F
5' GAATTAATACGACTCACTATAGGGAGACGACACAATCGCCATTATCG 3'
Ack Rev
5' GAATTAATACGACTCACTATAGGGAGAAGGAACTATCGCCTGCTCCC 3'

Gene: Src64b
NewSrc64b For
5' GAATTAATACGACTCACTATAGGGAGACAATGTGCAGACGGTCGGTG 3'
NewSrc64b Rev
5' GAATTAATACGACTCACTATAGGGAGAGAGCTTTTCGTGGCGGTTTT 3'

Gene: Myt1
Myt1 F
5' GAATTAATACGACTCACTATAGGGAGACGCGCCGTTTGGGGAAGAAT 3'
Myt1 Rev
5' GAATTAATACGACTCACTATAGGGAGAGCGGAAGATAGGGCGGAACA 3'

Gene: Slpr
Slpr F
5' GAATTAATACGACTCACTATAGGGAGACAACAACAGCATCTCCGCCAA 3'
Slpr Rev
5' GAATTAATACGACTCACTATAGGGAGATAACTGGTTGGGCAGTGCAA 3'

Gene: Fps85D
Fps85D F
5' GAATTAATACGACTCACTATAGGGAGAGCCTGGACAACAGCCACTAA 3'
Fps85D Rev
5' GAATTAATACGACTCACTATAGGGAGAGACAAACGTAGGGATTGCAT 3'

Gene: Twinfilin- Twf
Twf F
5' GAATTAATACGACTCACTATAGGGAGACTATCGCCGGCACAAGCAGGAT 3'
Twf Rev
5' GAATTAATACGACTCACTATAGGGAGACGCGAGCTCGTCGAGAAAAG 3'

Gene: Darkener of apricot –Doa
Doa F
5' GAATTAATACGACTCACTATAGGGAGATGTGAGCTCTTCAGTCTGAT 3'
Doa Rev
5' GAATTAATACGACTCACTATAGGGAGAGCAACATGTGCTCGAAGTAT 3'

Gene: CG10673
CG10673 F
5' GAATTAATACGACTCACTATAGGGAGAGAAATCCTGAAACAAGGCGC
CG10673 Rev
5' GAATTAATACGACTCACTATAGGGAGAAGATCCACACCCTTGTCCTC

Gene: Eph
Eph F
5' GAATTAATACGACTCACTATAGGGAGACTTGTCATGCAAGGAAAC ATTTAG 3
Eph Rev
5' GAATTAATACGACTCACTATAGGGAGATGTAATTCGGCTCATAGCCA 3'

Gene: CG31640
CG31640 F
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CG31640 Rev
5' GAATTAATACGACTCACTATAGGGAGAAAGTTGTAGGTCCTGGCGCG 3'

Gene: Ddr
Ddr F
5' GAATTAATACGACTCACTATAGGGAGAAGGACTATGCGGTGCCTCAC 3'
Ddr Rev
5' GAATTAATACGACTCACTATAGGGAGACGCTCCTCGTTAATGTCGGC 3'

Gene: Pvr
Pvr F
5' GAATTAATACGACTCACTATAGGGAGATTCTAACCAGAACGTACAAC
Pvr Rev
5' GAATTAATACGACTCACTATAGGGAGAATAGGAGCGTACTGTGCACT

Gene: Btk29a
Btk20a F
5' GAATTAATACGACTCACTATAGGGAGACCGCTTTCCGTGTGCTCATG 3'
Btk29a Rev

5' GAATTAATACGACTCACTATAGGGAGATGTCAGTTGTTGCGCTTCGT 3'

Gene: Ror
Ror F
5' GAATTAATACGACTCACTATAGGGAGAGGATATGAACGCAAATTGCC 3'
Ror Rev
5' GAATTAATACGACTCACTATAGGGAGACGTTGGCCACTCCTCTATAT 3'

Gene: Stam
Stam F
5' GAATTAATACGACTCACTATAGGGAGAAGTGCTGAAGAACTGGGCTG 3'
Stam Rev
5' GAATTAATACGACTCACTATAGGGAGACACATGAATGATCTCGCCGG 3'

Gene: Drl
Drl F
5' GAATTAATACGACTCACTATAGGGAGAGACAGTGCGCACGAGGAGTA 3'
Drl Rev
5' GAATTAATACGACTCACTATAGGGAGAATATGGTGGGCAGTCTCTGG 3'

Gene: Dnt
Dnt F
5' GAATTAATACGACTCACTATAGGGAGAGAAGATCTGTCTTATGAATGAC 3'
Dnt Rev
5' GAATTAATACGACTCACTATAGGGAGAATAAACTCGTCCAAACGTTC 3'

Gene: Ret
NewRet For
5' GAATTAATACGACTCACTATAGGGAGATAGTGTTTCCCAGAGTTCTA 3'
NewRet Rev
5' GAATTAATACGACTCACTATAGGGAGAGTTTAGCTTATGGCGTAAAT 3'

Gene: Torso
Torso F
5' GAATTAATACGACTCACTATAGGGAGAATGCTTATTTTCTACGCGAAG 3'
Torso Rev
5' GAATTAATACGACTCACTATAGGGAGAACAGCATCCACCCGGATTAT 3'

Gene:Off track-Otk
Otk F
5' GAATTAATACGACTCACTATAGGGAGAGCTATGTCTACCAGTCCAGT 3'
Otk Rev
5' GAATTAATACGACTCACTATAGGGAGAATAATGCTGGCAAAGCTTAG 3'

Gene:Drl-2
Drl-2 F

5' GAATTAATACGACTCACTATAGGGAGAATGGCCAGTCACGGGGAGAA 3'
Drl-2 Rev
5' GAATTAATACGACTCACTATAGGGAGATGGTGATGTGCAGCCCAAAC 3'

Gene: Nrk
Nrk F
5' GAATTAATACGACTCACTATAGGGAGAGTGGACCCCAATGCTGTCGA 3'
Nrk Rev
5' GAATTAATACGACTCACTATAGGGAGAACTTCTCCAGGCGTGCATTC 3'

Gene: Alk
Alk F
5' GAATTAATACGACTCACTATAGGGAGATGGTGGTGGTGCTGGCCA TCCTAT 3'
Alk Rev
5' GAATTAATACGACTCACTATAGGGAGAATCCGCAGATCCTAGGCCCTGA 3'

Gene: EGFR
EGFR F
5' GAATTAATACGACTCACTATAGGGAGAAGCACCTGGTCCTAGTCACA 3'
EGFR Rev
5' GAATTAATACGACTCACTATAGGGAGAACTCGTGATCGGAGCTCGTT 3'

Gene: Tie
Tie F
5' GAATTAATACGACTCACTATAGGGAGAATGTGTGTGTCGGTCTGTGC 3'
Tie Rev
5' GAATTAATACGACTCACTATAGGGAGAGGCTGAAAATTTCCGCTGAA 3'

Gene: CG10743
CG10743 F
5' GAATTAATACGACTCACTATAGGGAGAAACATTAATCAGAAGGCCTT 3'
CG10743 Rev
5' GAATTAATACGACTCACTATAGGGAGATTTCATTCTCACCGAACAAC 3'

Gene: Btl
Btl F
5' GAATTAATACGACTCACTATAGGGAGAGGCAAAAGTGCCGATCACGCTG 3'
Btl Rev
5' GAATTAATACGACTCACTATAGGGAGACGACTCTCATTCAGGGGCTG 3'

Gene: Htl
Htl F
5' GAATTAATACGACTCACTATAGGGAGATGGAGTTGGCGAGCCAGTCA 3'
Htl Rev
5' GAATTAATACGACTCACTATAGGGAGAGCCATTGCGATACCATGTGA 3'

Gene: InR
InR F
5' GAATTAATACGACTCACTATAGGGAGAATGTTCAATATGCCACGGGG 3'
InR Rev
5' GAATTAATACGACTCACTATAGGGAGAAGCAGCGATTGCAATGTTCG 3'

Gene: Cad96Ca
Cad96Ca F
5' GAATTAATACGACTCACTATAGGGAGATATCCACCGGTGCCCCAGAA 3'
Cad96Ca Rev
5' GAATTAATACGACTCACTATAGGGAGATTAGCGTCCTGCTGATGGCG 3'

Gene: Sev
Sev F
5' GAATTAATACGACTCACTATAGGGAGAGTTTTGGCAACAAAATGTAGA CCAC 3'
Sev Rev
5' GAATTAATACGACTCACTATAGGGAGATTGGTCTTTGGAGACGGGTT

Chapter 6

Discussion and Future Directions

Tina Tootle

Eyes absent, EYA, is one of a small but growing number of transcription factors possessing enzymatic activity, functioning as both a transcriptional coactivator with Sine oculis (SO) (Ohto et al. 1999; Silver et al. 2003) and a protein phosphatase (Figure 6-1) (Chapter 4) (Li et al. 2003; Rayapureddi et al. 2003; Tootle et al. 2003). Both of EYA's activities are required for *Drosophila* eye development, although the exact roles of and the interplay between these two activities are unknown. As post-translation modifications are commonly used to regulate the activities of both transcription factors and enzymes, such modifications may be used to differentially regulate the two activities of EYA.

During eye development, EYA is positively regulated by MAPK-mediated serine/threonine phosphorylation, as EYA's ability to induce ectopic eyes when overexpressed is inhibited by either mutating the phosphoacceptor site to alanine or decreasing the amount of MAPK, while mimicking phosphorylation by mutation to aspartic/glutamic acid or increasing the amount of activated MAPK results in increased induction of ectopic eyes (Hsiao et al., 2001). Serine/threonine phosphorylation of EYA also enhances EYA's ability to function as a transcriptional coactivator with SO (Silver et al., 2003). This suggests that the role of serine/threonine phosphorylation of EYA in the eye may be to regulate EYA's transcriptional activity, although it does not rule out the possibility that other facets of EYA function could also be modulated.

In addition to MAPK-mediated serine/threonine phosphorylation, EYA is tyrosine phosphorylated in *Drosophila* cultured cells and can dephosphorylate itself on tyrosine residues (Tootle et al., 2003). The sites and functional consequences of EYA's tyrosine phosphorylation are likely to be conserved as Murine EYA3 (MmEYA3) is also tyrosine phosphorylated (Chapter 5). EYA phosphatase mutants, EYA^{HAD}, exhibit increased levels of tyrosine phosphorylation,

Figure 6-1

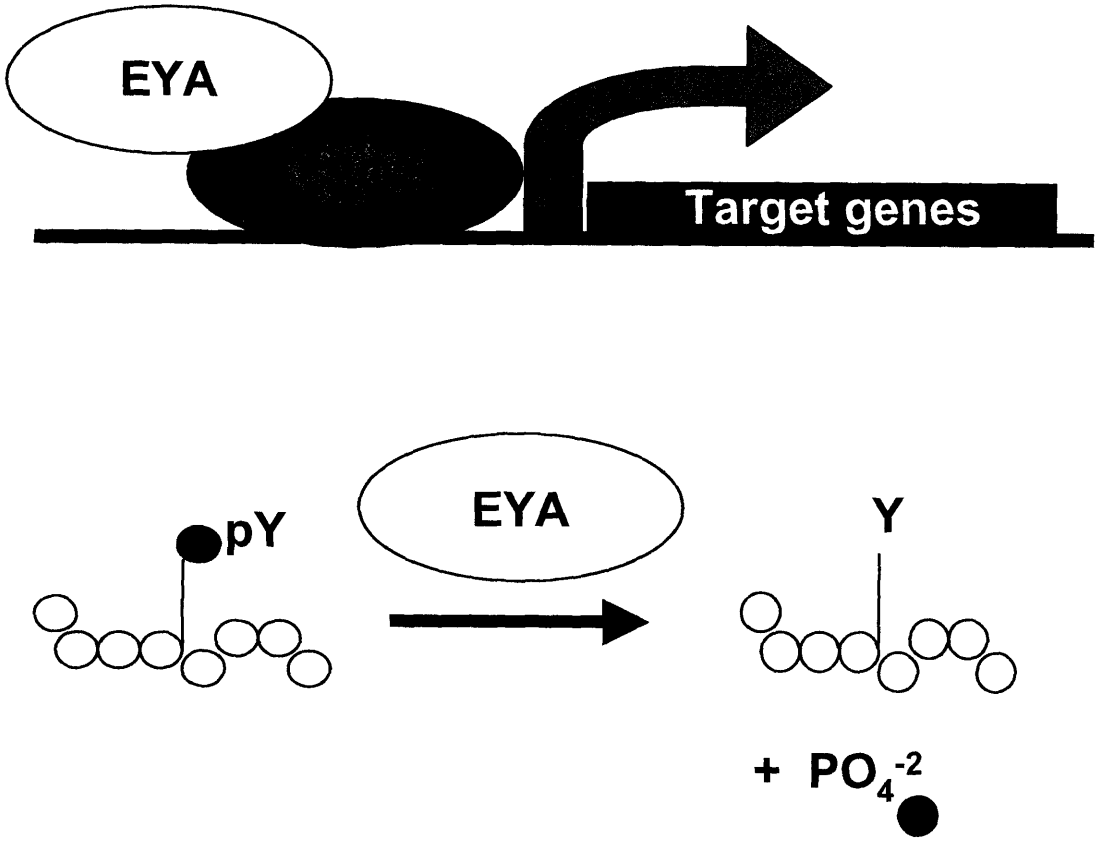


Figure 6-1

Schematic representation of EYA's two known functions. EYA possess two functions, one as a transcriptional coactivator, and one as a protein phosphatase.

and reduced ability to induce ectopic eyes or rescue the eyeless phenotype of the *eya*^{2} mutationt (Tootle et al., 2003). The observed increase in tyrosine phosphorylation of EYA^{HAD} mutants may contribute to their loss of activity in vivo, indicating the tyrosine phosphorylation may negatively regulate EYA. It is equally probable that EYA^{HAD} phenotypes do not result directly from increased tyrosine phosphorylation of EYA but rather are due to loss of dephosphorylation of other substrates. Thus, it is unclear how tyrosine phosphorylation and dephosphorylation of EYA are regulated or how such modifications regulate the two functions of EYA, as a transcriptional coactivator and a protein phosphatase.

As a scientific community our understanding of tyrosine phosphorylation and dephosphorylation of nuclear proteins is extremely limited. Analyses on EYA, how it is regulated by tyrosine phosphorylation and how it regulates the tyrosine phosphorylation of other transcriptional regulators, may yield insight into the roles which tyrosine phosphorylation and dephosphorylation generally play in regulating transcription.

The first step toward understanding the roles of tyrosine phosphorylation in regulating the two distinct functions of EYA is to determine the sites of phosphorylation. Figure 6-2A lists the conserved tyrosines and the sequences surrounding them in *Drosophila* EYA (DmEYA), and the corresponding sequences in MmEYA3. Utilizing the phosphorylation prediction program NetPhos, only four of the conserved tyrosines are likely to be phosphorylated, scoring ≥0.5 (Figure 6-2B). Three of these four tyrosines, two of which are Peptides 1 and 3 from Chapter 5, are within the ED, the domain predicted to be tyrosine phosphorylated by deletion analysis in Chapter 5. Thus both deletion analysis (Chapter 5) and NetPhos precidtions indicate EYA is likely to be tyrosine phosphorylated within the ED.

Figure 6-2

A

DmEYA-b			MmEYA3	
227	GSNLpYGCSS		99	ATQTpYGLPP
265	DYYYpYNSMQ			
284	YGIPpYAAAT			
326	YSSPpYAGYN		135	SNQDpYPTYT
329	PYAGpYNNFG		138	DYPTpYTILG
345	YNEQpYGNYY		146	GQNQpYQACY
348	QYGNpYYSPA			
349	YGNYpYSPAN		150	YQACpYPSSS
354	SPANpYSPYA			
357	NYSPpYAVSS			
509	LSGSpYANRY		262	LTGSpYAQKY
513	YANRpYTKDH		266	YAQKpYGKDP
567	DLSApYNFAT		320	DLSNpYSFST
606	LAFRpYRKIK		356	LAFRpYRKVR
613	IKDIpYNSYR		363	VREIpYDKHK
616	IYNSpYRGNV			
695	IENIpYSAHK	*	445	IENIpYSATK
719	RKSTpYVVIG	*	469	KKVTpYVVIG

Within ED2
*= Already synthesized.

B

		Score			Score
DmEYA-b	YSSPYAGYN	0.576	DmEYA-b	IKDIYNSYR	0.761
MmEYA1	SQQDYPSYP	0.894	MmEYA1	VKEIYNTYK	0.649
MmEYA2	VHQDYPSYP	0.313	MmEYA2	VKEMYNTYR	0.280
MmEYA3	SNQDYPTYT	0.873	MmEYA3	VREIYDKHK	0.497
HsEYA1	SQQDYPSYP	0.894	HsEYA1	VKEIYNTYK	0.649
HsEYA2	VHQDYPSYP	0.313	HsEYA2	VKEMYNTYK	0.378
HsEYA3	SNYDYPTYT	0.873	HsEYA3	VREIYDKHK	0.497
HsEYA3b	SNYDYPTYT	0.873	HsEYA3b		
HsEYA4	SQQDYPSYT	0.793	HsEYA4	VKELYNTYK	0.576

		Score			Score
DmEYA-b	IENIYSAHK	0.658	DmEYA-b	RKSTYVVIG	0.978
MmEYA1	IENIYSATK	0.651	mEYA1	RKVVYLLIG	0.477
MmEYA2	IENIYSATK	0.651	MmEYA2	RKAVYIVIG	0.813
MmEYA3	IENIYSATK	0.651	MmEYA3	KKVTYVVIG	0.962
HsEYA1	IENIYSATK	0.651	HsEYA1	RKVVYVVIG	0.939
HsEYA2	IENIYSATK	0.651	HsEYA2	RKAVYVVIG	0.928
HsEYA3	IENIYSATK	0.651	HsEYA3	KKVTYVVIG	0.962
HsEYA3b			HsEYA3b		
HsEYA4	IENIYSATK	0.651	HsEYA4	RKVVYVVIG	0.939

Peptide 1 Peptide 3

Figure 6-2

Conserved tyrosines between *Drosophila* and mammalian EYA homologs. A. List of the phosphotyrosine peptides of conserved tyrosine in DmEYA and MmEYA3. B. Four of conserved tyrosines between *Drosophila* and mammalian EYA homologs are predicted by NetPhos to be phosphorylated. Scores ≥0.5 indicate a high probability of being phosphorylated.

Intriguingly Peptide 3, which is the best substrate for EYA thus far, has the highest NetPhos scores overall. One caveat to these analyses is that NetPhos predictions are founded on a database of known phosphorylated sequences, and thus novel phosphorylation sequences would not be predicted (Blom et al. 1999). As EYA is the defining member of a novel class of protein phosphatases it is difficult to predict whether the sequences recognized by EYA will be similar to known sites or identify new motifs.

As the sites of phosphorylation within EYA are also likely to be sites of dephosphorylation by EYA (Chapter 4, Figure 4-5B), one method that can be utilized to determine which tyrosines are phosphorylated it to synthesize EYA phosphopeptides and use these peptides as substrates for EYA's phosphatase activity. The tyrosine residues within the phosphopeptides that are the best substrates for EYA are good candidates for sites of phosphorylation within EYA. In addition to yielding insight into which tyrosines are phosphorylated within EYA, the phosphopeptide analysis may be able to determine a consensus sequence that is dephosphorylated by EYA, and this consensus could be used to identify other putative substrates of EYA. One caveat to the phosphopeptide analysis is that although these peptides are sequences from EYA, the residues within a particular peptide might not be adjacent to one another in the three dimensional protein, and therefore, the peptide results may not have any relevance to the actual sites of phosphorylation within EYA.

A more direct approach to identify the sites of tyrosine phosphorylation is to digest EYA into small fragments and analyze these fragments by mass spectrometry. The success of this analysis will depend on the homogeneity of the sample, on what percentage of the protein sample is tyrosine phosphorylated, and the number and complexity of the sites that are phosphorylated. By utilizing a stable S2 cell line expressing an affinity tagged phosphatase mutant (EYA^{HAD}) a

highly tyrosine phosphorylated sample of EYA can be obtained. Therefore, mass spectrometry analysis of EYA^{HAD} should identify the sites of tyrosine phosphorylation, along with the sites of other post-translational modifications.

Both the phosphopeptide analysis and mass spectrometry should indicate which tyrosines within EYA are likely to be phosphorylated. To address the relevance of these findings, these tyrosines can be mutated, to phenylalanine to block phosphorylation and aspartic acid to mimic the negative charge of phosphorylation (Figure 6-3), and analyzed to see whether loss of the tyrosine residue affects the level of tyrosine phosphorylation. If no loss of phosphotyrosine signal can be detected by individual mutations, multiple tyrosines can be mutagenized at a time and analyzed in the same manner. By utilizing mutations that result in a decrease or complete loss of the phosphotyrosine signal, the effects of tyrosine phosphorylation on both of EYA's functions, as a transcriptional coactivator and a protein phosphatase, may be elucidated by the full spectrum of in vivo and in vitro assays that have been developed (Figure 6-4).

Post-translational modifications like tyrosine phosphorylation often affect protein-protein interactions. EYA is known to homodimerize and interact with SO, and may interact with DAC. By co-immunoprecipitation experiments one can determine whether loss of or mimicking tyrosine phosphorylation has any effect on EYA's protein-protein interactions. It is unlikely that tyrosine phosphorylation affects EYA's abilities to interact with itself or SO, as EYA^{HAD} mutants, which exhibit a high level of tyrosine phosphorylation, are still capable of homodimerizing and binding to SO. As EYA interacts with SO and DAC in yeast-two hybrid assays, it is also unlikely that tyrosine phosphorylation is required for these protein-protein interactions. These analyses should determine whether tyrosine phosphorylation of EYA affects its known protein-protein interactions.

Figure 6-3

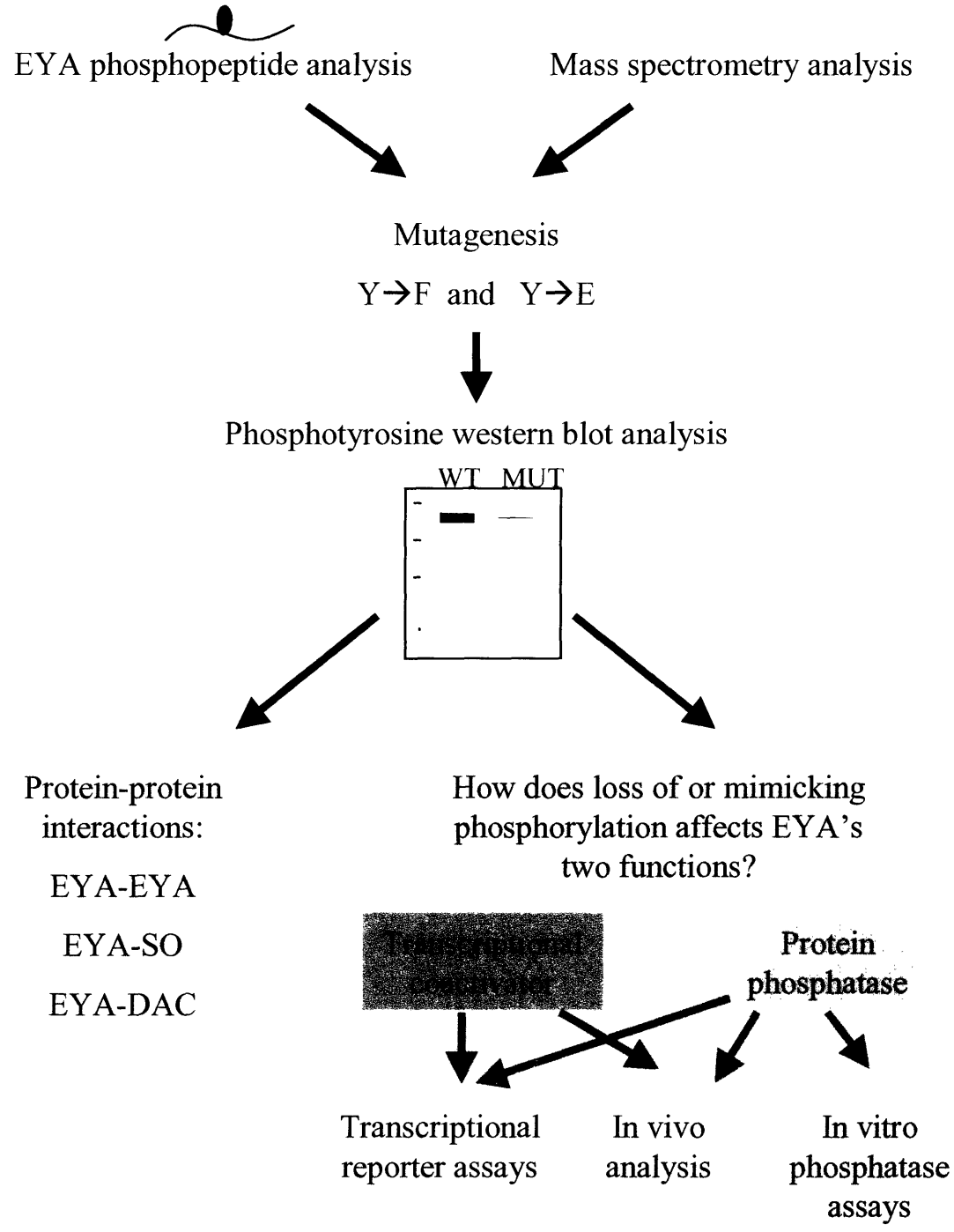

EYA phosphopeptide analysis
Mass spectrometry analysis
Mutagenesis
Y→F and Y→E
Phosphotyrosine western blot analysis
WT MUT
Protein-protein interactions:
EYA-EYA
EYA-SO
EYA-DAC
How does loss of or mimicking phosphorylation affects EYA's two functions?
Protein phosphatase
Transcriptional reporter assays
In vivo analysis
In vitro phosphatase assays

Figure 6-3

General schematic of how putative sites of tyrosine phosphorylation will be analyzed by mutagenesis. Tyrosines identified as potential sites of phosphorylation by either phosphopeptide analysis or mass spectrometry will be mutagenized to F to analyze the effects of loss of phosphorylation and E to analyze the effects of mimicking phosphorylation. These mutants will be analyzed for how they affect EYA known protein-protein interactions. In addition, by using multiple experimental avenues, including transcription assays, in vivo function analyses, and in vitro phosphatase assays, the mutants will be used to attempt to determine whether tyrosine phosphorylation affects/regulates one or both of EYA's activities.

Figure 6-4

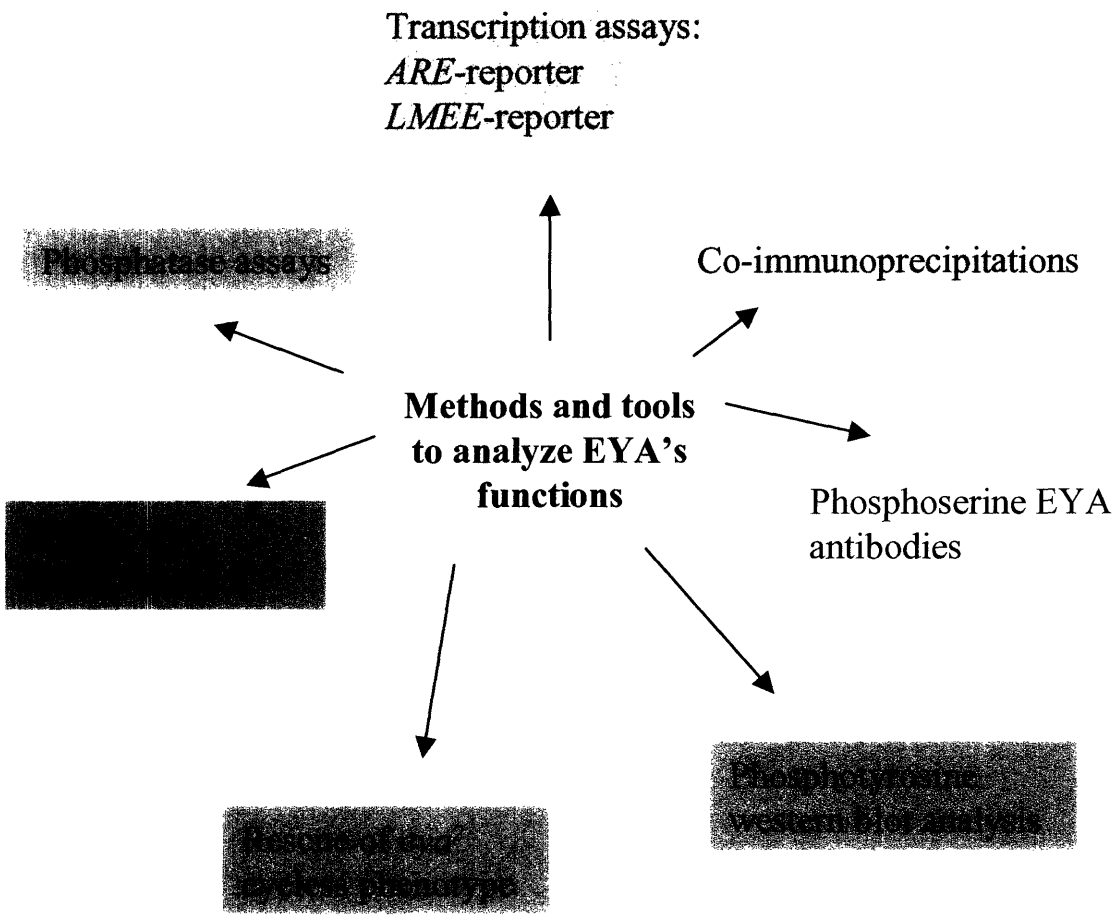


Figure 6-4

Schematic representation of the methods and tools we and others have developed to analyze EYA's two known functions.

The effects of both loss of and mimicking tyrosine phosphorylation on both EYA's ability to function as a transcriptional co-activator with SO and as a protein phosphatase can be examined by transcriptional reporter assays. Two distinct EYA-SO transcriptional reporter assays have been developed. The first reporter is a multimer of the known SIX family binding site termed *ARE* (Silver et al. 2003), while the second transcriptional reporter is a native response element from the *lozenge* promoter and is termed *LMEE* (S. Silver, personal communication; Yan et al. 2003). Utilizing the *ARE* reporter it has been shown that EYA functions as a transcriptional coactivator with SO (Silver et al. 2003). EYA's phosphatase function does not appear to be important for transcriptional activation as EYA^{HAD} mutants can still function as coactivators with SO in this context (Chapter 4, Figure 4-4) (Tootle et al., 2003). In addition, coexpression of the other RD network member DAC has no effect on EYA-SO mediated transcription using the *ARE*-reporter (Silver et al. 2003). Utilizing the *LMEE*-reporter, wild-type EYA functions as a transcriptional coactivator with SO, while EYA^{HAD} mutants exhibit varying degrees of coactivation (S. Silver, personal communication), indicating that phosphatase function has some role in mediating transactivation of this reporter. Intriguingly on the LMEE-reporter, DAC can function as a coactivator with both the EYA^{WT}-SO transcription factor and the EYA^{HAD}-SO transcription factors (S. Silver, personal communication). Therefore, coactivation of the *LMEE*-reporter by DAC is independent of EYA's phosphatase activity.

By using both of these reporter systems one can gain insight into whether loss of or mimicking tyrosine phosphorylation of EYA affects one or both of EYA's activities. Analysis with the *ARE*-reporter should provide information as to whether these mutations affect EYA's ability to act as a transcriptional coactivator with SO, as loss of phosphatase function has no affect on this read-out of transcriptional activity (Figure 6-5A). Conversely, studies using the

LMEE-reporter may indicate whether these mutations affect one or both of these activities, as EYA^{HAD} mutants function distinctly from EYA^{WT} (Figure 6-5B). If mutating the tyrosine residues within EYA solely inhibits transcriptional coactivator function then there will be decreased transcriptional activation with SO and loss of coactivation by DAC, while if the mutations only affect phosphatase function there will be normal coactivation with DAC and there may or may not be a decrease in transcriptional activation with SO (Figure 6-5C). If the mutations inhibit both activities of EYA there will be both a loss of coactivation with DAC and decreased transcriptional activation with SO. Analyses with these two transcriptional reporter systems will provide information on how loss of or mimicking tyrosine phosphorylation of EYA affect EYA's two functions, as a transcriptional coactivator with SO and as a protein phosphatase (Figure 6-5C).

To determine whether tyrosine phosphorylation of EYA is important for *Drosophila* eye development transgenic fly lines can be established to express both loss of and mimicking tyrosine phosphorylation EYA mutations under the control of the UAS/GAL4 system. These transgenic lines can be used to analyze how such mutations affect EYA's ability to induce ectopic eyes, as overexpression of wild-type EYA results in 50% of the flies exhibiting ectopic eyes (Hsiao et al. 2001). Additionally, it will be important to determine whether these mutations can rescue the eyeless phenotype of the eya^2 mutation. These experiments will show whether tyrosine phosphorylation is important for EYA function in the developing *Drosophila* eye, but as both of EYA's activities, as a transcriptional coactivator and a protein phosphatase, are required in vivo, they will not provide information as to which function is affected.

If the tyrosine phosphomimetic EYA mutations give any indication that they actually mimic phosphorylation, i.e. by behaving differently than loss of phosphorylation mutations, they

Figure 6-5

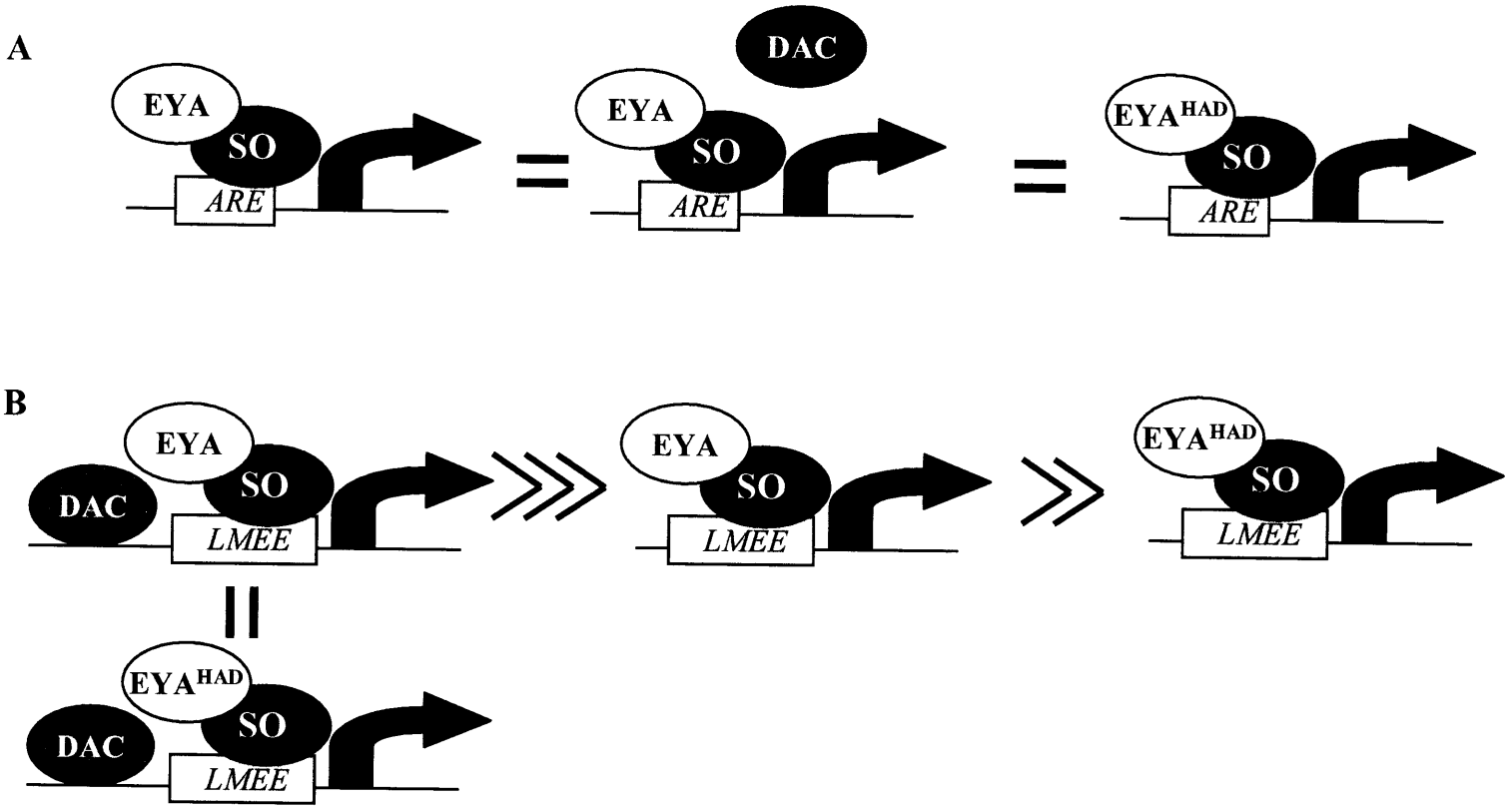


Figure 6-5

C

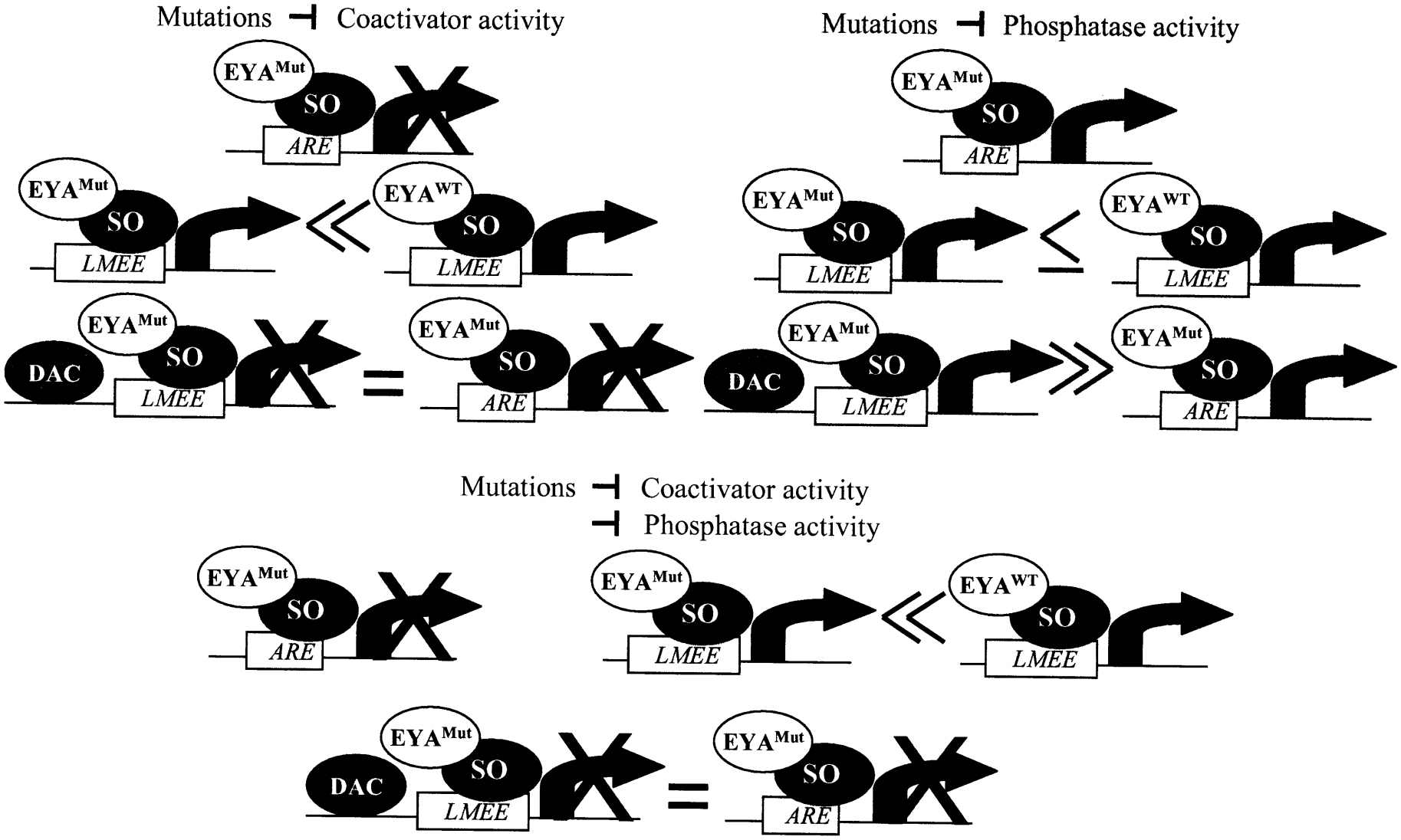

Mutations ⊣ Coactivator activity
EYA^Mut
SO
ARE
EYA^Mut
SO
LMEE
≪
EYA^WT
SO
LMEE
DAC
EYA^Mut
SO
LMEE
=
EYA^Mut
SO
ARE
Mutations ⊣ Phosphatase activity
EYA^Mut
SO
ARE
EYA^Mut
SO
LMEE
≤
EYA^WT
SO
LMEE
DAC
EYA^Mut
SO
LMEE
⋙
EYA^Mut
SO
ARE
Mutations ⊣ Coactivator activity
⊣ Phosphatase activity
EYA^Mut
SO
ARE
EYA^Mut
SO
LMEE
≪
EYA^WT
SO
LMEE
DAC
EYA^Mut
SO
LMEE
=
EYA^Mut
SO
ARE

Figure 6-5

Two transcriptional reporter systems can be used to determine how tyrosine phosphorylation affects both EYA's function as a transcriptional coactivator and as a protein phosphatase. A. Schematic illustrating the known outcomes of transcription assays utilizing the *ARE*-reporter. The EYA-SO transcription factor activates transcription, this activation is not affected by addition of DAC, or loss of EYA's phosphatase ability. B. Schematic illustrating the known outcomes of transcription assays utilizing the *LMEE*-reporter. The EYA-SO transcription factor activates transcription, this activation is enhanced by addition of DAC, inhibited to various degrees by loss of EYA's phosphatase ability. Interestingly, EYA's phosphatase activity is not required for DAC mediated enhancement of transcription. C. Schematic illustrating the predicted outcomes of transcription assays with both reporters in response to Y mutations. If mutating the tyrosines inhibits only coactivator activity then I would expect loss of or decrease in transcriptional activation with both reporters, and loss of DAC mediated enhancement of transcription from the *LMEE*-reporter. If mutating the tyrosine inhibits only phosphatase activity then I expect that there will be no affect of transcriptional response from the *ARE*-reporter, a decrease or loss of transcription and retention of DAC mediated enhancement of transcription from the *LMEE*-reporter. If mutating tyrosine inhibits both activities I would expect to find that transcription from both reporters is lost or reduced, and there will be no enhancement of transcription by addition of DAC using the *LMEE*-reporter.

can be used to directly look at how tyrosine phosphorylation affects EYA's protein phosphatase activity. The tyrosine to aspartic acid mutations can be made in MmEYA3, and the effect of such mutations on the enzyme's ability to dephosphorylate substrates can be analyzed. As a control it will be important to also test the tyrosine to phenylalanine mutations. It will be important to determine whether the mutations can dephosphorylate both the phosphopeptide substrates and immunoprecipitated tyrosine phosphorylated EYA, as tyrosine phosphorylation could only affect the dephosphorylation of one of the substrates and not the other.

By utilizing the variety of in vivo and in vitro assays described above (Figure 6-4), one may begin to understand how tyrosine phosphorylation of EYA regulates EYA's two activities, as a transcriptional coactivator and protein phosphatase. In addition, once the tyrosines that are phosphorylated have been identified, phosphotyrosine specific EYA antibodies can be generated. Such antibodies can be used to determine when and where EYA is tyrosine phosphorylated during development, yielding insight into why such modifications occur. The antibodies can also be used to identify proteins that interact with tyrosine phosphorylated EYA. These interactors are likely to have phosphotyrosine binding domains and play roles in mediating the effects of tyrosine phosphorylation of EYA.

In addition to determining the sites of tyrosine phosphorylation within EYA it is also important to identify the kinase(s) which tyrosine phosphorylate EYA. A variety of methods, in addition to the RNAi based screen discussed in Chapter 5, can be utilized to determine which kinase(s) is (are) responsible for tyrosine phosphorylating EYA (Figure 6-6). One method is to use EYA as a substrate for in vitro kinase assays with the kinases known to be expressed in *Drosophila* S2 insect cell culture, as EYA is tyrosine phosphorylated in these cells (Chapter 4). By utilizing the EYA phosphorylation mutants one should be able to determine which kinase

Figure 6-6

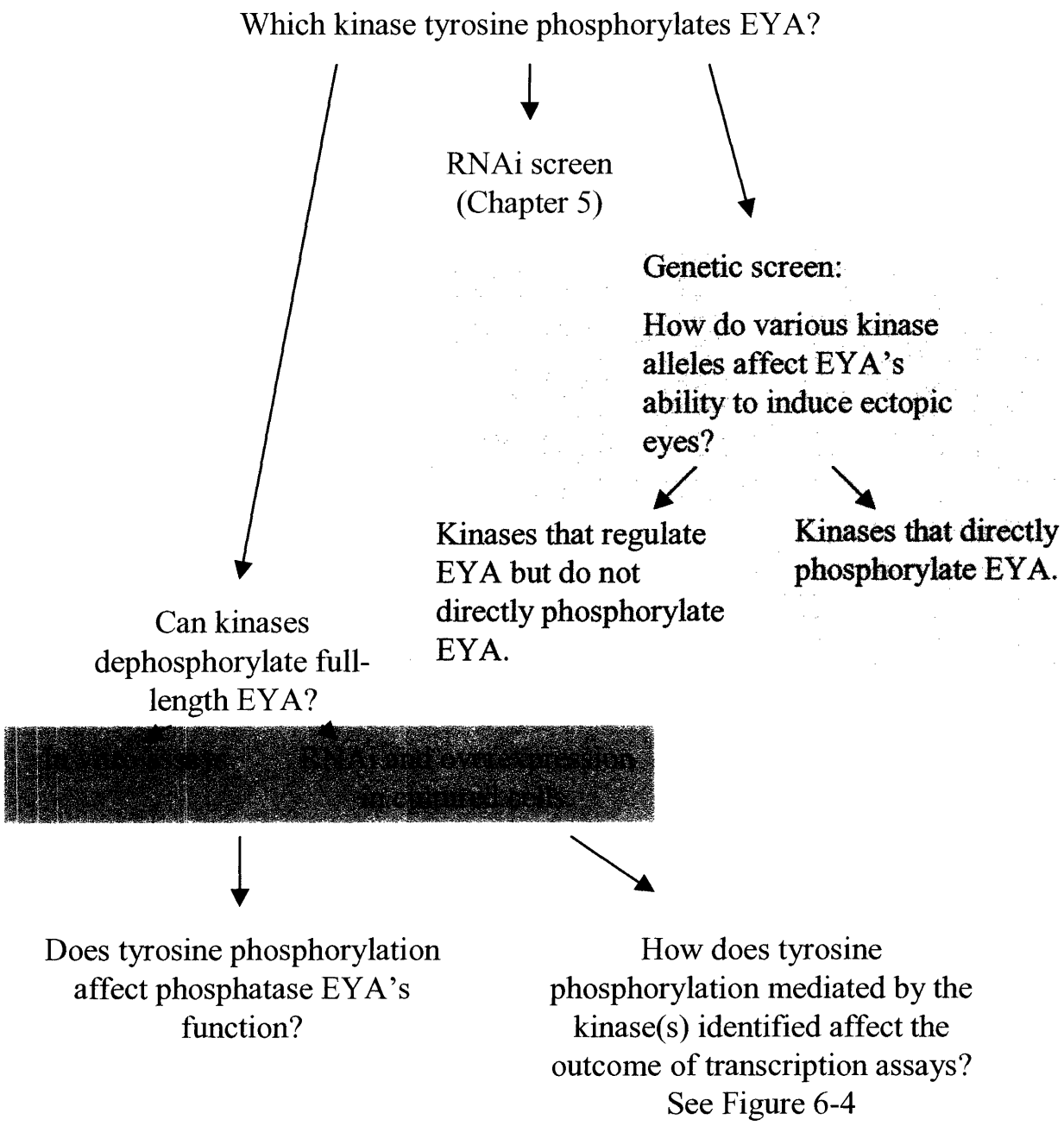

Which kinase tyrosine phosphorylates EYA?
RNAi screen
(Chapter 5)
Genetic screen:
How do various kinase alleles affect EYA's ability to induce ectopic eyes?
Kinases that regulate EYA but do not directly phosphorylate EYA.
Kinases that directly phosphorylate EYA.
Can kinases dephosphorylate full-length EYA?
Does tyrosine phosphorylation affect phosphatase EYA's function?
How does tyrosine phosphorylation mediated by the kinase(s) identified affect the outcome of transcription assays?
See Figure 6-4

Figure 6-6

Schematic illustrating the planned experiments to elucidate which kinase or kinases tyrosine phosphorylate EYA. If a kinase or kinases are found to phosphorylate EYA, the effect of phosphorylation on EYA's ability to function as a protein phosphatase can be analyzed.

phosphorylates which tyrosine within EYA. In addition a genetic modifier screen of ectopic eye induction by overexpression of EYA can be used to identify the kinase(s) which tyrosine phosphorylate EYA. As similar genetic interactions have successfully established the in vivo relevance of ERK MAPK-mediated phosphorylation of EYA (Hsiao et al. 2001), loss of function or activated tyrosine kinase alleles which alter EYA-mediated ectopic eye induction will indicate the kinases involved in regulating EYA. This screen may identify the kinase or kinases that directly tyrosine phosphorylate EYA. In addition, it will identify tyrosine kinases involved in indirectly regulating the functions of EYA during Drosophila eye development, yielding more insight into how RD network members are regulated by various signaling events. Kinases for which alleles exhibit a modification of the EYA overexpression phenotype can be tested by in vitro kinase assays with full-length EYA to determine whether the effect is due to direct phosphorylation.

The kinases that can phosphorylate EYA in vitro can then be analyzed to determine whether they possess such functions *Drosophila* cultured cells. Knocking down the expression of the kinase or kinases by RNAi, or utilizing dominant negative allele(s) of the kinase(s) responsible for tyrosine phosphorylating EYA, should result in a loss or decrease in the level tyrosine phosphorylation of EYA. Conversely, expression of an activated form of the kinase(s) should increase in the level of tyrosine phosphorylation of EYA. These effects should not be seen with EYA phosphorylation site mutations. Using this method one can identify which kinase or kinases tyrosine phosphorylate EYA in vivo.

The functional consequences of tyrosine phosphorylation by specific kinases can be analyzed using the same series of assays utilized to look at EYA phosphorylation mutants. How loss of the kinase(s) or expression of activated kinase(s) affects EYA's two activities, as a

transcription coactivator and a protein phosphatase, can be analyzed by the transcriptional reporter assays as described above. The results of the loss of phosphorylation mutants of EYA and the loss of kinase experiments should exhibit the same effect, while expression of activated kinase and the phosphomimetic EYA mutants may give the same results. The in vivo effects of kinase alleles, including loss of function, dominant negative, and activated alleles, can also be analyzed by ectopic eyes assays, and rescue of the *eya*^{2} eyeless phenotype. One can even determine the effect of specific tyrosine phosphorylation events on EYA's protein phosphatase activity by in vitro phosphorylating EYA with a particular kinase, and using this phosphorylated EYA in phosphatase assays. The above analyses on the kinase(s) which tyrosine phosphorylate EYA, in addition to assays on EYA phosphorylation site mutations, will yield insight into how tyrosine phosphorylation affects EYA's activities.

Having identified the kinase or kinases involved in mediating the tyrosine phosphorylation of EYA one can address the regulatory mechanisms that affect this phosphorylation. By analyzing where and when the kinase or kinases are coexpressed with EYA, one can determine the spatial and temporal regulation of tyrosine phosphorylation of EYA. In addition, it will be important to understand what upstream signaling events regulate the activity of the kinase(s) and thus regulate the tyrosine phosphorylation of EYA. This may provide insight into how the many signaling pathways implicated in regulating RD network members affect their activities.

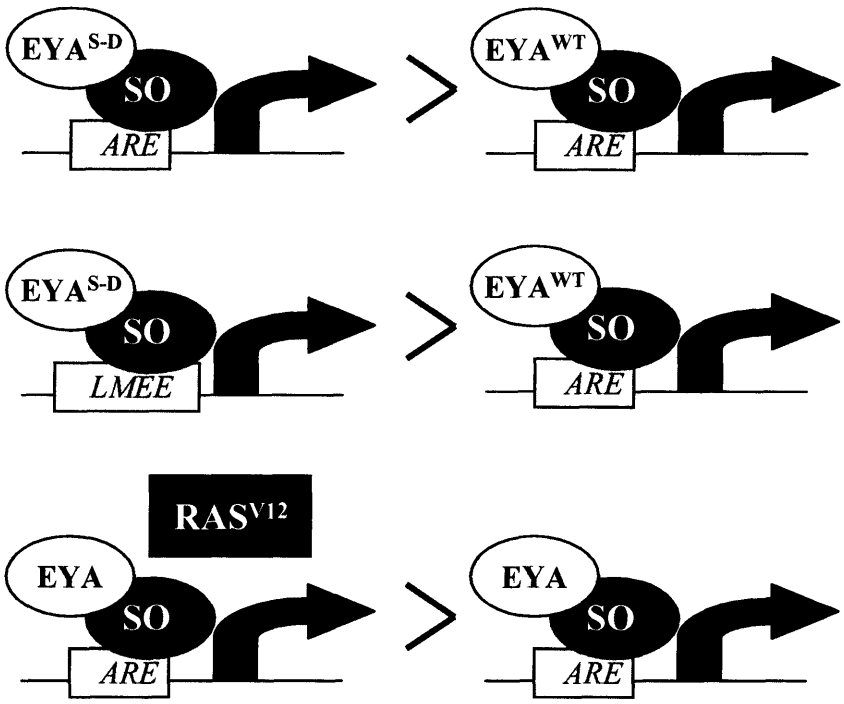
As different post-translational modifications can affect other modifications, it is important to determine whether/how serine/threonine phosphorylation affects tyrosine phosphorylation of EYA. The phosphotyrosine specific EYA antibodies can be used in conjunction with the already existing phosphoserine specific EYA antibodies (I. Rebay, personal

communication) for immunofluorescence, western blot, and co-immunoprecipitation analyses to determine whether both modified forms of EYA colocalize or are co-expressed throughout development, and whether both modifications can occur on EYA at the same time. Also RAS/MAPK signaling can be stimulated by a variety of means, and the effects of this on the tyrosine phosphorylation of EYA can be analyzed by phosphotyrosine western blot analysis.

In addition, both the transcriptional reporters, the *ARE*-reporter and the *LMEE*-reporter, can be used to analyze whether there is interplay between these phosphorylation events (Figure 6-7). Analysis with the *ARE*-reporter has shown that serine phosphomimetic EYA mutants exhibit increased transcriptional coactivator ability (Silver et al. 2003), while both addition of activated RAS and serine phosphomimetic EYA mutants exhibit increased activity with the *LMEE*-reporter (S. Silver, personal communication). Double mutants of loss of or mimicking tyrosine phosphorylation and loss of or mimicking serine phosphorylation can be analyzed with both transcriptional reporters. If both phosphorylation events function in the same direction than further transcriptional activation will be seen with the double phosphomimetic mutant (Figure 6-3). By using the *LMEE*-reporter one can look at the effects of activated RAS on the ability of EYA tyrosine phosphorylation mutants to coactivate transcription. These experimental approaches can be used to begin to understand whether/how serine/threonine phosphorylation affects the tyrosine phosphorylation of EYA.

It is debated as to whether EYA is a dual specific phosphatase and can dephosphorylate both serine/threonine and tyrosine residues (Li et al. 2003), or whether EYA is solely a protein tyrosine phosphatase (Chapter 4) (Rayapureddi et al. 2003; Tootle et al. 2003). To truly understand EYA's functions, the interplay of the separate functions, and how tyrosine phosphorylation regulates these functions, it must be clear what type of phosphatase EYA is.

Figure 6-7



If both S/T and Y phosphorylation positively regulate EYA's activity:

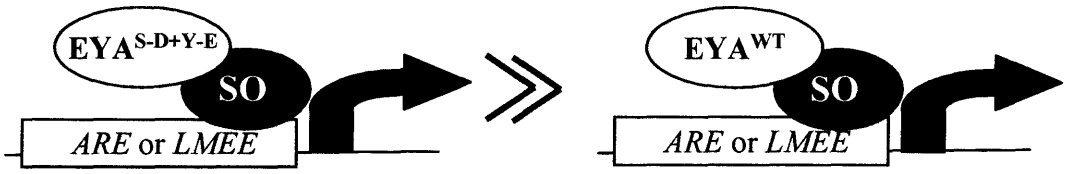


Figure 6-7

Schematic illustrating how S/T phosphorylation is known to affect transcription by the EYA-SO transcription factor on both reporters. Mimicking serine phosphorylation of EYA results in increased transcription from both reporters, while addition of activated RAS only increases transcription from the *LMEE*-reporter. If serine and tyrosine phosphorylation of EYA both positively regulate its activities I would expect to find that double phosphomimetic mutants will exhibit an even higher level of transcription.

Full length *Arabidopsis* Eya, which only contains an ED, is a protein tyrosine phosphatase (Rayapureddi et al. 2003), indicating that evolutionarily EYA is a protein tyrosine phosphatase and not a dual specific phosphatase. It is possible that the different conditions used for the in vitro phosphatase assays between the various groups result in the different activities detected. It is also possible that EYA evolved from a tyrosine specific phosphatase into a dual specific phosphatase, similar to its gain of transcriptional coactivator function. To verify which type of phosphatase EYA is, the conditions utilized by the group seeing dual specific phosphatase activity need to recapitulated to determine if EYA can indeed dephosphorylate both phospho-serine/threonine and -tyrosine peptides.

If EYA is a dual specific phosphatase it will be important to determine whether EYA can dephosphorylated itself and SO on serine/threonine residues. To determine if EYA can dephosphorylates itself at phospho-serine/threonine residues one can use the phosphoserine specific EYA antibodies and analyze whether bacterially purified EYA can dephosphorylated itself at phosphoserine residues. Knowing whether EYA can regulate itself in this manner will help us to interpret the effects of EYA phosphatase mutants.

The activity of SIX1, one of the human orthologs of SO, is regulated by S/T phosphorylation, and phosphorylated SIX1 runs as three distinct bands on western blots (Ford et al. 2000). Interestingly, immunoprecipitated SO also runs as a triplet on western blots (S. Silver, personal communication) and metabolic labeling has revealed that SO is phosphorylated in cultured cells (E. Davies, personal communication), indicating that SO is likely to be phosphorylated in a similar manner to SIX1. As EYA is known to interact with SO, and EYA may be a dual specific phosphatase, SO may be a substrate for EYA. It is important to first verify that the three SO bands seen on western blots are indeed due to differential

phosphorylation. After which, one can analyze whether incubation of serine/threonine phosphorylated SO with the phosphatase EYA results in the loss of any of the three bands. By this analysis I should be able to determine whether EYA can dephosphorylate SO.

EYA is a dual function protein, possessing both transcription coactivator and protein phosphatase activity, that is regulated by both serine/threonine and tyrosine phosphorylation. While there is some understanding of how serine/threonine phosphorylation regulates EYA, there is no information on how tyrosine phosphorylation affects EYA's functions. The above experimental avenues should help to determine which tyrosines are phosphorylated by which kinases, how individual phosphorylation events affect EYA's known functions by transcriptional, in vivo, and in vitro readouts of activity, the interplay of serine/threonine phosphorylation and tyrosine phosphorylation, and whether EYA is a dual specific phosphatase. Understanding the mechanistic details of how post-translation modifications, like tyrosine phosphorylation, affect EYA's functions will give us a better handle on how cells can differentially regulate the two functions of EYA.

Additionally as a community, our understanding of how tyrosine phosphorylation affects transcription factor function is severely limited. Analyses on how tyrosine phosphorylation individually affects specific transcription factors will yield insight into possible general mechanisms of tyrosine phosphorylation function and encourage others to analyze whether similar regulation is occurring with other transcription factors.

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Appendix I

Deficiency kit screen for mislocalizers of YAN

Tina Tootle, Xiao Tan, Karolina Fraczkowska and Ilaria Rebay

Results

In conjunction with the work presented in Chapter 2, a screen was undertaken to further pursue the mechanisms regulating the export of YAN. The deficiency kit, ~200 fly lines which sequentially delete small genomic regions, was screened for embryonic mislocalization of YAN by myself and two undergraduates, Xiao Tan and Karolina Fraczkowska. The dynamic embryonic expression pattern of YAN (Figure A-1) has been well characterized. Embryos were collected from individual lines and analyzed by immunofluorescence. We chose to focus on YAN expression in the ventral midline during embryonic stages 10-12 due to YAN's downregulation and presumed degradation in this tissue (Figure AI-1 C), but observed all stages of development.

If a deficiency line resulted in the deletion of a protein involved in the nuclear export of YAN, the expected phenotype would be nuclear YAN expression in the ventral midline, while loss of a protein involved in the degradation of YAN could result in cytoplasmic YAN expression. Twenty-five deficiencies were identified as mislocalizers (Figure AI-2). One class of mislocalizers exhibited the expected cytoplasmic staining (Figure AI-3A), while the other main class was unexpected and showed unusual clumping of YAN protein (Figure AI-3B). Only two deficiencies exhibited some nuclear retention of YAN in the ventral midline (Figure AI-3C). The largest class of mislocalizers is made up of a variety of phenotypes that exhibit aberrant YAN expression (Figure AI-3D).

Each deficiency deleted a large number of genes, making isolation of the gene or genes causing the mislocalization phenotype rather labor intensive. Due to the success of other experimental avenues, I did not pursue further the deficiencies exhibiting mislocalization of YAN.

Figure AI-1

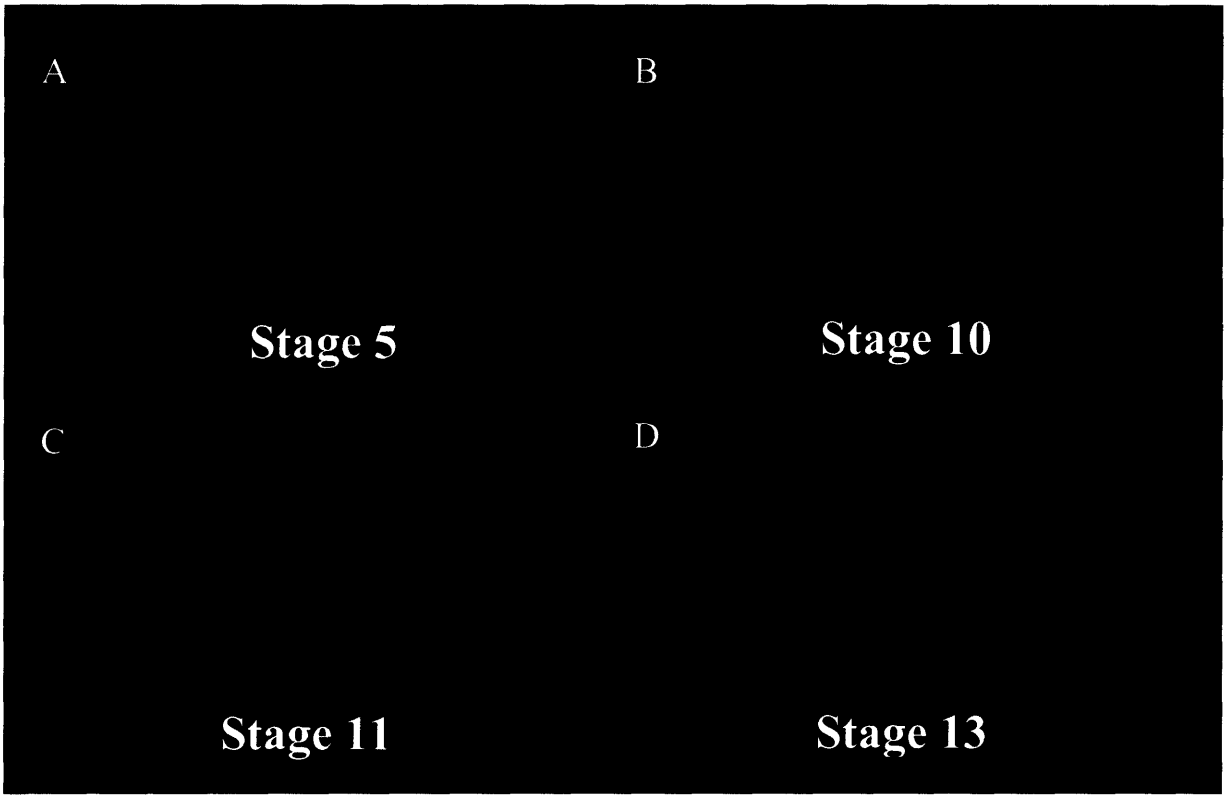

Figure AI-1

YAN expression pattern during embryogenesis. (A-D) Immunofluorescence, as described in Chapter 2, of *Drosophila* embryos collected for 6 hours at 25° and stained with mouse monoclonal YAN antibody (1:500) and CY3 secondary (1:2000). (A) Stage 5 embryo exhibiting ubiquitous nuclear YAN staining. (B) Ventral view of stage 10 embryo; nuclear YAN staining in mesoderm and ectoderm, while no YAN is present in the ventral midline or developing CNS. (C) Dorsal-lateral view of stage 11 embryo, expression is equivalent to Stage 10. (D) Dorsal-lateral view of stage 13 embryo; YAN expression is restricted to the peripheral nervous system.

Figure AI-2

DF	Description	Location	YAN mislocalization
125	Df(1)4b18, y[1] cv[1] v[1] nonA[4b18] f[1] car[1]/Basc	014B08;014C01	nuclear in midline and not w here it should be
167	Df(2L)TW161, cn[1] bw [1]/CyO	038A06-B01;040A04-B01	early embryos w ith only a few dispersed nuclei
282	Dp(1;Y)y[+]/y[1]; Df(2R)X58-12/SM5	058D01-02;059A	tw isted, nuclear and cytoplasmic
442	Df(2R)CX1, b[1] pr[1]/SM1	049C01-04;050C23-D02	no nuclear
948	Df(1)ct-J4, In(1)dl-49, f[1]/C(1)DX, y[1] w [1] f[1]; Dp(1;3)sn[13a1]/Ki[1]	007A02-03;007C01	nuclear everyw here
954	Df(1)v-L15/FM6	009B01-02;010A01-02	clumpy
993	Df(1)r-D1, v[1] f[1]/C(1)DX, y[1] w [1] f[1]; Dp(1;4)r[+]*/+	014C02-04;015B02-C01	w eird - general defect?
1045	Df(2L)Mdh, cn[1]/Dp(2;2)Mdh3, cn[1] ! see comment	030D-30F;031F	clumpy
1702	Df(2R)X1, Mef2[X1]/CyO, Adh[nB]	046C;047A01	cytoplasmic
1888	Df(2R)ST1, Adh[n5] pr[1] cn[*]/CyO	042B03-05;043E15-18	early embryos missing nuclear staining in patches
1962	Df(3R)p-XT103, ru[1] st[1] e[1] ca[1]/TM3, Ser[1]	085A02;085C01-02	missing YAN in patches
2993	Df(3L)st-f13, Ki[1] roe[1] p[p]/TM6B, Tb[1]	072C01-D01;073A03-04	clumpy
3007	Df(3R)ry615/TM3, Sb[1] Ser[1]	087B11-13;087E08-11	enlarged nuclei
3084	Df(2L)ast2/SM1	021D01-02;022B02-03	light cytoplasmic
3127	Df(3L)ri-79c/TM3, Sb[1]	077B-C;077F-78A	gaps in staining
3138	Df(2L)b87e25/CyO	034B12-C01;035B10-C01	fuzzy staining, Nuclear and cytoplasmic
3546	Df(3R)B81, P{ry[+t7.2]=RP49}F2-80A e[1]/TM3, Sb[1]; Dp(3;1)67A	099C08;100F05	clumping along midline
3548	Df(2L)al/In(2L)Cy, Cy[1]	021B08-C01;021C08-D01	early embryo missing nuclei
3560	Df(1)v-N48, f[*]/Dp(1;Y)y[+]v[+]#3/C(1)DX, y[1] f[1]	009F;010C03-05	clumpy
3686	Df(3L)GN24/TM8, l(3)DTS4[1]	063F04-07;064C13-15	small and w eird
4431	Df(3R)DG2/TM2	089E01-F04;091B01-B02	cytoplasmic
4955	Df(2L)XE-2750/CyO, P{ry[+t7.2]=sevRas1.V12}FK1	028B02;028D03	cytoplasmic in midline
4959	Df(2L)C'/CyO	040h35;040h38L	cytoplasmic in midline
4960	Df(2R)CB21/CyO; ry[506]	048E;049A	cytoplasmic in midline
5281	Df(1)dx81, w [*]/Dp(1;Y)dx[+]1/C(1)M5	005C03-10;006C03-12	clumpy

Figure AI-2

Table of deficiency lines exhibiting mislocalization of YAN.

Figure AI-3

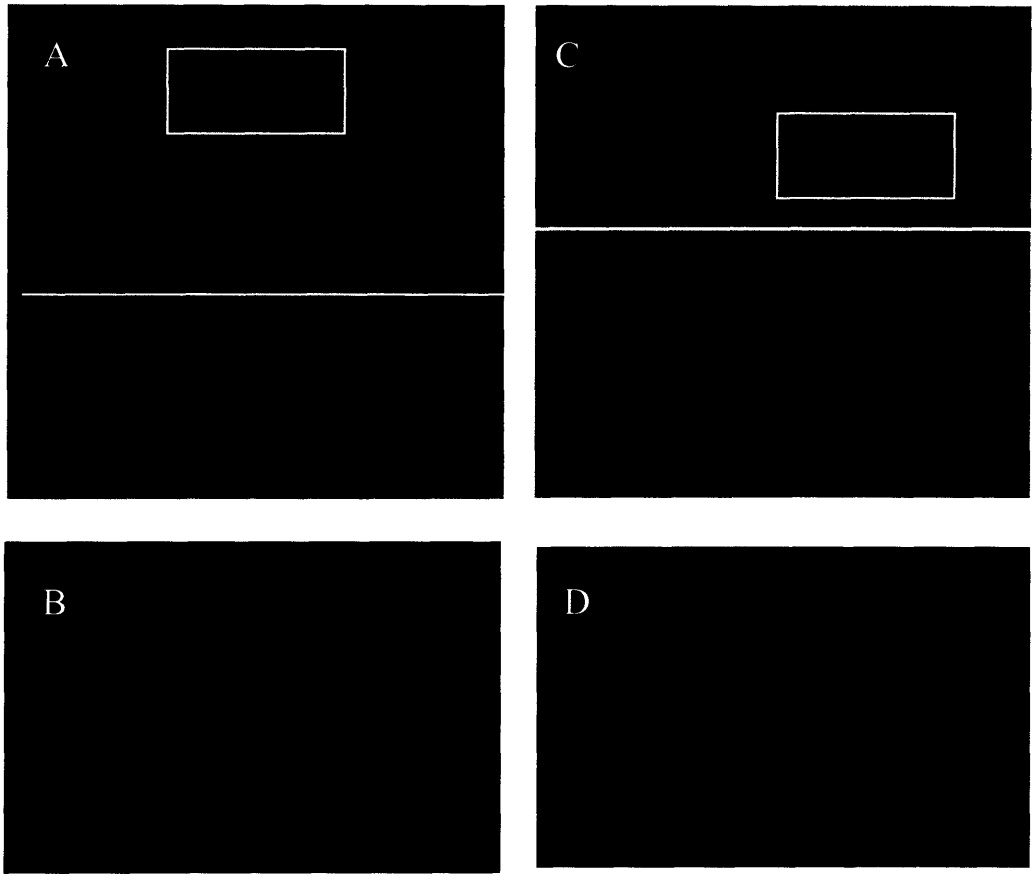

Figure AI-3

Examples of mislocalization of YAN. (A) Cytoplasmic YAN localization. (B) Retention of nuclear YAN in the ventral midline. (C) Clumping YAN expression. (D) An example of general defects in YAN localization, no nuclear YAN expression.

Appendix II

YAN regulation by JNK and p38 MAPKs

Tina Tootle and Ilaria Rebay

Results

Others had shown that the MAPK JNK phosphorylates and inactivates YAN during embryonic dorsal closure (Riesgo-Escovar and Hafen 1997). In addition it had previously been shown by in vitro kinase assays that ERK, JNK, and p38a/p38b MAPKs phosphorylate YAN (F. Hsiao, personal communication). As ERK-mediated phosphorylation results in export of YAN from the nucleus to the cytoplasm (Tootle et al. 2003), we wanted to determine whether phosphorylation by JNK and p38 had similar effects. JNK and p38 MAPKs are stress-activated kinases.

To determine how phosphorylation of YAN by JNK and p38 MAPKs affects YAN localization we transiently transfected various constructs of YAN, including wild-type and phosphorylation mutants, along with the respective kinase. We activated JNK and p38 MAPKs by heat shock. This analysis showed that like ERK, JNK-mediated phosphorylation of YAN, particularly at the first phosphorylation site results in cytoplasmic localization of YAN (Figure AII-1A). Phosphorylation by p38a or p38b does not affect YAN localization, as YAN remains nuclear (Figure AII-1B). Therefore, it seems like regulation of YAN by ERK and JNK MAPKs occurs by the same mechanism, while regulation of YAN by p38 MAPKs is happening by a different method.

References

Riesgo-Escovar, J.R. and E. Hafen. 1997. Drosophila Jun kinase regulates expression of decapentaplegic via the ETS-domain protein Aop and the AP-1 transcription factor DJun during dorsal closure. *Genes Dev* **11**: 1717-27.

Tootle, T.L., P.S. Lee, and I. Rebay. 2003. CRM1-mediated nuclear export and regulated activity of the Receptor Tyrosine Kinase antagonist YAN require specific interactions with MAE. *Development* **130**: 845-857.

Figure AII-1

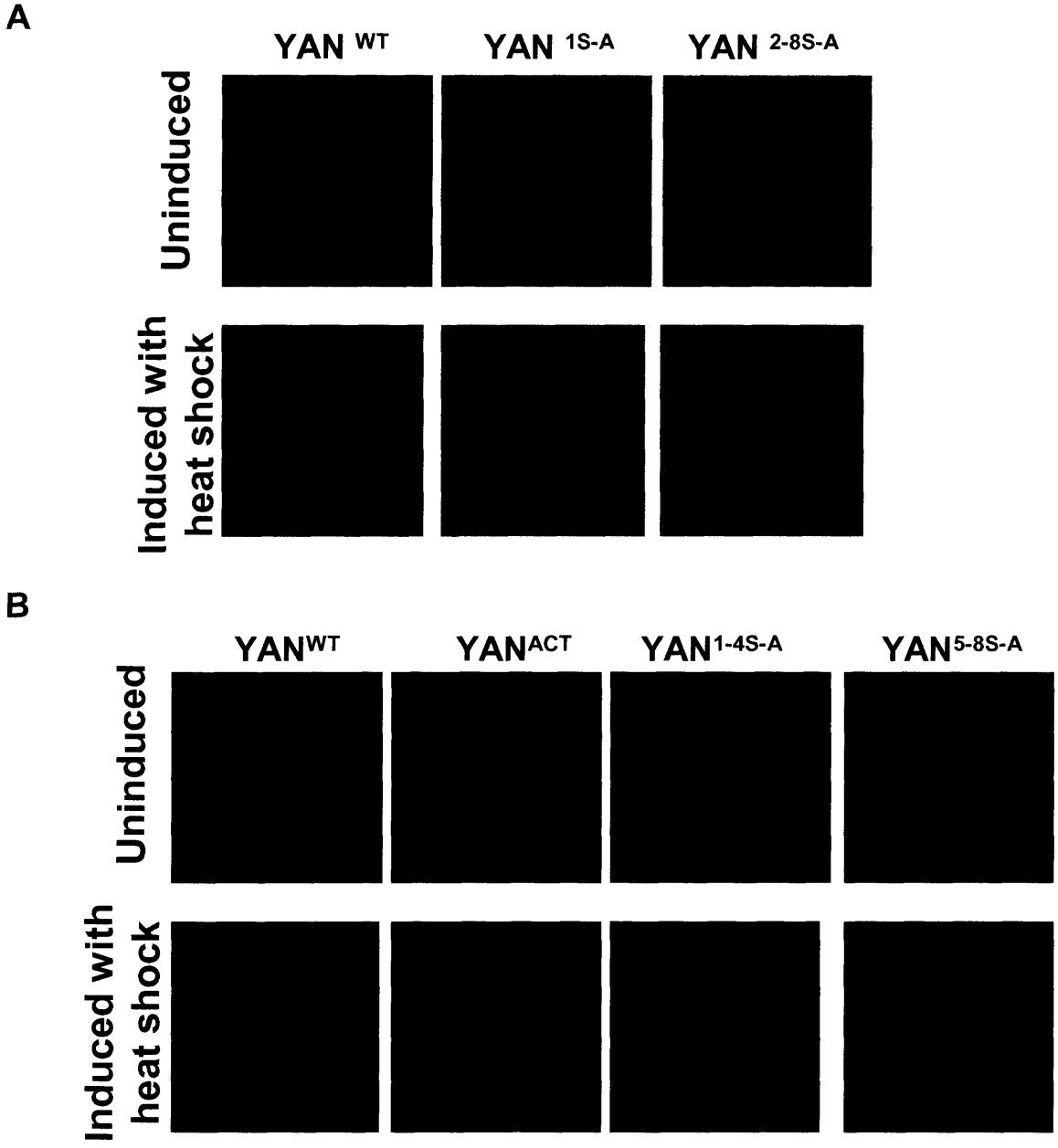
A
YAN WT
YAN 1S-A
YAN 2-8S-A
Uninduced
Induced with heat shock
B
YANWT
YANACT
YAN1-4S-A
YAN5-8S-A
Uninduced
Induced with heat shock

Figure AII-1

JNK but not p38 mediated phosphorylation of YAN leads to cytoplasmic localization of YAN. Transient transfections of the YAN construct listed along with the MAPK, JNK in A and p38b in B. Kinases were activated by 10 minutes of heat shock at 30°C. Results were analyzed by immunohistochemistry with anti-YAN MAb 8B12 at 1:250 with CY3 conjugated goat anti-mouse secondary (1:10000). A) Activated JNK leads to cytoplasmic localization of YAN^{WT} and $YAN^{2\text{-}8S\text{-}A}$, while $YAN^{1S\text{-}A}$ remains nuclear in the presence of activated JNK. B) Activated p38b and activated p38a (same as with p38b, data not shown) has no effect on YAN localization, as YAN remains nuclear.

Appendix III

More insights into the roles of MAE in regulating YAN and PNT.

Tina Tootle and Ilaria Rebay

Introduction

One pathway used reiteratively throughout development is the receptor tyrosine kinase (RTK) signaling network. RTKs signal through the evolutionarily conserved GTPase RAS and the mitogen activated protein kinase (MAPK) cascade. Among the best characterized downstream targets of activated MAPK are the *Drosophila* ETS-domain transcription factors encoded by *pointed (pnt)* and *yan*. In response to RTK activation, MAPK-mediated phosphorylation abrogates YAN repressor activity, 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.

We have shown, Chapter 2, that nuclear export of YAN is a necessary step in its downregulation, is mediated by CRM1, and is dependent on both YAN's nuclear export sequences (NESs) and pointed domain (PD) (Tootle et al. 2003). MAPK-mediated phosphorylation of YAN and PNT, and the ensuing abrogation of YAN's repressor activity and activation of transcription by PNT, is thought to be facilitated by a protein called Modulator of the Activity of ETS (MAE). Mechanistically, MAE binds to YAN or PNT via their respective PDs (Baker et al. 2001). A PD is protein-protein interaction motif found at the N-terminus of YAN or PNT and the C-terminus of MAE. We have demonstrated that MAE has a separate role in mediating export, independent of its role in mediating phosphorylation (Tootle et al. 2003). In addition, overexpression of MAE inhibits YAN mediated transcription repression (Tootle et al. 2003) and completely inhibits PNT mediated activation of transcription (Tootle et al. 2003; Yamada et al. 2003). Therefore, it has been proposed that MAE plays multiple roles in downregulation of both YAN and PNT.

Further work, Chapter 3, has shown that overexpression of MAE in *Drosophila* cultured cells inhibits nuclear export of YAN. However, overexpression in the eye does not result in YAN being aberrantly retained in the nuclei, but instead it results in an array of phenotypes that can be rescued by overexpression of PNT. Thus MAE appears to play a role in downregulation of PNT in vivo. Interesting, we find that *mae* transcription is regulated by YAN and PNT, adding further complexity to the signaling cascade. Many questions remain about MAE's roles in regulating YAN and PNT.

Results and Discussion

Overexpression of MAE blocks YAN downregulation

Based on our previous finding that overexpression of MAE inhibits YAN's ability to repress transcription (Tootle et al. 2003), we decided to investigate further the underlying mechanisms. One possibility was that overexpression of MAE might disrupt YAN's subcellular localization. In *Drosophila* S2 cultured cells YAN localizes to the nucleus (Figure AII-1A), and upon activation of the RAS/MAPK cascade is exported to the cytoplasm (Figure AIII-1B) (Rebay and Rubin 1995). Previous analyses studying *mae* loss-of-function revealed that MAE is necessary for nuclear export of YAN, as YAN remains restricted to the nucleus in the absence of *mae* in both cultured cells and in vivo (Tootle et al. 2003). Therefore, we expected that overexpression of MAE would either not disrupt Yan localization or perhaps lead to premature export and down-regulation of YAN in the absence of signaling. As predicted, no effect on nuclear YAN localization in the absence of pathway activation was observed (Figure AIII-1C) but surprisingly, overexpression of MAE prevented RAS^{V12} induced nuclear export of YAN (Figure AIII-1D). In order to rule out the possibility that the MYC epitope tag on MAE was

interfering with normal MAE function to produce a misleading phenotype, we confirmed the results using a non-tagged MAE construct (Figure AIII-1E,F). Addition of dsRNA (RNAi) against *mae* restored export (Figure AIII-1G, H), suggesting that precisely regulated levels of MAE are critical for proper YAN regulation. Thus both loss and overexpression of *mae* result in aberrant nuclear restriction of YAN in *Drosophila* cultured cells.

Why might overexpression of MAE restrict YAN to the nucleus? One possibility is that MAE overexpression results in a YAN-MAE complex that is somehow different from the YAN-MAE complex formed under lower levels of endogenous MAE expression, and that is therefore inefficiently phosphorylated by activated ERK. Because phosphorylation is necessary for nuclear export (Rebay and Rubin 1995), such a complex would be retained in the nucleus. To test this hypothesis, we overexpressed activated ERK (ERK^{SEM}), in the presence of RAS^{V12} and MAE, reasoning that an excess of activated ERK might more efficiently phosphorylate YAN. Overexpression of ERK^{SEM} partially restored YAN export (Figure AIII-1K), consistent with the hypothesis that excess MAE results in a YAN-MAE complex that is refractory to ERK-mediated phosphorylation.

To test this hypothesis further, we asked whether co-overexpression of PNT-P2 could also overcome the MAE-mediated restriction of YAN to the nucleus. The rationale is that if MAE overexpression results in aberrant YAN-MAE complexes that are no longer efficiently phosphorylated by ERK and therefore cannot be exported, then by introducing a second MAE binding partner, PNT-P2 (Baker et al. 2001; Tootle et al. 2003), we should be able to titrate out sufficient MAE to restore a "normal" RAS/MAPK-responsive YAN-MAE complex. As predicted we found that cotransfecting PNT-P2, YAN and MAE partially restored YAN export

Figure AIII-1

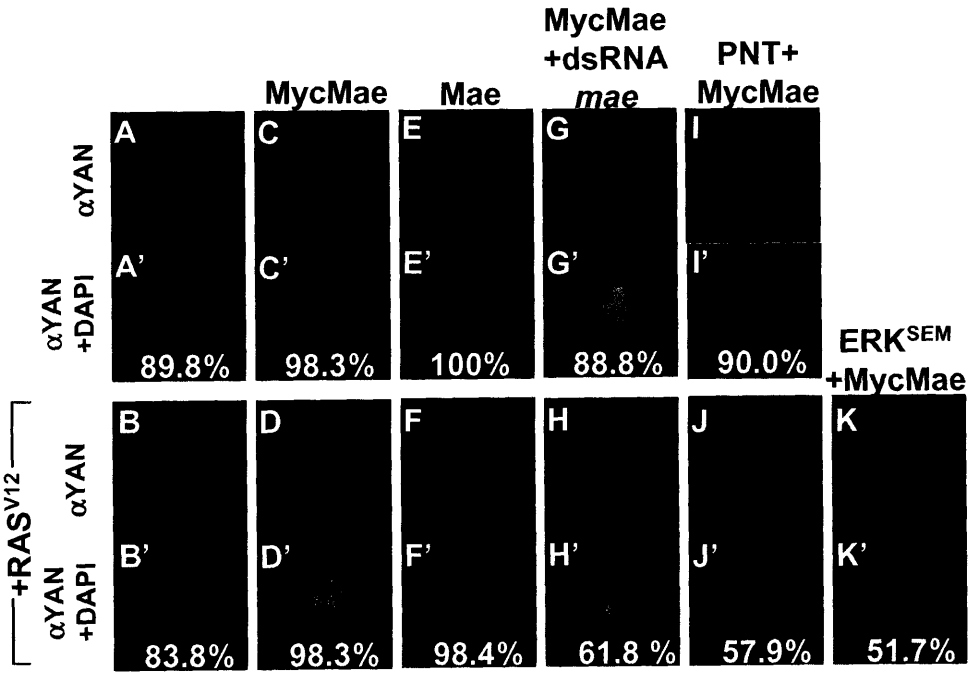

Figure AIII-1

MAE-YAN complex in insect cell culture is refractory to normal YAN downregulation. (A-K) Transient transfections of *Drosophila* S2 insect cultured cells expressing YAN and the indicated constructs stained with anti-YAN MAb 8B12 at 1:250 with CY3 conjugated goat anti-mouse secondary (1:10000). (A'-K') DAPI (1:10000) staining overlaying YAN antibody staining of the same cells. The percentage of transfected cells exhibiting nuclear localization (A, C-G, I) or exclusively cytoplasmic localization (B, H, J, K) is indicated, n≥250. (A, C, E, G, I) YAN localization in the absence of RAS^{V12}. (B, D, F, H, J, K) YAN localization in the presence of RAS^{V12}. (C, E, G, I) Coexpression of Myc-MAE, MAE, MAE+dsRNA mae, and MAE+PNT-P2 has no effect on the nuclear localization of YAN in the absence of signaling. (D, E) Coexpression of Myc-MAE or untagged MAE along with RAS^{V12} results in nuclear retention of YAN. (H, J) Coexpression of either PNT-P2 or ERK^{SEM} along with RAS^{V12} restores nuclear export of YAN. Overexpression of MAE results in a MAE-YAN complex that cannot be downregulated by the endogenous machinery, and thus it requires overexpression of other EGFR signaling components to restore proper downregulation of YAN.

in the presence of RAS^{V12} (Figure AIII-1J). Therefore, MAE-mediated restriction of YAN to the nucleus can be overcome by either overexpression of ERK^{SEM} or PNT-P2, suggesting that precise regulation of the amount of MAE available to complex with YAN is critical in ensuring RAS/MAPK mediated phosphorylation and export of YAN.

To confirm the in vivo relevance of MAE overexpression restricting YAN to the nucleus, we generated UAS MYC-MAE transgenic lines and used ELAV GAL4 to drive expression in the developing embryonic CNS. In wild-type germband extended embryos, nuclear YAN expression is rapidly cleared from the cells of the developing brain and ventral nerve cord, but remains expressed at high levels in the epidermis and mesoderm (Figure AIII-2A, A'). If downregulation of YAN is blocked, for example by expressing a constitutively "active" form, YAN^{ACT}, which is refractory to RAS/MAPK signaling, Yan remains nuclear and normal induction of neuronal markers is inhibited (Figure AIII-2A") (Rebay and Rubin 1995). As predicted from the cell culture results, MAE expressing embryos exhibit a YAN^{ACT}-like phenotype, with strong nuclear YAN expression (Figure AIII-2B, B") and inhibition of neuronal markers (Figure AIII-2B") in the developing brain and ventral nerve cord. Thus both in cell culture and in *Drosophila*, overexpression of *mae* inhibits YAN downregulation. However, in contrast to YAN^{ACT}, whose expression results in fully penetrant lethality, the effects of MAE overexpression appear to be partially overcome with time, as some ELAV GAL4; UAS Myc-MAE flies eclose, and exhibit a rough eye phenotype (data not shown). This led us to look at germband retracted embryos (stage 15/16, Figure AIII-2C, C'). While most of the embryos examined appeared dead, exhibiting no staining pattern, there were properly developing, although YAN expression was weaker, germband retracted embryos (Figure AIII-2D, D'). Analysis of embryonic viability revealed that 22.5% of ELAV GAL4; UAS Myc-MAE embryos

Figure AIII-2

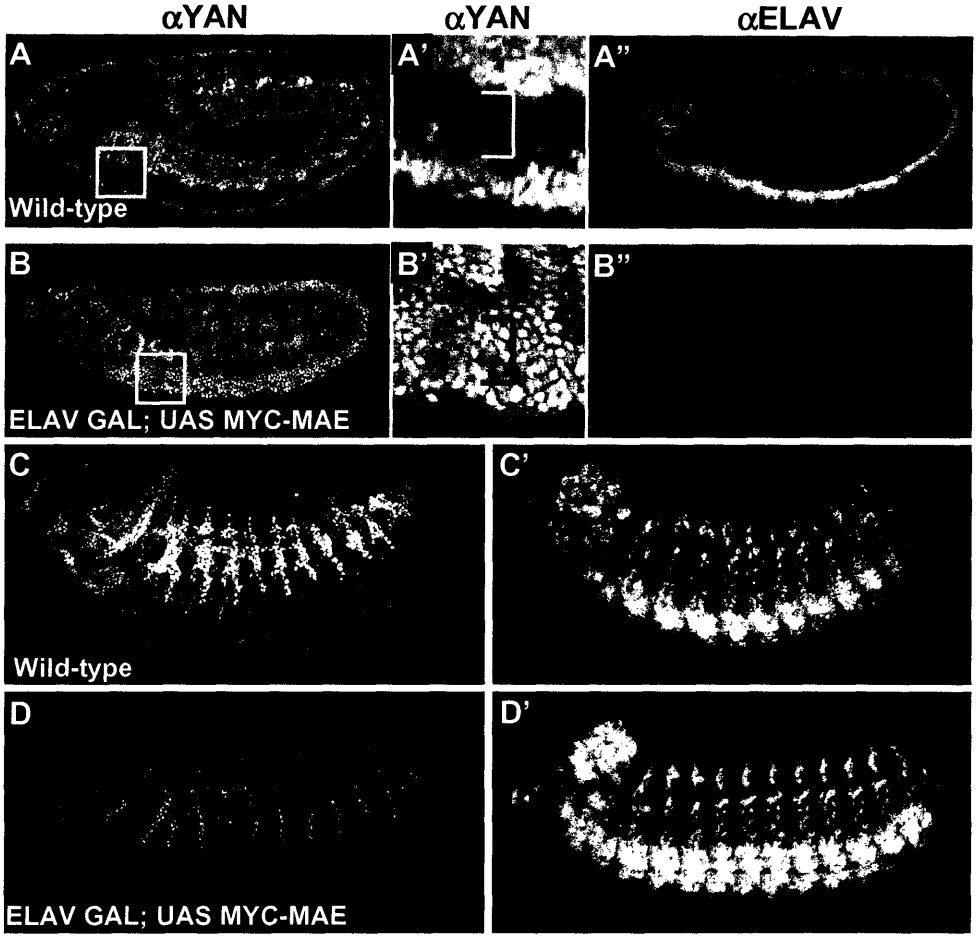

Figure AIII-2

Overexpression of MAE initially restricts YAN to the nucleus in the embryonic CNS. (A-D") Confocal images of *Drosophila* embryos double labeled with anti-YAN MAb8B12 (1:750) , CY3 goat anti-mouse (1:1000) (A-D, A'-B') and rat anti-ELAV MAb 7E8A10 (1:500), and CY2 conjugated goat anti-rat (1:2000) (A"-B". C'-D'). (A', B') (A-B") Germband extended *Drosophila* embryos. Higher magnification views of regions boxed in A-B with normal or failed YAN downregulation highlighted by bracket. ELAV GAL4 was used to drive expression of (B, D) UAS Myc-MAE. Overexpression of MAE restricts YAN to the nucleus and delays neuronal differentiation (B-B") in germband retracted embryos. However, this appears to be a developmental delay as later stages reveal normal YAN localization and proper neuronal differentiation (D-D').

develop to larvae compared to 45.5% of ELAV GAL4 embryos. Of the 22.5% of the ELAV GAL4; UAS Myc-MAE larvae only 43.4% mature to adults. One explanation for why the restriction of YAN to the nucleus by MAE is overcome with time is that enough activated ERK builds up to disrupt the complex, resulting in export and presumably degradation of YAN.

Neither in cultured cells nor in the embryonic developing CNS is YAN aberrantly downregulated by ectopic MAE expression. This could be due formation of a MAE-YAN complex that is refractory to signaling, as suggested by the cell culture experiments. Such a complex could occur naturally at sites of high levels of MAE expression, although there is no evidence for this, or it could be an unnatural phenomena caused by overexpression. However, both loss of function mutations and overexpression of transcriptional regulators are associated with the development of various human diseases including cancer. Thus it is possible that the MAE-YAN complex formed during overexpression of MAE could have implications for human diseases, as loss of function and chromosomal translocations fusing the PD of the human homolog of YAN, TEL, are associated with leukemia and solid tumor development, and the likely homolog of MAE, TEL2b, is known to interact with TEL.

YAN phosphorylation mutants

In an attempt to further understand the roles of MAE in regulating YAN's activity, we analyzed how overexpression of MAE affects the localization of YAN phosphorylation mutants in insect cultured cells. YAN has nine MAPK phosphorylation consensus sequences, P-X-S/T-P. The first consensus site has been shown to be the critical phosphorylation site for YAN regulation (Rebay and Rubin 1995). We found that like wild-type YAN, $YAN^{2\text{-}8S\text{-}A}$, which is nuclear in the absence of high levels of RAS/MAPK signaling and is exported upon signaling, is restricted to the nucleus by MAE in the presence RAS/MAPK signaling (Figure AIII-3).

YAN^{1S-A} always localizes to the nucleus and is unresponsive to RAS/MAPK signaling; overexpression of MAE has no effect on YAN^{1S-A} localization (Figure AIII-3). These data are in agreement with the work of others (Baker et al. 2001) suggesting that MAE plays an integral role in mediating phosphorylation of YAN at the first phosphorylation site.

We next examined the effects of MAE overexpression on the YAN phosphorylation mutants' abilities to repress transcription (Figure AIII-4). We have previously shown that PNT-P1 drives transcription of an EBS-luciferase reporter and co-expression of YAN results in transcriptional repression; this repression is relieved by addition of RAS^{V12}, MAE, or both. When we express YAN^{ACT}, which can not be phosphorylated and thus can not be downregulated by RAS^{V12}, we see that neither RAS^{V12} or MAE, alone or together, significantly relieves the transcriptional repression. This result with MAE is in disagreement with previously published work (Baker et al. 2001) and with repeats of this experiment in the Rebay lab by Pavithra Vivekanand. Both phosphorylation mutants YAN^{1S-A} and YAN^{2-8S-A} exhibit reduced response to RAS^{V12}, most likely due to reduced levels of phosphorylation. These mutants exhibit the same extent of removal of repression by addition of MAE as they do in response to RAS^{V12}, but interestingly addition of both MAE and RAS^{V12} restores the level of response to wild-type levels. This indicates that MAE and RAS^{V12} play independent and additive roles in downregulating YAN. YAN^{S-D}, which mimics the negative charges of phosphorylation and can not be phosphorylated, is also unresponsive to RAS^{V12}, but is almost completely wild-type in its response to addition of MAE. Addition of both RAS^{V12} and MAE do not synergize to remove YAN^{S-D} mediated repression even further. Again, these results further support the idea that MAE is playing a phosphorylation independent role in mediating the downregulation of YAN.

Figure AIII-3

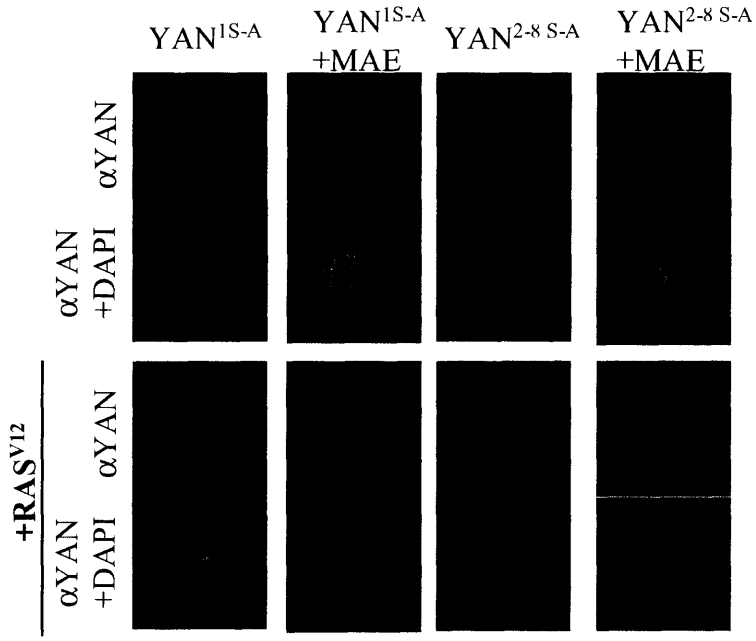

Figure AIII-3

MAE restricts YAN^{2S-A} to the nucleus. *Drosophila* S2 insect cultured cells were transiently transfected with the constructs as labeled and analyzed by immunohistochemistry with anti-YAN MAb 8B12 at 1:250 with CY3 conjugated goat anti-mouse secondary (1:10000) and DAPI (100 μg/ml at 1:5000).

Figure AIII-4

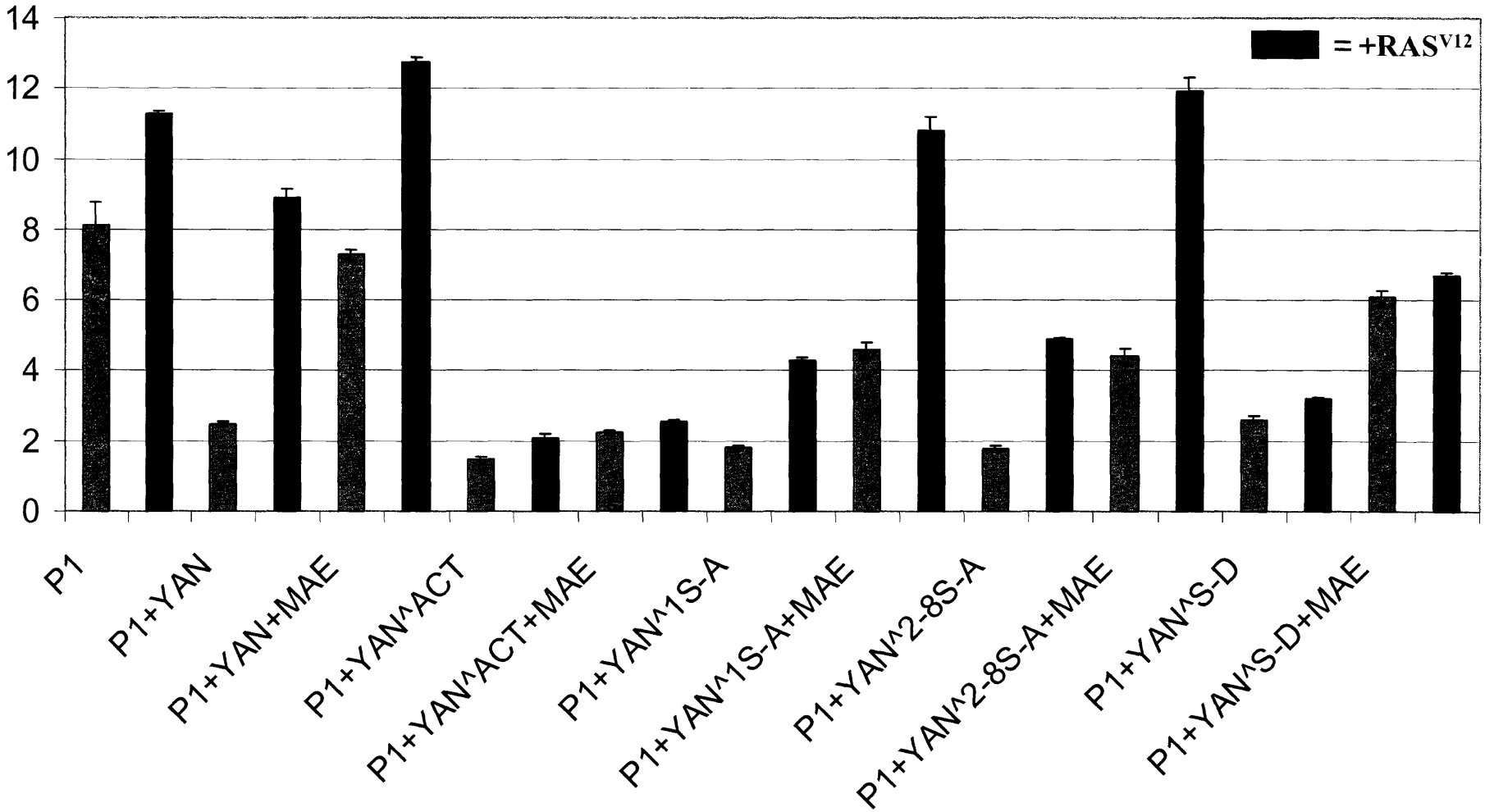

Figure AIII-4

MAE plays a phosphorylation independent role in regulating YAN's transcriptional activity. Transcription assays were performed in *Drosophila* S2 insect cultured cells as described in Chapter 2 (Tootle et al. 2003). As previously shown YAN represses transcription and this repression is relieved by addition of RAS^{V12}, or MAE; addition of both RAS^{V12} and MAE synergizes to remove repression even further. YAN^{1S-A}, which knocks out the key phosphorylation site for regulating YAN, and YAN^{2-8S-A}, which knocks out seven phosphorylation sites but leaves the first, key site intact, are only partially responsive to the addition of RAS^{V12} or MAE, yet addition of both still synergizes to remove repression further. YAN^{S-D}, which mimics the negative charge of phosphorylation at eight of the phosphorylation sites, represses transcription but is not RAS^{V12} responsive. It is however responsive to MAE. The synergism upon addition of both RAS^{V12} and MAE, along with YAN^{S-D} being only responsive to MAE, indicates that MAE has a role or roles in regulating YAN activity separate from its previously identified role in mediating the phosphorylation of YAN.

Together these data indicate that the synergism seen with wild-type, and the S-A mutants of YAN upon addition of MAE and RAS^{V12} is due to separate effects on YAN.

This analysis on the effects of MAE on YAN phosphorylation mutants, supports the previous results (Chapter 2; Tootle et al. 2003), and suggest that MAE plays phosphorylation independent roles in mediating the downregulation of YAN.

The effects of MAE on PNT

As we have shown in Chapter 3, the overexpression of MAE phenotypes in the developing *Drosophila* eye are rescued by overexpression of PNT-P2. This suggests that the phenotype of MAE overexpression is due to loss of PNT function. The most intriguing phenotype of MAE overexpression is the loss of basal YAN expression in third instar larval eye discs. As null *pnt* mutations are embryonic lethal, we utilized an allelic combination of *pnt* mutants that results in viable escapers and analyzed YAN expression in third instar larval eye imaginal discs, revealing wild-type YAN expression (Figure AIII-5). This result suggests that loss of *pnt* function does not result in decreased YAN expression, however this result could simply be due to the hypomorphic nature of these alleles. To really determine the role of PNT in regulating basal YAN expression will require clonal analysis of *pnt* null alleles. Overexpression of PNT-P2 by itself results in increased basal YAN expression (Figure AIII-6), but does not inhibit photoreceptor or cone cell differentiation. This suggests the PNT does play a role in regulation of YAN expression, but as with all overexpression results this could be due to unnatural affects of excess PNT. As discussed in Chapter 3, these data support the model that a complex regulatory network with multiple feedback loops regulates YAN, PNT, and MAE expression and activity.

Figure AIII-5

Figure AIII-5

Hypomorphic *pnt*-/- flies exhibit normal YAN expression in third instar eye imaginal discs. $pnt^{3680\Delta21}/pnt^{78d5\Delta82}$ allelic combination results in viable escaper flies. Eye discs were dissected and stained as described in Chapter 3.

Figure AIII-6

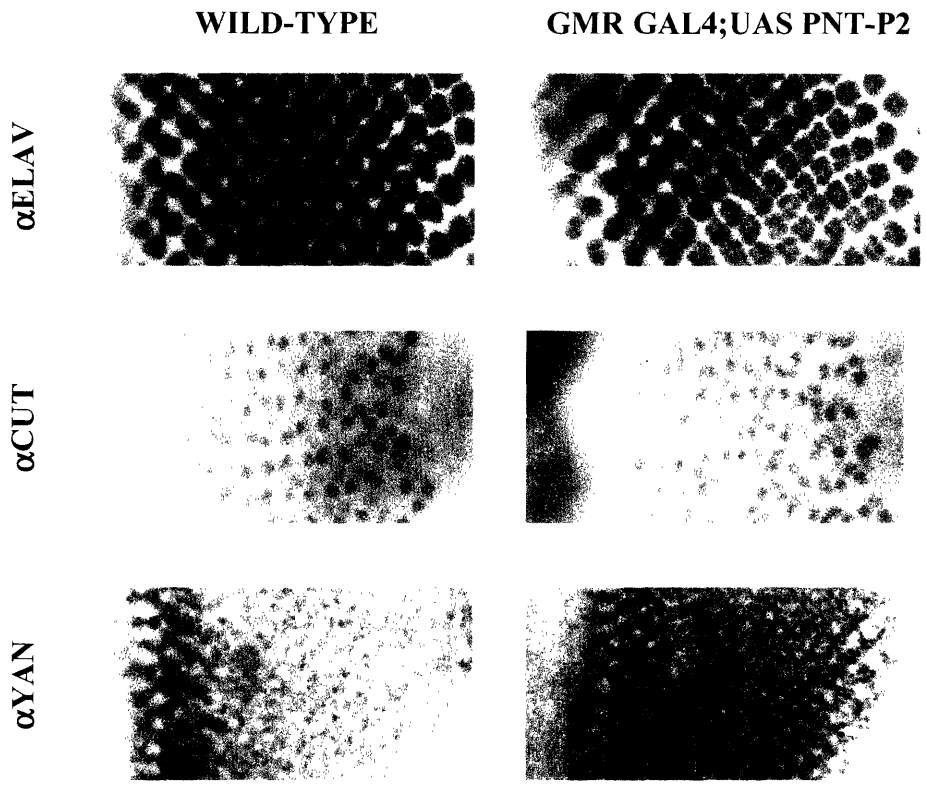

Figure AIII-6

Overexpression of PNT-P2 results in increased basal YAN expression in third instar larval eye imaginal discs with no loss in photoreceptor (anti-ELAV) or cone cell recruitment (anti-cut). Eye discs were dissected and stained as described in Chapter 3.

pnt mutants result in aberrant changes in midline glia, resulting in the loss of separation of the anterior and posterior commissures (Klambt 1993). As overexpression of MAE inhibits PNT-P2 function, both in transcriptional reporter assays (Chapter 2, Tootle et al, 2003; Yamada et al. 2003) and in the developing *Drosophila* eye (Chapter 3), we asked whether overexpression of MAE in the midline glia would exhibit a *pnt-/-* phenotype. Overexpression of MAE in the midline glia exhibited a wild-type phenotype (data not shown). This indicates that in midline glia overexpression of MAE does not inhibit PNT function. A possible reason for this could be that MAE is not normally expressed in this cell type and other regulatory proteins prevent MAE-PNT interactions.

Concluding remarks

While these data do not currently add to our understanding of the roles of MAE in regulating YAN and PNT, in conjunction with future experiments in this area these data may help to further elucidate the activities of MAE.

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