Cell. Mol. Life Sci. (2013) 70:1897–1913
DOI 10.1007/s00018-012-1144-9

Cellular and Molecular Life Sciences

REVIEW

The Eyes Absent proteins in development and disease

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Received: 5 June 2012 / Revised: 24 July 2012 / Accepted: 20 August 2012 / Published online: 13 September 2012

Abstract The Eyes Absent (EYA) proteins, first described in the context of fly eye development, are now implicated in processes as disparate as organ development, innate immunity, DNA damage repair, photoperiodism, angiogenesis, and cancer metastasis. These functions are associated with an unusual combination of biochemical activities: tyrosine phosphatase and threonine phosphatase activities in separate domains, and transactivation potential when associated with a DNA-binding partner. *EYA* mutations are linked to multiorgan developmental disorders, as well as to adult diseases ranging from dilated cardiomyopathy to late-onset sensorineural hearing loss. With the growing understanding of EYA biochemical and cellular activity, biological function, and association with disease, comes the possibility that the EYA proteins are amenable to the design of targeted therapeutics. The availability of structural information, direct links to disease states, available animal models, and the fact that they utilize unconventional reaction mechanisms that could allow specificity, suggest that EYAs are well-positioned for drug discovery efforts. This review provides a summary of EYA structure, activity, and function, as they relate to development and disease, with particular emphasis on recent findings.

Keywords EYA · Eyes absent · Angiogenesis · Cancer · Organ development · Cell migration

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Introduction

The Eyes Absent (EYA) proteins are components of a conserved regulatory network involved in cell-fate determination in organisms ranging from insects to humans. In *Drosophila*, where most of the initial characterization was conducted, this network is referred to as the retinal determination network and is composed of the genes twin of eyeless (*toy*), eyeless (*ey*), sine oculis (*so*), eyes absent (*eya*) and dachshund (*dac*). These genes are regulated by interconnected feedback loops and their protein products form complexes that play critical roles in fly eye development [1–5]. In higher animals, an analogous network variably composed of genes belonging to the *Pax* (corresponding to *toy* and *ey*), *Six* (corresponding to *so*), *Eya* (corresponding to *eya*), and *Dach* (corresponding to *dac*) families play key regulatory roles in the development of multiple organs including the eye, muscle, ears, heart, lungs, endocrine glands, placodes, pharyngeal pouches, craniofacial skeleton, and parathyroid. This network is often referred to as the Pax-Six-Eya-Dach network (PSEDN) to better reflect the vertebrate genes/proteins involved. Because of its deployment in multiple developmental contexts and its high degree of conservation through animal evolution, the PSEDN has been extensively studied.

A unique feature of the EYA proteins is that they combine several biochemical activities in a single polypeptide: independent protein tyrosine phosphatase (PTP) and threonine phosphatase domains, and a transcriptional activation domain. Each of these activities has been linked to distinct cellular functions, biological roles, and disease states. There is now an emerging understanding of the molecular mechanisms and regulation of EYA's biochemical activities, the cellular pathways influenced by

EYAs, and the biological context in which these processes occur. This is leading to a better understanding of the role of EYAs in both normal development and in disease, thereby raising the possibility that EYAs might be attractive molecular targets for therapeutic intervention. Here we focus on the EYA proteins, their structure, mechanism of action, and association with human development and disease.

EYA protein architecture and activities

Vertebrates encode four EYA proteins (EYA1–4) that are characterized by a conserved C-terminal 271 amino-acid domain commonly referred to as the EYA domain (ED) (Fig. 1). Animal EYA proteins have a poorly conserved N-terminal domain (NTD) that ranges in size between 266 and 320 amino acids in vertebrates, but is 491 amino acids long in *Drosophila*. In contrast, plant EYA does not have an NTD.

The conserved EYA domain

The ED was originally described as a protein interaction domain involved in binding the SIX and DACH proteins [6–8]. It was later recognized that the ED also includes the sequence motifs characteristic of the large haloacid dehalogenase class of enzymes [7, 9, 10] and that it has tyrosine phosphatase activity [9, 10]. This observation linked the PSEDN to signal transduction and altered the conventional view that it was a purely transcriptional network. The EYAs represent a novel mechanistic class of PTPs as they do not have the signature Cys-containing motif that traditionally defines the large PTP family (reviewed in [11, 12]). Instead EYAs use an aspartate residue as a nucleophile and require a divalent metal ion in the active site to catalyze tyrosine phosphate hydrolysis. To date, the only other tyrosine phosphatase shown to share this reaction mechanism is the TFIIF associating component of CTD phosphatase/small CTD phosphatase (FCP1/SCP) [13].

Three-dimensional structural data are available for the EYA2 ED [14]. Like most HAD family enzymes, the EYA2 ED is composed of two subdomains: a "core" catalytic domain that includes all three conserved motifs that bear the catalytic residues, and an inserted helical "cap" domain (Fig. 1b). A divalent metal ion is found in the active site. The cap domain is positioned such that it forms part of the catalytic center, and is thus likely to participate in substrate binding and/or selection.

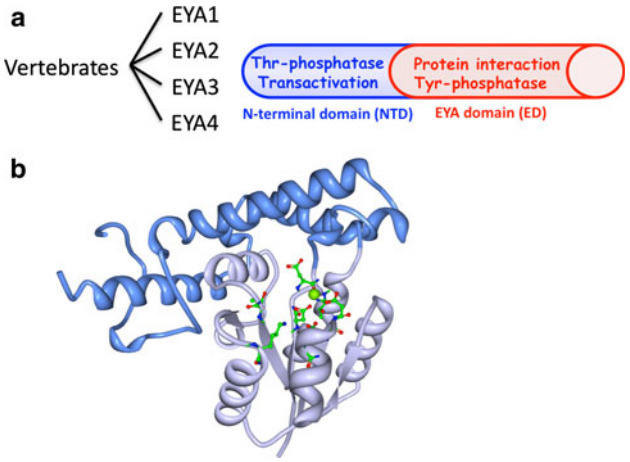

Fig. 1 Molecular architecture of the EYA proteins. **a** There are four vertebrate EYA proteins. They have a well-conserved C-terminal domain called the EYA domain (*ED*) and a poorly conserved N-terminal domain (*NTD*). The ED participates in protein interactions, notably with the SIX family of proteins that are part of the PSEDN. The ED also has tyrosine phosphatase activity. The NTD has transactivation and threonine phosphatase activities. **b** The three-dimensional structure of the EYA2 ED as determined by X-ray crystallography [14]. It has two subdomains referred to as the core (*light blue*) and cap (*dark blue*) domains. The active site residues are shown in *green*. A divalent metal ion (*green sphere*) in the active site participates in the catalytic reaction

The N-terminal domain

While the amino acid sequence in the NTD is very poorly conserved (Table 1), in all cases it is rich in Pro, Ser and Thr residues reminiscent of the proline/serine/threonine-rich transactivation domains [15]. Xu et al. [16] used a classical GAL4–DNA binding domain fusion of the NTD to demonstrate that it could modestly activate the expression of a CAT reporter. Subsequently Ohto et al. [6] showed that the EYA proteins can be localized on DNA via the homeodomain-containing SIX proteins and can activate transcription from SIX-responsive elements. Thus the prevailing model (Fig. 2) holds that the intrinsically cytosolic EYA proteins are translocated to the nucleus as complexes with SIX proteins, and that the SIX–EYA complex can activate transcription, whereas the SIX proteins by themselves are generally transcriptional repressors.

Most recently the NTD has been shown to have threonine phosphatase activity [17, 18]. While the NTD bears no sequence resemblance to any known family of Thr phosphatases, Alanine replacement of a set of four relatively conserved tyrosine residues disrupts threonine phosphatase activity [17]. Whether this observation reflects involvement of the tyrosine residues in the catalytic function, or general disruption of the three-dimensional structure of the NTD remains to be determined.

Despite the apparent conservation of biochemical function across species, the NTDs of bilaterian EYAs bear little homology at the amino acid level to EYAs from protostomes. Furthermore, plant EYAs have no true NTD,

Table 1 Amino acid sequence conservation among human EYA1, EYA2, EYA3, EYA4, and *Drosophila* (dm) Eya

	EYA1		EYA2		EYA3		EYA4		Dm Eya	
	Percentage identity	Percentage similarity	Percentage identity	Percentage similarity	Percentage identity	Percentage similarity	Percentage identity	Percentage similarity	Percentage identity	Percentage similarity
EYA1			*82.7*	*92.3*	*72.1*	*84.9*	*88.6*	*95.2*	*69.1*	*83.6*
EYA2	**39.4**	**55.6**			*68.4*	*82.7*	*79.8*	*91.2*	*64.4*	*82.5*
EYA3	**26.8**	**44.8**	**28.8**	**36.5**			*73.2*	*85.7*	*61.8*	*77.5*
EYA4	**54.3**	**68.9**	**29.7**	**43.9**	**25.3**	**48.7**			*78.2*	*84.4*
Dm Eya	**11.2**	**22.8**	**6.7**	**16.9**	**7.7**	**21.8**	**13.8**	**25.5**		

Italics EDs, **bold** NTDs.

Accession numbers for the protein sequences: EYA1 NP_000494.2, EYA2 NP_005235.3, EYA3 NP_001981.2, EYA4 AAH41063.1, dm Eya AAF52400.

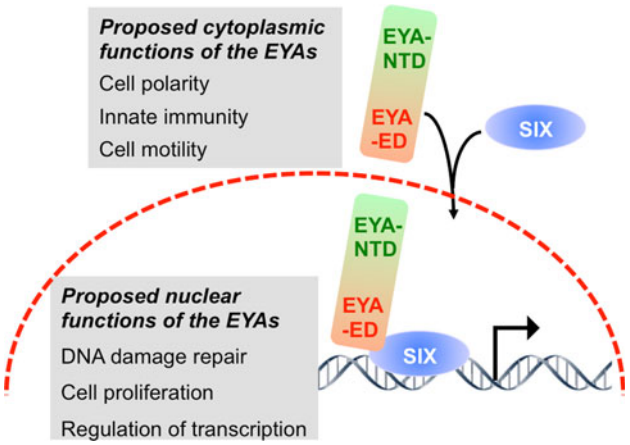

Fig. 2 EYA subcellular localization and functions. The EYAs are intrinsically cytoplasmic proteins. Upon interaction with the SIX proteins (with the exception of SIX3) they are translocated into the nucleus where they are localized on DNA via the homeodomain of the SIX proteins, and convert the SIX proteins into transcriptional activators. The proposed cellular functions for cytoplasmic and nuclear EYA are shown in the *gray boxes* and discussed in the text

but just a short stretch of 18–23 residues [19]. This raises the possibility that an ancestral plant EYA PTP gained an NTD and a transcriptional function in animals. Pertinent to this is the observation that plants do not encode orthologues of the other members of the PSEDN, and thus there is no conservation of this transcriptional regulatory cascade between plants and animals.

EYA: a protein phosphatase with dual specificity

Conventional dual specificity phosphatases (DUSPs) are characterized by their ability to dephosphorylate both phosphotyrosine and phosphoserine/threonine residues within the same substrate using the same catalytic domain [20, 21]. In contrast, EYA's threonine phosphatase and tyrosine phosphatase activities exist in separate domains, and there is no evidence yet that EYAs can dephosphorylate both phosphothreonine and phosphotyrosine residues in the same substrate. Hence, EYAs do not fit the classical definition of DUSPs, but rather represent a unique and unusual class of phosphoprotein phosphatases with dual specificity. It remains to be established whether the NTD and EDs regulate each other's catalytic activities.

To date the only truly validated substrate for the EYA tyrosine phosphatase activity is the minor histone protein H2AX (described in detail below) [22, 23]. No substrate has yet been identified for the threonine phosphatase activity. Given the growing list of cellular roles that are emerging for the EYA family of proteins, it is highly likely that multiple EYA substrates will be identified in the future.

EYA: a transcriptional activator-phosphatase

Early studies in *Drosophila* characterized the transcriptional role of EYA through genetic and/or biochemical interaction with the SO/SIX [24] and DAC/DACH [25] classes of transcription factors. SIX proteins physically interact with, and actively translocate EYAs from the cytoplasm to the nucleus [6, 24, 26]. Since EYA has no recognized DNA binding activity, but possesses a transactivation domain, it is widely accepted that EYAs act as transcriptional coactivators upon recruitment by the SIX protein [6, 16, 27]. A direct interaction between Eya and Dac is thought to underlie the synergistic induction of ectopic eyes in *Drosophila* [25], and a similar interaction has been reported between mouse EYA2 and DACH2 [26]; however, a later study revealed that the interaction between EYA and DACH may be mediated by the CREB binding protein (CBP) [8]. DACH is believed to be a transcriptional repressor that binds directly to specific DNA sites

Table 2 Experimentally validated EYA binding proteins [86]

EYA binding partner	Validation method	Functional outcome	References
SIX/So and DACH/Dac	Yeast two-hybrid, genetics, immunoprecipitation	Nuclear translocation of EYA by SIX; increase in SIX transcriptional activity by EYA; alleviation of DACH repressor activity toward SIX; increase in SIX-DNA binding affinity	[8, 25, 86, 153]
Gαz and Gαi	Yeast two-hybrid, immunoprecipitation, immunofluorescence	Recruitment of EYA to plasma membrane and prevention of SIX-mediated nuclear translocation	[46, 154]
SIPL1 and RBCK1	Yeast two-hybrid, immunoprecipitation	Enhancement of the transactivation potential of the SIX–EYA complex	[49]
aPKC-zeta	Immunoprecipitation	Dephosphorylation of aPKC-zeta and NUMB; regulation of polarity in the lung epithelium	[50]
ATM/ATR	Immunoprecipitation	Phosphorylation of EYA; interaction of EYA with γH2AX	[22, 51]
γH2AX	Immunoprecipitation, immunofluorescence	Dephosphorylation of pY142-γH2AX; initiation of DNA damage repair	[22, 23]
Abl kinase	Immunoprecipitation, genetics	Phosphorylation of Eya and retention in the cytoplasm	[52]
Nemo kinase	Immunoprecipitation, genetics	Phosphorylation of Eya; potentiation of transcriptional activity of the Eya–So complex	[53]
IPS-1, STING, NLRX1	Immunoprecipitation	Dephosphorylation of a phospho-Ser/Thr substrate; enhancement of innate immune response	[17, 44]
SOX2	Immunoprecipitation	Possibly acts with EYA1 in the generation of progenitor cells in the otocyst	[54]

[28, 29] and recruits the coregulator transcriptional elongation regulator 1 (TCERG1) [30]. The DACH–CBP–EYA complex acts as a transactivator [8]. Similarly, multiple studies suggest that the SIX proteins are either repressors of transcription or weak activators. In either case, the presence of EYA converts them into strong activators of transcription [6, 7, 31, 32]. Moreover, no SIX- and DACH-independent transcriptional activities of EYA have yet been clearly described.

While GAL4–DNA-binding domain fusions of the EYA-NTD can activate transcription on their own [16], transactivation by a SIX–EYA complex is dependent on the EYA tyrosine phosphatase activity [7]. This observation is intriguing and implies that the N-terminal transactivation activity is somehow regulated by the C-terminal phosphatase activity, or that a substrate of the SIX–EYA complex is involved in transcriptional activation. The precise mechanism underlying this observation is yet to be determined. There has been some suggestion that the phosphatase activity of the EYAs may participate in a cytoplasmic cellular function while the transactivation activity of the SIX–EYA complex represents a nuclear function. This is borne out by evidence that cytoplasmic EYA4 plays a role in innate immunity via its threonine phosphatase activity [17] and that cytoplasmic EYA3 promotes cell motility via its tyrosine phosphatase activity [33]. However, the role of the EYA tyrosine phosphatase activity in DNA damage repair [22] is clearly a nuclear function.

In the *Drosophila* eye, EYA target genes include *lozenge*, *hedgehog*, *eyeless*, *so* and *atonal* (reviewed in [34]). In vertebrates, EYA target genes are implicated in the development of multiple organs and include Na^+/K^+ *ATPase α1 subunit*, *myogenin*, *Igfbp5*, *aldolase A*, c-*myc*, *Gdnf*, *cyclin A1*, *cyclin D1*, *Slc12a2*, *p27Kip1*, *muscle creatine kinase*, *ezrin* and *Six2* [7, 35–43]. In the immune response to viral infection, EYA4 promotes the expression of interferon-beta (IFN-β) and CXCL10 [17, 44].

EYA binding proteins

In a study aimed at mapping the interactome of the worm *Caenorhabditis elegans,* EYA was found to have an unusually large number of interacting partners [45]. EYA binding proteins supported by experimental evidence are listed in Table 2. The SIX and DACH transcription factors remain the best-characterized and functionally validated EYA-binding proteins. Nuclear translocation of EYA can be prevented by EYA interaction with the alpha subunits of Gz and Gi proteins [46]. This might be relevant to eye development, as a mouse knockout of a G-coupled protein receptor recapitulates anterior ocular defects seen in patients with mutations in the *EYA1* gene [47, 48]. On the other hand, EYA's cotranscriptional activator function can be enhanced by its interaction with SIPL1 (shank-interacting protein-like 1) and RBCK1 (RBCC protein interacting with PKC1), and this interaction is important in craniofacial development in mouse and zebrafish [49].

In lung epithelial morphogenesis, EYA1 forms a complex with several polarity proteins including PAR3, PAR6, NUMB, LGN and MLNSC, through its direct binding to the zeta isoform of the atypical protein kinase C (aPKC-zeta) [50]. In response to DNA damage, EYA3 is phosphorylated by the ataxia telangiectasia mutated (ATM)/ATM Rad3-related (ATR) kinase, and upon activation, can bind to and dephosphorylate H2AX, a prerequisite for DNA damage repair [22, 51].

In *Drosophila* eye development, the interaction of Eya with the Abelson (Abl) tyrosine kinase (which phosphorylates Eya) is required for Eya to function as a cytoplasmic protein phosphatase [52]. The *Drosophila* MAP kinase family member Nemo regulates retinal determination genes by phosphorylating Eya, thereby potentiating the transcriptional activity of the Eya–So complex [53]. In its role as stimulator of the innate immune response against viruses, EYA4 forms a complex with the IFN-β promoter stimulator 1 (IPS-1), the stimulator of IFN genes (STING) and the nucleotide-binding oligomerization domain leucine-rich repeat containing X1 (NLRX1) in immune cells [17, 44, 52]. EYA1 also forms a complex with the transcription factor SOX2 in the sensory epithelium of the inner ear and in the future organ of Corti, implicating this interaction in the development of sensory progenitors as well as hair cell differentiation [54]. SOX2 is the only DNA-binding protein (other than the SIX/So proteins) thus far identified as an EYA interaction partner, raising the possibility that a SOX2–EYA1 complex could regulate expression of SOX2-dependent genes. While such SOX2–EYA1 targets are yet to be described, SOX2, SIX1 and EYA1 are known to interact and regulate *Atoh1* expression through both SOX2 and SIX1 binding sites in the *Atoh1* enhancer, and to specify hair cell fate in the developing ear [55].

Cellular functions of the EYAs

EYA in cell proliferation and survival

Embryonic growth and patterning is dependent upon the proper balance between proliferation and cell death. *Drosophila* germ-line *eya* mutations are embryonic lethal, and eye-specific mutations are characterized by massive cell death in the eye primordium [3, 4]. In *C. elegans*, loss of *Eya1* by RNAi and deletion mutations result in early larval lethality with incomplete penetrance, and late embryos display pharyngeal malformation and excess cell death in the anterior region [56]. Zygotic depletion of *Xeya3* induces local apoptosis in the anterior neural plate, and overexpression of exogenous *Xeya3* is able to enlarge the neural plate by causing an over-proliferation of neural precursor cells [57]. Mice mutant for *Eya* display abnormal apoptosis and reduced cell proliferation during the development of multiple tissues including the kidney, muscle, and ear [7, 58, 59]. EYA has also been shown to regulate proliferation through negative modulation of Sonic hedgehog signaling in mouse lung epithelium [60]. Although the developmental role of the EYA proteins appears to be overwhelmingly antiapoptotic and proproliferative, there are instances in which it has a proapoptotic function. Overexpression of *Eya* triggers apoptotic cell death in 32D.3 murine myeloid cells [61], and the *C. elegans* SIX protein (CEH-34) and EYA1 cooperate to promote programmed cell death of a particular pharyngeal neuron by directly activating expression of the proapoptotic protein egl-1 [62].

EYA in cell migration

The process of cell migration is fundamental to embryogenesis, as the initial morula ball segregates into the three main germ layers and cells move relative to each other to shape and populate different tissues. The promigratory function of EYA was first reported in *Drosophila*, since mutant embryos displayed defects in germ cell migration and head morphogenesis [1, 63]. In *C. elegans*, *Eya1* mutants are characterized by a loss of directionality in the migration of gonad cells [56]. In vertebrates, *Eya* transcripts are expressed in migrating cells, including muscle precursor cells, neural crest cells and their derivatives [57, 64–67], suggesting that EYA proteins may play a role in developmental cell migration. Overexpression of *Eya*s in breast epithelial cell lines increases single cell migration [33, 68], and siRNA-mediated depletion of *EYA4* in malignant peripheral nerve sheath tumor (MPNST) cells [69], or *EYA3* in endothelial cells [68], reduces their migration in Transwell assays. While all of these observations support a role for EYAs in promoting cell migration, the precise cellular signaling pathways involved have not yet been delineated. However, links between EYA phosphatase activity and processes fundamental to cell motility such as GTPase activation and cytoskeletal architecture have been observed in mammary epithelial cells [33].

EYA in DNA damage repair

The most validated substrate for the EYA tyrosine phosphatase activity thus far is the minor histone protein H2AX that links EYA to DNA damage repair [22, 23]. In both mouse embryonic kidney and human embryonic kidney cell lines, H2AX is dephosphorylated at the C-terminal tyrosine 142 (pY142) by EYA1, EYA2, and EYA3. This permits recruitment of the MDC1/MRN repair complex

and tips the balance towards survival rather than cell death. While phosphorylation of H2AX at Ser139 following DNA damage has been well-established (reviewed in [70]), phosphorylation at tyrosine 142 has only recently been uncovered. Xiao et al. [71] have reported that the William-Beuren syndrome transcription factor (WSTF; also known as BAZ1B) phosphorylates H2AX tyrosine 142 utilizing a novel kinase domain. Furthermore, Xiao et al. [71] showed that H2AX is constitutively phosphorylated at tyrosine 142 in mouse embryonic fibroblasts and that Y142 phosphorylation levels fall, while Ser139 phosphorylation levels rise, upon DNA damage. However, Xie et al. [72] could not detect H2AX Y142 phosphorylation using mass spectrometric techniques, and they did not observe differences between the abilities of wild-type and Y142F H2AX to promote either MDC1 focus formation or homologous recombination in mouse embryonic stem cells. This discrepancy could reflect true cell-specific and/or condition-specific differences of biological interest, or merely technical differences in the referenced studies.

EYA and angiogenesis

Endothelial cell migration, a process promoted by EYA, is a prerequisite for angiogenesis—the formation of new blood vessels. EYA tyrosine phosphatase activity has recently been shown to contribute to sprouting angiogenesis in cell culture-based assays, ex vivo assays, and in a zebrafish model system [68]. Although angiogenesis is essential for vascular development throughout embryogenesis and in the formation of functionally distinct, tissue-specific vascular beds, there is only one report of a vascular defect upon *Eya* deletion; Eya1−/− embryos show hemorrhage around the large pulmonary vessels attributed to vascular smooth muscle defects that weaken the blood vessels [60]. It is possible that compensation by other isoforms of *Eya* mask more widespread vascular phenotypes, or that the phenotypes are subtle and thus escaped notice.

The mechanism by which EYA modulates angiogenesis has not yet been defined. However, recent evidence links the DNA damage response to hypoxia-induced angiogenesis [73]. Specifically, hypoxia induces replicative stress and stalled replication forks. This activates the ATR kinase, leading to phosphorylation of H2AX (to yield γ-H2AX; phosphorylated on Ser139), the recruitment of MDC1 and DNA repair. Thus the proliferative potential of endothelial cells is restored leading to neovascularization. Economopoulou et al. [73] have shown that inactivation of H2AX substantially impairs neovascularization in several hypoxic conditions including retinal disease, hindlimb ischemia and tumor growth. Given the established link between EYA and H2AX-mediated recruitment of MDC1 and other MRN components, it is possible that activation of EYA in hypoxic conditions, both during normal development and in disease, promotes DNA damage repair via H2AX tyrosine dephosphorylation. This in turn could promote angiogenesis.

Salient to this discussion, a recent report shows that SIX1 in breast cancer cells promotes lymphangiogenesis by upregulating *VEGF-C* expression [74]. A SIX1 binding site was reported in the *VEGF-C* promoter. As in other contexts, SIX1 activates *VEGF-C* transcription only in the presence of EYA2, thus implicating EYA2 in promoting lymphangiogenesis. This mechanism is likely to be distinct from the angiogenic role for EYA in endothelial cells described by Tadjuidje et al. [68].

EYA and developmental cell polarity

Oocyte polarity is a prerequisite to normal cleavage and the establishment of proper cell fate in animal embryos. Tissue polarity is important for proper differentiation of embryonic tissue and homeostasis of adult tissues [75, 76]. *Drosophila* Eya is a key repressor of polar cell fate during oogenesis [77], and EYA1 is involved in the control of cell polarity and mitotic spindle orientation in lung epithelium [50]. Therefore, EYA may play a role in polarity not only during the early cleavage stage, but also later in development when highly specialized epithelial tissues differentiate. aPKC-zeta is a proposed EYA1 tyrosine phosphatase substrate reported to play a role in specifying the apical–basal polarity of lung epithelial cells [60] since aPKC-zeta tyrosine phosphorylation levels are increased upon *Eya1* knockdown. However, the literature provides little information on either tyrosine phosphorylation site(s) on aPKC-zeta or the role of aPKC-zeta tyrosine phosphorylation, hence the molecular mechanism by which EYA1 specifies cell polarity remains unclear.

EYA in innate immunity

Okabe et al. [17] identified EYA4 as a regulator of the innate immune response to challenge from either cytosolic nucleic acids or undigested DNA from apoptotic cells. In vitro, EYA4 leads to phosphorylation of IRF3 and upregulation of NF-κB, and enhanced induction of IFN-β and CXCL10. These authors propose that EYA4 initiates this response as a complex with RIG-1, TNF receptor-associated death domain, TNF receptor-associated factor 3 and NEMO (IKKγ). They further showed that the N-terminal threonine phosphatase activity of EYA4 is required for this function. While a role for EYA in nucleic acid-induced immunity is yet to be established in vivo, EYA as a mediator of such a response, possibly by dephosphorylating and inactivating an inhibitory kinase, is an attractive possibility.

Eyes Absent in development

In *Drosophila,* specific recessive *eya* mutations result in elimination of compound eyes in viable flies, while *eya* null mutations are embryonic lethal [4, 63, 78]. This could imply that Eya is required in other embryonic tissues and prior to eye development. In support of this, *eya* transcripts are expressed during embryogenesis of plants [19], squid and worms [56, 79], flies [3] and vertebrates [57, 64, 67, 80–83]. Expression data from flies and vertebrates are consistent with a requirement for EYA function during the entire process of animal embryogenesis, beginning from the oocyte in which the transcript is deposited during oogenesis [57, 77]. This correlates with the numerous defects observed upon genetic inactivation of *Eya* genes in mice (Table 3). Here we outline the role of EYA in the development of organs where there is a clearly established association with human developmental disorders.

EYA in eye development

Early studies showed that *eya* mutation leads to a lack of compound eyes in flies with otherwise normal head structures [84, 85]. Furthermore, forced ectopic expression of *eya* is sufficient to induce retinal development [5]. Despite this strong link between *eya* and retinal development in *Drosophila*, a no-eye phenotype has thus far not been reported in mouse *Eya1*, *Eya2*, *Eya3* and *Eya4* mutants. It is possible that functional redundancy in the vertebrate eye, where multiple *Eyas* are expressed in overlapping patterns, may underlie this discrepancy. In particular, *Eya1* and *Eya2* are expressed in a complementary fashion in the developing retina, with the lens placode being the only optic structure that only expresses *Eya1* [16, 64]. *EYA1* mutations have been reported in human patients with congenital cataracts [47], suggesting a role for EYA in human eye development. Moreover, transgenes of mouse *Eya1*, *Eya2* and *Eya3* can rescue the eyeless phenotype in the *Drosophila eya* mutant [86], suggesting that vertebrate EYAs are indeed endowed with eye specification potency. The recently uncovered role for EYAs in angiogenesis and cell migration may also point to roles in developmental retinal angiogenesis that will be uncovered upon more detailed and targeted analyses.

EYA in kidney development

EYA1 and *EYA2* are expressed in fetal and adult human kidneys, respectively [79, 87]. Mouse *Eya1* is expressed in the developing kidney, and *Eya3* is expressed in the adult kidney [81, 87, 88], chick *Eya1* is expressed in the nephrogenous mesenchyme [89], and *Xenopus eya2* is expressed in the nephric mesoderm [82]. Moreover, *Eya1*-mutant homozygous mice lack kidneys, and molecular analysis suggests that *Eya1* expression in the metanephric mesenchyme is required for the expression of the gene encoding *glial-derived neurotrophic factor* (*Gdnf*), which in turn is required to direct ureteric bud outgrowth [59, 90]. *Six1*-deficient mice lack kidneys, but have ureters. Detailed analysis has revealed that the initial expression of *Gdnf* is not lost; therefore, the ureteric bud emerges from the Wolffian duct, but fails to invade the mesenchyme, which subsequently leads to apoptosis in the mesenchyme [91, 92]. *Six2*-deficient mice have hypoplastic kidneys due to depletion of the progenitor cell population within the metanephric mesenchyme as a result of a premature and ectopic differentiation of mesenchymal cells into epithelia [93]. Since EYA1 is required very early in kidney development, a comparison of *Eya1*, *Gdnf*, *Six1* and *Pax2* mutant mice led to the suggestion that *Eya1* probably functions at the top of the genetic hierarchy controlling kidney organogenesis [90]. Surprisingly, no *Eya1* expression has been reported in the developing kidney of frog or fish [66, 67, 94]. Thus the function of EYA1 in nephrogenesis might be a characteristic of higher vertebrates, or another EYA may undertake this function in lower vertebrates as suggested by the expression of *eya2* in *Xenopus* nephric mesoderm.

Table 3 Phenotypes of *Eya* mutant mice

Mouse mutant	Phenotype	References
Eya1−/−	Die at birth; craniofacial and skeletal defects; ear malformation; dysmorphic/absent kidneys; thymus and parathyroid agenesis; thyroid hypoplasia; open eyelids; hypoplastic lungs	[59, 60, 155]
Eya2−/−	No external phenotype; viable and fertile	[156]
Eya1−/−	No diaphram; severe limb muscle hypoplasia	[156]
Eya2−/+		
Eya3−/−	Viable and fertile with no external phenotype; reduced body length	[108]
Eya4−/−	Die shortly after birth; abnormal ear development and hearing deficiency; males sterile or reduced fertility	[105]

EYA in ear development

Disorders of the auditory system coexist with renal anomalies in humans with mutations of *EYA* genes [95–98], and *EYA4* mutations cause late onset deafness [99]. Expression and loss of function data support a role for EYA in the development of the auditory system. The otic vesicle develops from the otic placode. In zebrafish, amphibians and chick, all placodes originate from a common precursor domain, the preplacodal region, marked by the expression of *Six1* and *Six4*, and *Eya1* and *Eya2* [100]. In zebrafish, Eya1 is found in the developing otic primordium as early as the placodal stage [49, 66], and mutations in the *eya1* gene or morpholino-mediated knockdown of the protein cause the dog-eared phenotype which is characterized by a defect in the formation of the inner ear [101]. In *Xenopus, eya1* and *eya2* transcripts are expressed in the otic placode [67, 82], and immunostaining reveals that the eya1 protein displays a distinctive expression pattern during multiple stages of ear development [102]. Moreover, overexpression of mutant forms of *eya1* mRNA cause *Xenopus* embryos to develop with dysmorphogenesis of the otic vesicle, defects in the establishment of sensory tissue and defective otic innervations [103]. Mouse *Eya1* is expressed in the developing otic vesicle [64] and *Eya1*-deficient mice lack ears [59]. It is suggested that EYA1 is required in a dose-dependent manner for proper ear development because hypomorphic mutations of the *Eya1* gene result in inner ear malformations and hearing defects in mice and humans [54]. Furthermore, EYA1 and SIX1 can induce the putative neurosensory stem cells in the cochlea (GER cells) to differentiate into hair cells [55], while coexpressed EYA1, SIX1 and SOX2 can induce GER cells to differentiate into neurons [104]. Thus EYA1 (along with SIX1) initiates neuronal development in the inner ear. On the other hand, EYA4 is involved in middle ear development, with *Eya4−/−* mice having abnormal middle ear cavities and eustachian tube dysmorphology [105]. They are also more prone to otitis media, and could represent a valuable animal model for this common childhood disease.

EYA in heart development and function

Mutations in *EYA4* cause dilated cardiomyopathy, a disorder characterized by ventricular dilation and impaired systolic function resulting in congestive heart failure and arrhythmia [106]. Cardiofacial syndrome, a combination of an asymmetric crying face and heart defects, associated with an *EYA1* mutation has also been reported [107]. Consistent with these findings, transcripts of *EYA1* and *EYA2* are expressed in the adult human heart [79, 81, 87, 106]. In zebrafish, *eya4* is expressed in both the embryonic and adult heart, and antisense morpholino-mediated depletion of *eya4* causes a progressive ventral swelling due to pericardial edema, suggestive of cardiovascular dysfunction. Further analyses have revealed that *eya4*-depleted embryos have smaller ventricles than controls [106]. Although there is no report of cardiac expression of Eyes Absent during mouse embryogenesis, *Eya2* and *Eya3* are expressed in the adult heart [79, 81, 108]. Moreover, *Eya3*-deficient young adult mice show a defect in the electrophysiology of the heart, possibly suggesting a role for EYA3 in heart function [108]. EYA2 appears to be an important regulator of both pathological and physiological hypertrophy. *Eya2* is upregulated during regression of cardiac hypertrophy and blocks phenylephrine-induced development of cardiomyocyte hypertrophy in vitro. Similarly, *Eya2* is upregulated during recovery following transverse aortic constriction (a treatment that causes prominent cardiac hypertrophy) [109], suggesting that EYA2 may function during the regression of hypertrophy. Transgene-mediated overexpression of *Eya2* inhibits development of cardiac hypertrophy, and prevents wall thinning, ventricular dilation and deterioration of cardiac function as well as fibrosis and inflammation in the heart under long-term pressure overload. In addition, this prevention of pathological hypertrophy and heart failure by EYA2 correlates with the elevation of genes involved in mitochondrial biogenesis and metabolism in transgenic mice [110]. *Eya2* expression is upregulated in hearts with swimming exercise-induced physiological hypertrophy [111].

In summary, although there is no solid evidence for the involvement of EYAs in mammalian heart development, there is considerable evidence that EYA is critical for cardiac function. Pathological and physiological cardiac hypertrophy are known to have different molecular signatures [112], and the involvement of EYA in both suggests that EYA could play a cardioprotective role.

EYA in craniofacial development

The craniofacial complex (including the head, face and oral cavity) is mostly formed of tissue derived from the neural crest and mesoderm. Many congenital craniofacial malformations display major anomalies in neural crest cell patterning, but often they arise as a secondary consequence of anomalies in other tissues with which the neural crest cells interact during their development and migration (reviewed in [113]). Cranial placodes and neural crest cells also depend upon each other for proper development. Craniofacial anomalies are part of the phenotypic characteristics of *EYA* deficiency-related syndromes such as cardiofacial and otofaciocervical syndromes, indicative of a role for EYA in craniofacial development [97, 107, 114, 115]. Consistent with the human phenotypes, *Eya*

Table 4 EYA and human disease

Disease	Gene	Type of misregulation	References
Branchiootorenal syndrome	*EYA1*	Loss of function mutations	[87, 95]
Otofaciocervical syndrome	*EYA1*	Loss of function mutations	[97]
Cardiofacial syndrome	*EYA1*	Loss of function mutations	[107]
Congenital cataract	*EYA1*	Loss of function mutations	[47]
Wilms' tumor	*EYA1*	Overexpression	[137]
EBV-negative gastric cancer	*EYA1*	Frequent methylation	[138]
Late-onset deafness	*EYA4*	Loss of function mutations	[99]
Dilated cardiomyopathy	*EYA4*	Deletion	[106]
Esophageal adenocarcinoma	*EYA4*	Frequent methylation (overexpression)	[128]
Colon cancer	*EYA4*	Overexpression	[130]
Colorectal cancer	*EYA4*	Overexpression	[131]
Colorectal cancer	*EYA2*	Silencing methylation	[139]
Epithelial ovarian cancer	*EYA2*	Overexpression	[133]
Lung adenocarcinoma	*EYA2*	Overexpression	[134]
Pancreatic ductal adenocarcinoma	*EYA3*	Frequent deletion	[140]

transcripts are expressed in cranial placodes and/or their derivatives during the embryogenesis of lower vertebrates. In *Xenopus*, *eya1* is expressed in all neurogenic placodes [67, 94], and reducing eya1 protein levels by injection of morpholino antisense oligonucleotides leads to reduced expression of neuronal marker genes such as *neuroD* in all neurogenic placodes (reviewed in [116]). *Eya2* is expressed in multiple cranial placodes [82], and *Eya3* is expressed in migrating cranial neural crest [57]. Zebrafish *eya1* is expressed in cranial placodes [66], and chick *EYA1* and *EYA2* are expressed in cranial placodes and derivatives [65, 89]. In mice, *Eya1* and *Eya2* are extensively expressed in cranial placodes, [64], and *Eya4* is expressed in the nasal placode [80]. Moreover, EYA1 and SIX1 have been directly implicated as promoters of the early steps of neurogenesis in mouse cranial placodes [117, 118]. A role for EYA in craniofacial development is further supported by the work of Landgraf et al. who showed that Eya1 interacts with Sipl1 and Rbck1, proteins important in craniofacial development whose knockdown causes zebrafish embryos to develop with a branchiootorenal (BOR) syndrome-like phenotype [49].

EYA and photoperiodism

An exciting new development is the observation that EYA3 is the first and strongest molecular response that is activated by a long photoperiod (light cycle) in birds [119], mice [120] and sheep [121]. It appears that *Eya3* expression in the pars tuberalis always occurs about 12 h after the onset of a dark phase, and is directly suppressed by darkness [122]. Along with SIX1 and TEF, EYA3 synergizes to induce thyroid-stimulating hormone β (Tsh-β) expression in both sheep and mice. Tsh-β contributes to the conversion of the inactive thyroid hormone (T4) to its active form T3, which in turn influences the activity of neurons producing gonadotropin-releasing hormone that regulate levels of follicle-stimulating hormone and luteinizing hormone. Hence, photoperiod-regulated EYA3 levels can directly influence the reproductive cycles of seasonally breeding animals. This places the EYA and SIX proteins at the heart of a conserved transcriptional photoperiodic response in the pars tuberalis that mediates a rapid response to changes in day length. Rhythmic cycling of *Eya* expression has also been observed in corals where transcription appears to be under the control of an endogenous light-entrained clock [123]. Here the diurnal pattern of *Eya* expression continues in constant darkness and cycling of *Eya* expression has been observed in both larvae and adult corals. Since the reproductive cycles and other physiological functions of animals are linked to seasonal environmental changes, including light cycles, these observations open up a new biological context in which EYA proteins play a fundamentally important role.

Eyes Absent in human disease

Mutations in *EYA* genes have long been associated with human developmental disorders (Table 4). In addition there is now growing evidence that overexpression of *EYAs* is associated with several malignancies. Below we outline the documented links between EYA proteins and human diseases.

EYA in BOR and BOR-related syndromes

The first human Eya homologue (EYA1) was identified by positional cloning in the search for the gene responsible for BOR syndrome [87, 95, 98]. The autosomal dominant BOR syndrome with a prevalence of 1:40,000 is characterized by hearing loss, branchial fistulae, preauricular pits or tags, and renal abnormalities [124]. These multiorgan defects are recapitulated in *Xenopus* embryos in which the endogenous eya1 protein is depleted and replaced with exogenous protein bearing mutations similar to those seen in patients with BOR syndrome [103], suggesting that the observed human phenotypes are a direct consequence of the reported mutations. Some *EYA1* mutations encountered in BOR patients have been shown to disrupt EYA1–SIX [125] and EYA1–SOX2 [54] interactions, but most of them affect the enzymatic activity of EYA without affecting protein translation or stability [103, 126]. In addition to BOR syndrome, *EYA1* mutation has been reported in patients with congenital cataracts and ocular anterior segment anomalies [47]. A point mutation of the *EYA1* gene leading to aberrant mRNA maturation has been reported in the otofaciocervical syndrome, which is characterized by trophic alterations of face and shoulder girdles in addition to the malformations seen in BOR [97]. A deletion within the *EYA1* gene that leads to a premature truncation of the protein has been reported in cardiofacial syndrome, associated with an asymmetric crying face and congenital heart defects [107]. Frame-shift insertion and point mutations that create a truncation of EYA4 C-terminal domain are associated with late-onset deafness [99], and deletion of the whole ED as well as part of the transactivation domain of EYA4 has been identified in patients with a form of dilated cardiomyopathy and heart failure preceded by sensorineural hearing loss [106].

In light of the extensive involvement of the EYAs in organ development, it is rather surprising that the list of human diseases associated with their loss of function is so short, albeit characterized by defects in multiple organs. One explanation may be redundancy arising from overlapping expression patterns of *EYA* transcripts. Furthermore, a survey of the expression profile of human *EYA* transcripts using the UniGene database [127] reveals that EYAs are present throughout human development from the embryoid body stage through adulthood, which suggests that they may also function very early in development. Therefore, one cannot exclude the possibility that some human loss of function mutations of EYA may go unnoticed due to early embryonic lethality, a phenomenon that has been reported in flies and worms [1, 56].

EYA in cancer and related pathologies

Overexpression of *EYAs* has been reported in various human cancers (Table 4). *EYA4* is overexpressed in MPNST [69], esophageal adenocarcinoma, and colon and colorectal cancers [128–131]. In esophageal squamous cell carcinoma, its expression level in the peripheral blood correlates with disease progression [132]. Moreover, depletion of *EYA4* induces MPNST cells to undergo necrosis [69], suggesting that it promotes the survival of those tumor cells. *EYA2* is overexpressed in epithelial ovarian cancers and lung adenocarcinoma [133, 134], and its overexpression has been correlated with poor prognosis. *EYA2* is also overexpressed in breast cancers [133, 135] and correlates with a worse prognosis. *EYA* overexpression in mammary epithelial cells promotes transformation, migration and invasion [33], and EYA2 is required to mediate the prometastatic function of SIX1 in an established human breast epithelial cancer cell line [135]. Loss of a portion of chromosome 2 (2q37), encoding a microRNA (miR562) that regulates *EYA1*, has been reported in Wilms' tumors and *EYA1* mRNA has also been shown to be overexpressed in these tumors [136, 137]. EYA1 may also be involved in gastric cancer, as its gene is often methylated in Epstein-Barr virus-negative gastric cancers [138]. Despite this tendency of EYAs to be oncogenic, some reports suggest that the outcome of the deregulation of *EYA* expression may be context-dependent. For example, silencing methylations of the *EYA2* gene have been reported in colorectal cancers as opposed to normal tissues [139], the *EYA3* gene is frequently deleted in certain pancreatic ductal adenocarcinomas [140], and overexpression of *EYA*s (including human *EYA2*) can trigger the apoptotic program (an antitumorigenic process) in a murine myeloid cell line [61].

Since the EYA proteins have multiple biochemical activities, and these are apparently associated with different cellular functions, it is likely that EYAs contribute to tumor growth, metastasis, and angiogenesis by different mechanisms. Another intriguing possibility, in light of the proposed role of the EYA tyrosine phosphatase activity in DNA damage repair, is that elevated EYA levels might contribute to increased resistance to DNA-damaging therapeutic regimens commonly used in cancer treatment.

Interestingly, there appears to be a coordinated misregulation of *EYA*, *SIX* and *DACH* gene expression in many cancers. Specifically in breast cancer [134, 135] and MPNST [69], levels of SIX1 and either EYA2 or EYA4 are elevated while DACH levels are reduced [141–144]. This is consistent with the reported tumor-suppressor functions of the DACH proteins, and the tumor-promoting properties of the SIX and EYA proteins. The PSEDN may thus represent an instance in which a pathway involved in fetal organogenesis promotes tumorigenesis when reinstated in the adult.

EYA: a novel therapeutic target?

While most EYA-associated developmental disorders are linked to loss-of-function mutations, there is growing

evidence that elevation of EYA levels (gain of function) is associated with cancers. In both in vivo and in vitro experiments EYAs promote proliferation and invasiveness of tumor cells [33, 68, 69, 133, 145]. Furthermore the tyrosine phosphatase activity is specifically associated with cell migration/invasion and angiogenesis [33, 68], and removal of endogenous EYA can prevent the metastasis of tumor cells overexpressing SIX1 [135], suggesting that even when they are not overexpressed EYAs are still required for metastasis. Together these observations would imply that inhibition of the EYA tyrosine phosphatase activity could be useful as a targeted mode of cancer therapy.

Cell proliferation and migration are prerequisites for angiogenesis, the generation of new blood vessels from preexisting ones. A role for angiogenesis in cancer progression has been long-established. Newly formed blood vessels not only supply the tumor cells with oxygen and nutrients that they need for growth, but also serve as routes for the dissemination of cancer cells during metastasis (for review, see [146]). The EYA tyrosine phosphatase activity has been shown to contribute to angiogenesis using in vitro, ex vivo and in vivo assays [68]. Hence EYA proteins could be positive contributors to tumor growth through their angiogenic function. Pathological neovascularization is not limited to tumor angiogenesis; it is also seen in proliferative retinopathies, arthritis and vascular tumors as a response to a local hypoxic environment [147]. In all these instances inhibition of the EYA PTP could be useful.

There is growing evidence that tumor cells with greater metastatic potential frequently show activation of DNA repair pathways. This has been reported for melanomas, bladder cancer and breast cancers [148, 149]. Furthermore, solid tumors are frequently treated with DNA-damaging therapies both in the form of chemotherapeutics and ionizing radiation. Since EYA promotes DNA damage repair, it is quite likely that elevated EYA levels would reduce the effectiveness of such treatment. Hence, EYA inhibitors could potentially be useful in combination regimens as sensitizing agents.

EYA as a druggable target

While the association between EYA tyrosine phosphatase activity and various disease states suggests that it is a good target for drug development, the identification of specific inhibitors of PTPs has been historically difficult. This is largely because there are over 100 PTPs encoded in the genome that share similar active site architecture, making the discovery of selective ligands/inhibitors challenging. EYAs do not suffer from this problem, being the only known members of their mechanistic class, and having an active site that is stereochemically distinct from that of all other PTPs. A general consensus (recent reviewed in [150]) in the field of drug development is that a "druggable target" must have two important features: (1) be linked to a disease and (2) have the potential to bind, with high affinity, a small molecule having the appropriate chemical properties. As described in this review, EYA has both of these properties. Being an enzyme it has a defined active site that can be targeted by small molecules. Structural information is available [14] permitting rational design of inhibitors, and specific EYA inhibitors have been identified. The most extensively validated EYA PTP inhibitors include the compounds benzbromarone and benzarone [68]. While their IC50 values are modest (<10 μM) in enzymatic assays, these compounds exhibit potency in cellular assays inhibiting cell motility and tubulogenesis in vitro, and angiogenic sprouting ex vivo. They have also been shown to be effective in an in vivo assay measuring vascular development in zebrafish. Hence, these compounds are proof-of-principle that inhibition of EYA can attenuate angiogenesis and are also valuable leads for the development of more potent and effective EYA inhibitors. This class of compounds also has the advantage that because of their history of usage for gout treatment, their long-term toxicity, pharmacokinetic and pharmacodynamics profiles are well-established. As a result they are excellent candidates for the old compound–new target–new use paradigm.

Other classes of EYA inhibitors identified through virtual screening have been reported [151]. These compounds bind in the active site and chelate the essential divalent metal ion of EYA2. While they have not yet been tested in cellular or in vivo assays, these studies point to the feasibility of both in silico and experimental screening for EYA-inhibitory small-molecule compounds.

Conclusions

EYAs are an unusual class of proteins that combine tyrosine phosphatase, threonine phosphatase and transactivation activities. Along with other components of the PSEDN, Eyes Absents have been conserved among widely disparate animal species ranging from amphioxus to humans [152]. EYAs are required for the development of multiple organs and have correspondingly been implicated in multiple developmental disorders. Increasing evidence points to an oncogenic, prometastatic and angiogenic function for the EYAs, and based on its proposed role in angiogenesis, it is likely that EYAs will be associated with vascular disorders.

Key questions regarding EYA regulation and function remain. The range of biological processes associated with EYAs (including, but not restricted to, cell proliferation

and migration, angiogenesis, DNA damage repair, innate immunity, photoperiodism) would imply that the EYA proteins are an integral part of core signaling processes in the cell. Moreover, since many of these activities are described in the adult and not the developing embryo, EYAs clearly have a role in the maintenance of function. Other than H2AX, no substrate for the EYA's two phosphatase activities has been thoroughly validated. Undoubtedly, yet-unidentified substrates exist and in light of the multiple cellular roles of EYAs they are likely to exist in both the nucleus and the cytoplasm. Pertinent to this, the mechanism by which EYA's subcellular localization is regulated is of interest. It is generally believed that EYA translocation to the nucleus is predicated on the formation of a SIX-EYA complex. Furthermore, since EYA has no intrinsic DNA-binding ability, it needs to be localized on DNA by a binding partner such as the SIX proteins. Hence SIX–EYA complexes must act in concert both in a transcriptional regulation role and in any proposed nuclear phosphatase activity such as DNA damage repair. Thus it would be reasonable to assume that any nuclear function of the EYAs is SIX-dependent, or that unidentified EYA binding partners facilitate the movement of this acidic protein into the nucleus. The converse also appears to be true in some instances: the prometastatic and lymphangiogenic functions of SIX1 are EYA-dependent, and quite likely dependent on EYA tyrosine phosphatase activity. And finally, with the association between EYAs and an ever-growing number of disease states including developmental disorders, cardiac conditions, innate immune responses, pathological angiogenesis, and tumor growth and metastasis, inhibition of EYA tyrosine phosphatase activity is an attractive target for drug development.

Acknowledgments This work was supported by a grant from the National Institutes of Health (NEI014648) to R.S.H.

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