The Eyes Absent proteins in development and in developmental disorders

Upendra Kumar Soni, Kaushik Roychoudhury and Rashmi S. Hegde

Division of Developmental Biology, Cincinnati Children’s Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, OH 45229, U.S.A.

Correspondence: Rashmi S. Hegde (rashmi.hegde@cchmc.org)

The Eyes Absent (EYA) transactivator-phosphatase proteins are important contributors to cell-fate determination processes and to the development of multiple organs. The transcriptional regulatory activity as well as the protein tyrosine phosphatase activities of the EYA proteins can independently contribute to proliferation, differentiation, morphogenesis and tissue homeostasis in different contexts. Aberrant EYA levels or activity are associated with numerous syndromic and non-syndromic developmental disorders, as well as cancers. Commensurate with the multiplicity of biochemical activities carried out by the EYA proteins, they impact upon a range of cellular signaling pathways. Here, we provide a broad overview of the roles played by EYA proteins in development, and highlight the molecular signaling pathways known to be linked with EYA-associated organ development and developmental disorders.

Introduction

The Eyes Absent (EYA) proteins are part of the well-conserved retinal determination gene network (RDGN) that contributes to cell-fate determination processes in multiple tissues and species (reviewed in [1–4]). In addition to the EYA proteins, the RDGN consists of the master regulatory transcription factor PAX6 (eyeless ey and twin of eyeless toy in flies), the SIX family of homeodomain transcription factors (SIX1–6; sine oculis and optix in flies), and the transcription factor DACH (dachshund). Interactions between components of the RDGN are summarized in Figure 1. While the RDGN was first described in Drosophila eye development [5], surveys of the eukaryote genome suggest that the RDGN was employed in two different contexts early in evolution, eye development and oocyte development [6]. In vertebrates, the proteins coded by members of the RDGN are widely implicated in cell survival and differentiation during tissue specification. Here, we describe our current understanding of the roles played by EYA proteins during development and in tissue homeostasis. Particular emphasis is placed on linking the multiple biochemical activities of the EYA proteins with their developmental roles whenever possible. We will highlight how the EYA proteins interface with other signaling pathways involved in organ development, the emerging evidence that EYA3 contributes to photoperiodism, and mutations in the Eya genes associated with human disorders.

EYA protein architecture and its placement in the RDGN

Mammals have four Eya paralogs (Eya1–4) and their expression begins during embryonic development. Eya expression is much more restricted to specific adult tissues, with Eya3 having the most wide-spread expression. The intrinsically cytoplasmic EYA proteins physically partner with the SIX proteins and are then translocated to the nucleus where they play an essential role in the regulation of gene expression driven by the RDGN (Figure 1b).
All animal EYA proteins are characterized by a highly conserved C-terminal ~270 amino-acid domain referred to as the EYA domain (ED) (Figure 1a). Interaction with the SIX proteins occurs through the ED and there is strong evidence that ED has tyrosine phosphatase activity [7–9]. However, ED bears no structural or sequence similarity to the classical protein tyrosine phosphatases (PTP). This places the EYA proteins in the unique position of being PTPs that do not employ the nucleophilic Cysteine residue-mediated catalytic mechanism characteristic of the classical PTPs (recently reviewed in [3]). Few EYA-PTP substrates have yet been identified (recently reviewed in [3]). The best-characterized of these is the DNA damage repair-associated histone protein H2AX [10,11]. Dephosphorylation of the terminal C-tyrosine of H2AX promotes DNA damage repair and survival of cells. In addition, the tumor suppressor estrogen receptor-β and the cytoskeleton modulating protein WDR1 have also been identified as likely EYA-PTP substrates [12,13]. The PTP activity of the EYA proteins also promotes cell motility possibly via the Cdc42/Rac GTPases [14,15], but no EYA substrate has yet been identified in this context. The N-terminal domain of the EYA proteins has an independent transactivation function localized to a Pro/Ser/Thr-rich ~210 amino-acid segment in Drosophila Eya [16]. In addition, a threonine phosphatase activity (either intrinsic or via interaction with protein phosphatase 2A (PP2A)) is associated with the N-terminal domain [17–22].

This juxtaposition of distinct biochemical activities in a single polypeptide places the EYA protein in the unique position of participating directly in both the regulation of gene transcription and in signal transduction...
processes. A schematic overview of molecular pathways impacted by EYA proteins as they participate in the processes described below is provided in Figure 2.

**EYA in eye development**

Loss of the *eya* gene in *Drosophila* eye progenitor cells anterior to the furrow (*eya*2 mutant flies) resulted in viable but completely eyeless flies due to apoptosis of eye progenitor cells [23]. The introduction of mouse *Eya2* in progenitor cells prior to furrow formation in the *eya*2 mutant background was able to restore eye formation, speaking to a high degree of conservation of function for these EYA proteins through evolution [24]. Initial reports suggested that MAPK-catalyzed phosphorylation of Eya positively regulates *fly* eye development [25–27]. However, subsequent investigation revealed that MAPK-mediated Eya phosphorylation was dispensable for normal eye development or survival [28].

Whether the tyrosine phosphatase activity of *Drosophila* Eya contributes to eye development has been addressed multiple times with varying conclusions. The original studies using overexpression or mis-expression showed that phosphatase-dead forms of Eya were defective in the rescue of the *eya*-null eyeless phenotype [8,9]. However, when genomic transgenes mimicking endogenous spatial and temporal levels of Eya were used in rescue experiments, phosphatase-dead Eya complemented the *eya* null alleles [29]. When such dose-controlled rescue experiments were conducted in genetically sensitized backgrounds (heterozygosity for other RDGN components *sine oculis* or *dachshund*), the phosphatase-dead form of Eya was not able to support normal retinal development [30]. Hence the current thinking is that *Drosophila* Eya is not essential on its own, but rather contributes to the ‘robustness’ of RDGN output in fly eye development [30].

It is not straightforward to extrapolate from fly eye development to the more complicated process of vertebrate eye development. In flies, development initiates at a single imaginal disc epithelium. In contrast, in vertebrates, the optic vesicle evaginates from the forebrain and forms the optic stalk, the retina and the retinal pigment epithelium, while the lens and cornea derive from the surface ectoderm. *Eya1* is seen earliest in the ectoderm-derived lens placode, *Eya2* in the retina, and *Eya3* in the optic vesicle. While no gross eye phenotype has yet been reported upon knockout of any *Eya* gene in mice, there is one report of congenital cataracts and anterior segment anomalies in humans with an *Eya1* mutation [31]. *Eya1*−/− mice die at birth with severe cranio-facial and skeletal defects. They also lack thymus, parathyroid glands, ears and kidneys [32]. Except for open eyelids, there appear to be no eye development defects. *Eya2*−/− and *Eya3*−/− mice have no obvious phenotype and are viable and fertile [33,34]. No eye phenotype has been recorded in double *Eya1*−/−/*Eya2*−/−
mice [33]. The only reported ocular effect upon loss of an EYA protein is a mild delay in the development of the post-natal mouse retinal vasculature upon endothelial-specific deletion of Eya3 [35].

In this context, it is relevant that the loss of other components of the RDGN have variable effects on vertebrate eye development. Six3-null mice lack all forebrain structures including eyes [36]. Loss of Dach1, which is expressed in the developing retina, results in no eye phenotype [37]. Pax6−/− mice die at birth with a complete disruption of eye development [38], and heterozygous Pax6 mutation in humans results in small eyes [38]. Dosage effects of the Pax6 gene on eye formation have been demonstrated through the use of the CRISPR/Cas system to generate a mosaic mixture of mutant and wild-type mice [39]. Notably, lens development from the surface ectoderm requires higher Pax6 gene dosage than retinal development from the optic vesicle [39]. These observations suggest that the roles of the RDGN proteins in eye development are more complex than can be deciphered from simple loss-of-function experiments, and are likely to be influenced by genetic background, somatic mosaicism and perhaps environmental factors.

**EYA in ear development**

EYA1 contributes to multiple stages of inner ear development. The inner ear has six sensory organs and each one has sensory hair cells as well as non-sensory supporting cells. Eya1 and the transcription factor Sox2 are co-expressed in the otocyst where pro-sensory epithelia are formed and play a role in specifying the sensory cell lineage [40]. EYA1 along with SIX1 and the BRG1-associated SW1/SNF chromatin remodeling complex (BRG1-BAF) induce Sox2, and thereby promote the specification of a pro-neurosensorial-restricted progenitor cell fate from an ectodermal cell [41]. The model proposed by these studies is that EYA1 bridges SIX1 and the BRG1-BAF complex, thus inducing the formation of a compact structure that enables binding to distant cis-regulatory elements, and hence Sox2 induction [41]. Together, EYA1 and SOX2 are required for the expression of sensory markers including Jag1, Lfng, Bmp4, and Fgfs, and for cell cycle progression of these pro-sensory progenitors. Once the progenitors become committed to a hair cell fate, Sox2 expression is no longer detected. However, Eya1 continues to be expressed in differentiating hair cells along with Atoh1. Consistent with this, Eya1-null mice completely lack inner ear sensory organs. There is evidence that dose-dependent EYA1 activity is necessary for the development of different parts of the ear [40], mirroring the variation in inner ear malformation seen in patients with Eya1 mutation-associated developmental disorders (described below). According to an Eya2lox/lox knock-in reporter study Eya2 is also expressed in differentiating hair cells in the sensory epithelia of the inner ear and Eya2−/− mice have a mild hearing deficit [41]. These new studies once again point to the likelihood that Eya genes can act synergistically during development, as well the possibility that they can compensate for each other and complicate loss-of-function studies. No studies have yet specifically queried the involvement of the C-terminal PTP activity of the EYA proteins in ear development.

**EYA in muscle development**

EYA proteins are implicated in various stages of trunk myogenesis. As in the case of the ear, the different Eyas are expressed at different timepoints during muscle development, and they work in conjunction with at least three SIX proteins. During early myocyte specification and myogenesis, Eya1 and Eya2 are expressed in the developing limb buds. In vitro reporter assays show that the N termini of these EYA proteins are sufficient to drive transactivation [42]. While some studies conclude that there is no requirement for EYA1 or SIX1 in early myogenesis [43] and that Eya1 knockout mice show no muscle phenotype [44], others report that Eya1 knockout mice have delayed myogenesis between e11 and e13, and that Eya1+/−/Eya2−/− mice have severe muscular abnormalities [33]. EYA1 also plays an important role in post-natal muscle development where it can switch a slow-twitch muscle to a fast-twitch phenotype by replacing the myosin I and IIA heavy chain isoforms to the absence of all hypaxial muscles and a severe reduction in epaxial muscle in SIX1−/−/Eya1−/− mice [44]. The EYA1-SIX1 complex activates SIX1 target genes involved in myogenesis including Pax3, MyoD and Myogenin [33]. Indeed, ectopic expression of Eya1 and Six1 (along with Esrrb (Estrogen related-receptor-beta) and Pax3) is sufficient to induce myogenic stem cells with differentiation capacity in vitro and in vivo [47]. In flies, eya and so induce tinman expression (ortholog of mammalian NKX2-5), which in turn activates the JAK/STAT pathway to initiate myocyte differentiation [48]. In Caenorhabditis elegans, myogenic vs non-myogenic fate is determined by the SIX-EYA complex downstream of WNT signaling [49].
No specific examination of whether the tyrosine phosphatase function of the EYA proteins contributes to muscle development has been reported, and the implication in all of the studies cited above is that EYA acts as an activator of transcription in complex with the SIX proteins.

**EYA in kidney development**

Eya1<sup>−/−</sup> mice have renal abnormalities while Eya1<sup>+/−</sup> mice completely lack kidneys [32]. Ureteric bud outgrowth and consequently metanephric induction are compromised in the absence of EYA1. Downstream of Eya1, Gdnf expression is down-regulated in Eya1-null mice. Eya1 is one of two genes (the other is Osr1) that are required for the initial formation of the metanephric mesenchyme which condenses to form a precursor cell population. These precursors can either differentiate (epithelialization to form the renal vesicle, the precursor of the nephron), or self-renew to maintain a progenitor pool at the tip of the ureteric bud (cap mesenchyme). A balance between self-renewal and differentiation is crucial to appropriate kidney development. Eya1 (and Six2) are expressed in the cap mesenchyme throughout nephrogenesis representing an essential multipotent cell population [50].

Nuclear EYA1 in nephron progenitors stabilizes MYC, which in turn promotes the proliferation of undifferentiated cells. This occurs through dephosphorylation of Thr58 on MYC, thus preventing MYC degradation and increasing MYC half-life. EYA1-mediated MYC stabilization induces nephron progenitor cell proliferation [50]. Threonine phosphatase activity in the N-terminal domain of the EYA proteins had been previously shown to play a role in the innate immune response [18]. While no structural or sequence similarity with known phosphatase families could be found in the poorly conserved N-terminal domain of the EYAs, threonine phosphatase activity was confirmed by several follow-up studies [17,19]. However, a recent report has raised the possibility that the threonine phosphatase activity ascribed to the EYA proteins is actually mediated through an interaction with the PP2A B55α subunit [21]. But in glioblastoma stem cells EYA1 directly interacts with MYC, and the EYA1-MYC axis regulates GSC proliferation, migration and self-renewal [51]. Regardless of the mechanism through which it occurs (either directly mediated by EYA or via PP2A), links between EYA proteins and MYC stabilization continue to emerge in contexts beyond kidney development (Figure 2).

**EYA proteins in other developmental contexts**

Knockout studies show that the development of several neural-crest derived cranial structures are EYA1-dependent. Eya1<sup>−/−</sup> embryos have defective pharyngeal arches and lack proximal arch structures contributing to a broad spectrum of defects in cranio-facial morphogenesis [52]. Interestingly, this is another developmental context in which a putative EYA1 threonine phosphatase activity is implicated: threonine dephosphorylation of the Notch intracellular domain (NICD) is believed to stabilize the NICD and thus enhance Notch signaling, a crucial contributor to cranio-facial development (Figure 2).

EYA1 is also required for the morphogenesis of organs derived from the third pharyngeal pouch (thymus, parathyroid, thyroid). Eya1<sup>−/−</sup> mice display thyroid hypoplasia and the thymus and parathyroid fail to form [53]. Eya1<sup>−/−</sup> mutants also have a spectrum of abnormalities in cardiovascular development, the most common being aortic arch defects [54]. This is exacerbated in Six1<sup>−/−</sup>/Eya1<sup>−/−</sup> double mutants (cardiac outflow tract and aortic arch defects), partially due to a reduction in fibroblast growth factor 8 (Fgf8) levels [54].

EYA1, along with SIX1, contributes to hindbrain development through its ability to promote Nrp1 transcription and favor Gli activators over Gli repressors following sonic hedgehog (Shh) stimulation [55]. In keeping with this role, Eya1 is expressed in Shh-subtype medulloblastomas and promotes tumor growth [55]. A Shh-Eya1 axis is also involved in promoting symmetric cell divisions during cerebellar development [22]. This is believed to occur through dephosphorylation of Thr410 and thus inactivation of the atypical protein kinase aPKCζ [22], once again implicating an EYA-linked threonine phosphatase activity in development.

A recent report shows that Eya1 is expressed in the bitter taste receptor cells of the oral epithelium and in the differentiating taste bud cells in circumvallate and fungiform papillae and the soft palate [56]. The signaling pathway(s) through which EYA contributes to the generation and differentiation of taste buds (specifically bitter cells) remains to be established.

**EYA in photoperiodism**

An intriguing association between EYA3 and photoperiodism is emerging largely through studies on large seasonal animals and birds. Photoperiodism is a phenomenon through which functional and behavioral responses accommodate changes in the length of dark and light cycles, thus allowing plants and animals to adjust to...
seasonal variation. In animals, photoperiodism impacts phenomena such as growth, metabolism, migration, reproductive behavior, and hibernation. In mammals, light-sensitive retinal ganglion cells (RGCs) transmit light information to the suprachiasmatic nucleus (SCN) in the hypothalamus (Figure 3). This signal is then transmitted via light-sensitive neurons to the pineal gland, where nocturnal melatonin is synthesized. The length of the melatonin signal is consequently proportional to the length of the dark period, or night, and is read by the pars tuberalis (PT) in the pituitary gland [57]. Long days (summer) induce thyroid stimulating hormone (TSH; a heterodimer of $\alpha$ and $\beta$ subunits) in the PT. TSH in turn promotes thyroid hormone production and activity in the basal hypothalamus, thus interfacing with the regulation of crucial season-guided physiology such as reproduction.

An EYA3-SIX1-TEF complex up-regulates TSH$\beta$ transcription in the PT [58]. EYA3 expression in the PT is rhythmic, peaking transiently 12.5 h after the onset of darkness or melatonin expression. As a result, EYA3 levels rise at first light in the summer and during the final hours of darkness in the winter.

![Figure 3. EYA3 in photoperiodism.](image)

A simplified schematic showing how light signaling through the eye is transmitted through the SCN and inhibits melatonin release by the pineal gland. Hence melatonin is exclusively secreted at night. TSH levels in the thyrotrophic cells of the PT display melatonin-dependent photoperiodic changes; high TSH under long photoperiod, and low levels under short photoperiod. EYA3 promotes transcription of the TSH$\beta$ subunit and thus acts as an ‘on-switch’ for TSH, making EYA3 a central player in photoperiodic time measurement [91]. EYA3 is induced 12.5 h after the onset of melatonin expression. As a result, EYA3 levels rise at first light in the summer and during the final hours of darkness in the winter.

Developmental disorders associated with EYA mutations

The importance of EYA proteins (and the RDGN network) during crucial stages of embryonic development is reflected in the numerous developmental disorders associated with Eya mutations. These are typically manifest as defects in eye, ear, neck, crano-facial, and heart development (summarized in Table 1). Below we describe the developmental defects frequently associated with Eya mutations and, when available, links between the biochemical activities of the EYA proteins and these disease states.
Table 1. A summary of *Eya* mutations associated with developmental disorders

| Disease/affected organ(S) | GENE | Mutations | References |
|---------------------------|------|-----------|------------|
| Branchio-oto-renal Syndrome 1 (BOR)/disrupted development of tissues in neck, malformation of ears and kidneys | *EYA1* | Deletions, insertions, mis-sense mutations | [61,64–66,69,70] |
| Branchio-oto-syndrome 1 (BOS)/disrupted development of tissues in neck, malformation of ears | *EYA1* | Insertions, deletions | [66] |
| Oto-facio-cervical Syndrome (OFC)/facial dysmorphism, ear anomalies, branchial cysts or fistulae, anomalies of the vertebrae and shoulder girdle, mild intellectual disability | *EYA1* | Deletions, splice site mutations | [63,67] |
| Anterior segment anomalies, congenital cataracts/defects of the cornea, lens, iris and aqueous humor | *EYA1* | Mutations, substitutions | [31] |
| Cardio-facial Syndrome/asymmetric crying face and heart defect | *EYA1* | Deletions, frame-shifts | [73] |
| Late-onset deafness (non-syndromic) | *EYA4* | Truncations, mutations | [77,78] |
| Dilated cardiomyopathy with sensorineural hearing loss/progressive defect in cardiac muscle, defects affecting inner ear and auditory nerve | *EYA4* | Deletions | [75,80] |
| Oculo-auriculo-vertebral Spectrum (Goldenhar Syndrome)/deformity of external ear and small ipsilateral half of face, vertebral anomalies | *EYA3* | Mis-sense mutation | [81] |

*Eya1* is one of the three known causative genes for BOR (Branchio-oto-renal syndrome also known as Melnick–Fraser syndrome; the other two genes being RDGN members *SIX1* and *SIX5* [61,62]). BOR is an autosomal dominant disorder characterized by malformations of the neck, ears and kidney. These include (to varying degrees) branchial fistulae or cysts, hearing loss as a result of inner, middle and outer-ear malformations, and kidney abnormalities ranging from hypoplasia to agenesis. Features of BOR partially overlap with otofaciocervical syndrome (OFC), a rare developmental defect characterized by distinctive facial features (triangular face, broad forehead, narrow nose and mandible, high arched palate), prominent ears, long neck, branchial fistulae or cysts, sloping shoulders and clavicles, hearing impairment and mild intellectual defects. *Eya1* mutations are also associated with OFC [63]. Furthermore, *Eya1* mutations are sometimes seen in patients with congenital cataracts and ocular segment anomalies, sometimes associated with BOR [31]. Deletions, mis-sense mutations as well as complex rearrangements of the *Eya1* gene are reported in BOR and OFC patients [61,64–70]. Most of the mutations are unique to individual families and no apparent genotype–phenotype correlation has yet emerged. Several experimental studies tried to link individual mis-sense mutations to biochemical activities, variably reporting impaired tyrosine phosphatase activity as well as impaired interaction with the SIX proteins [71,72].

*Eya1* mutations have been reported in patients with congenital cardiac abnormalities [73,74], and mutation of *Eya4* is associated with dilated cardiomyopathy [75]. Genetic studies using compound deletions in mice show that EYA1 and SIX1 play a role in cardiovascular development, and it is proposed that a *Tbx1-Six1/Eya1-Fgf8* regulatory cascade underlies a common mechanism important for the morphogenesis of both the heart and the face [54]. This data has led to the speculation that the pathogenesis of BOR and cardio-facial syndrome could be linked to *Eya1* and *Six1* mutations via a common molecular program [54].

*Eya4* mutations are reported in several families with non-syndromic autosomal dominant post-lingual progressive hearing loss (late-onset deafness) [76–79]. Most of these mutations result in truncated EYA4 protein lacking most, or all, of the conserved ED (tyrosine phosphatase) domain. There is some evidence that while loss of just the tyrosine phosphatase domain causes non-syndromic sensorineural hearing loss, disruption of both the ED and the N-terminal domains is associated with syndromic (dilated cardiomyopathy with sensorineural hearing loss) disease [75,78]. One clinically actionable extension of these studies on the pleiotropic effects of *Eya4* gene variants is described in a recent study where a ‘genotype-first’ approach was used to identify as-yet undiagnosed cardiac conduction disorders in adults presenting with hearing loss [80].

A recurrent mutation in *Eya3* (resulting in an Asn358Ser substitution) causing oculo-auriculo-vertebral spectrum has recently been reported [81]. This mutation appeared to increase the half-life of *Eya3* but have no impact on tyrosine phosphatase activity. This report notwithstanding, in general *Eya2* and *Eya3* mutations are
rarely been linked with developmental disorders. This could be a consequence of redundancy, since while both genes are broadly expressed during development, they are most often co-expressed with other Eya genes.

Conclusions

Many decades after Drosophila EYA was first described, insights into the roles of EYAs in normal development, physiology and disease continue to emerge. Not described here, but of emerging clinical importance, is the therapeutic potential of targeting the EYA PTP activity for diseases ranging from cancer [82–84] to pulmonary arterial hypertension [85] and proliferative retinopathies [35]. Much remains to be clarified regarding EYA protein function and regulation. The question of whether the N-terminal domain and C-terminal domain activities of the EYA proteins impact on each other is still a matter of some debate. The fact that interaction with the SIX proteins is mediated by the ED domain, while transactivation potential exists in the N-terminal domain suggests that, at least for the transactivation activity in the context of the RDGN, the two domains are functionally linked. It is curious that the PTP activity of the EYA proteins has not yet been linked to any major developmental role, other than contributing to post-natal vascular development in the murine retina [35]. This is particularly notable since the EYA-PTP activity promotes DNA damage repair [10] and cell motility [14,15], two processes important in development: embryonic development involves rapid cell proliferation inevitably accompanied by DNA damage, and cell motility plays a crucial role in morphogenesis. It is likely that subtle developmental defects attributable to the PTP activity will be uncovered upon more careful examination in the future.

How did the EYA proteins come to have so many biochemical activities in a single polypeptide? It is interesting to speculate about where these activities originated. Notably, plants have an EYA protein that is comprised solely of the ED domain [7], and no significant amino-acid sequence homology exists between the EYA N-terminal domain and any other known protein sequence [4]. It has been suggested that some animal EYA proteins with an ED-like domain have very weak, or no, tyrosine phosphatase activity [86]. But this has not been experimentally queried. Could the EYA proteins we see in vertebrates have emerged from an ancestral ED domain-containing protein that may, or may not, have had tyrosine phosphatase activity? Does the N-terminal domain have intrinsic threonine phosphatase activity? Evolutionary biochemists suggest that all enzymes are promiscuous (have an average of 10 promiscuous activities [87–89]). Perhaps the putative threonine phosphatase activity of the EYA proteins is one such instance of evolution in progress? In this review, we have attempted to match the observed biochemical activities of the EYA proteins with their developmental roles, as understood from our vantage point at one moment in the evolutionary journey of the EYA proteins.

Perspectives

- EYA transactivator-phosphatase proteins play crucial roles in embryonic development and in tissue homeostasis. Mutations or mis-expression of EYA are linked to human genetic disorders and cancers, offering opportunities for the development of therapeutics and genetic diagnoses.

- The vast majority of the studies thus far implicate the EYA transactivation function in cell-fate determination. However, a growing body of evidence suggests that the tyrosine phosphatase activity (and the putative threonine phosphatase activity) could contribute to disease states. These observations are now being exploited in efforts to devise EYA-targeted therapeutics.

- Beyond the further examination of the myriad signaling pathways impacted by EYA-mediated transactivation and dephosphorylation events, examination of the molecular evolution of this unusual protein is of great interest. Such studies could be important contributors to our understanding of how phosphorylation-based signaling mechanisms evolved in general.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.
Funding
This work was supported through grants from the NIH NCIC R01-CA207068 and NHLBI HL152094 to RSH.

Author Contributions
US helped write, edit, and generate figures, R.K. helped write and edit, R.S.H. wrote this article and generated figures.

Abbreviations
BOR, branchio-oto-renal; EYA, Eyes Absent; NICD, Notch intracellular domain; OFC, otofaciocervical syndrome; PP2A, protein phosphatase 2A; PTP, protein tyrosine phosphatases; RDGN, retinal determination gene network; TSH, thyroid stimulating hormone.

References
1. Kumar, J.P. (2009) The molecular circuitry governing retinal determination. Biochim. Biophys. Acta 1789, 306–314. https://doi.org/10.1016/j.bbadg.2008.10.001
2. Silver, S.J. and Rebay, I. (2005) Signaling circuitries in development: insights from the retinal determination gene network. Development (Cambridge, England) 132, 3–13. https://doi.org/10.1242/dev.01539
3. Hegde, R.S., Roychoudhury, K. and Pandey, R.N. (2020) The multi-functional eyes absent proteins. Crit. Rev. Biochem. Mol. Biol. 55, 372–385. https://doi.org/10.1080/10409238.2020.1796922
4. Tadjuidje, E. and Hegde, R.S. (2013) The Eyes Absent proteins in development and disease. Cell. Mol. Life Sci. 70, 1897–1913. https://doi.org/10.1007/s00018-012-1144-9
5. Halder, G., Callaerts, P., Flister, S., Walldorf, U., Kloter, U. and Gehring, W.J. (1998) Eyeless initiates the expression of both sine oculis and eyes absent during Drosophila compound eye development. Development (Cambridge, England) 125, 2181–2191. https://doi.org/10.1242/dev.125.12.2181
6. Gazzaniga, D.F., Suga, H., Schmid, V. and Gehring, W.J. (2012) The ‘‘eyes absent’’ (eya) gene in the eye-bearing hydrozoan jellyfish Cladiella radiataurum: conservation of the retinal determination network. J. Exp. Zool. B Mol. Dev. Evol. 318, 257–267. https://doi.org/10.1002/jz.b.22442
7. Rayapureddi, J.P., Kattamuri, C., Chan, F.H. and Hegde, R.S. (2005) Characterization of a plant, tyrosine-specific phosphatase of the aspartyl class. Biochemistry 44, 751–758. https://doi.org/10.1021/bi0418794
8. Rayapureddi, J.P., Kattamuri, C., Steinmetz, B.D., Frankfort, B.J., Ostrin, E.J., Mardon, G. et al. (2003) Eys absent represents a class of protein tyrosine phosphatases. Nature 426, 295–298. https://doi.org/10.1038/nature02093
9. Toole, T.L., Silver, S.J., Davies, E.L., Newman, V., Latek, R.R., Mills, I.A. et al. (2003) The transcription factor Eys absent is a protein tyrosine phosphatase. Nature 426, 299–302. https://doi.org/10.1038/nature02097
10. Cook, P.J., Ju, B.G., Telese, F., Wang, X., Glass, C.K. and Rosenfeld, M.G. (2009) Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. Nature 458, 591–596. https://doi.org/10.1038/nature07849
11. Krishnan, N., Jeong, D.G., Jung, S.K., Ryu, S.E., Xiao, A., Allis, C.D. et al. (2009) Dephosphorylation of the C-terminal tyrosyl residue of the DNA damage-related histone H2AX is mediated by the protein phosphatase eyes absent. J. Biol. Chem. 284, 16066–16070. https://doi.org/10.1074/jbc.C000032200
12. Yuan, B., Cheng, L., Chiang, H.C., Xu, X., Han, Y., Su, H. et al. (2014) A phosphotyrosine switch determines the antitumor activity of ERbeta. J. Clin. Invest. 124, 3378–3390. https://doi.org/10.1172/JCI74085
13. Mentel, M., Ionescu, A.E., Puscalau-Girtu, I., Helm, M.S., Badea, R.A., Rizzoli, S.O. et al. (2018) WDR1 is a novel EYA3 substrate and its cationic phosphatase of the aspartyl class. FEBS Lett. 585, 3143. https://doi.org/10.1111/2042-2042.13072
14. Silver, S.J., Davies, E.L., Doyon, L. and Rebay, I. (2003) Functional dissection of eyes absent reveals new modes of regulation within the retinal determination network. J. Biol. Chem. 278, 59610–59615. https://doi.org/10.1074/jbc.M207100200
15. Liu, X., Sano, T., Guan, Y., Nagata, S., Hoffmann, J.A. and Fukuyama, H. (2012) Drosophila EYA regulates the immune response against DNA through an evolutionarily conserved threonine phosphatase motif. PLoS One 7, e42725. https://doi.org/10.1371/journal.pone.0042725
16. Okabe, Y., Sano, T. and Hegde, R.S. (2012) The Eya tyrosine phosphatase activity is pro-angiogenic and is inhibited by Benzobromarone. PLoS One 7, e34806. https://doi.org/10.1371/journal.pone.0034806
17. Liu, X., Sano, T., Guan, Y., Nagata, S., Hoffmann, J.A. and Fukuyama, H. (2012) Drosophila EYA regulates the immune response against DNA through an evolutionarily conserved threonine phosphatase motif. PLoS One 7, e42725. https://doi.org/10.1371/journal.pone.0042725
18. Okabe, Y., Sano, T. and Hegde, R.S. (2009) Regulation of the innate immune response by the threonine-phosphatase of Eyes absent. Nature 460, 520–524. https://doi.org/10.1038/nature08138
19. Sano, T. and Nagata, S. (2011) Characterization of the threonine-phosphatase of mouse eyes absent 3. FEBS Lett. 585, 2714–2719. https://doi.org/10.1016/j.febslet.2011.07.029
20. Vartulli, R.L., Zhou, H., Zhang, L., Powers, R.K., Klariquist, J., Rudra, P. et al. (2018) Eya3 promotes breast tumor-associated immune suppression via threonine-phosphatase-mediated PD-L1 upregulation. J. Clin. Invest. 128, 2535–2550. https://doi.org/10.1172/JCI96784
21. Zhang, L., Zhou, H., Li, X., Vartulli, R.L., Rowe, M., Xing, Y. et al. (2018) Eya3 partners with PP2A to induce c-Myc stabilization and tumor progression. Nat. Commun. 9, 1047. https://doi.org/10.1038/s41467-018-03327-4
22. Merk, D.J., Zhou, P., Cohen, S.M., Pazyra-Murphy, M.F., Hwang, G.H., Rehn, K.J. et al. (2021) The Eya1 phosphatase mediates Shh-driven symmetric cell division of cerebellar granule cell precursors. Dev. Neurosci. 42, 170–186. https://doi.org/10.1159/000512976
23. Bonini, N.M., Leiserson, W.M. and Benzer, S. (1993) The eyes absent gene: genetic control of cell survival and differentiation in the developing Drosophila eye. Cell 72, 379–395. https://doi.org/10.1016/0092-8674(93)90115-7
24. Bonini, N.M., Bui, Q.T., Gray-Board, G.L. and Warrick, J.M. (1997) The Drosophila eyes absent gene directs ectopic eye formation in a pathway conserved between flies and vertebrates. Development (Cambridge, England) 124, 4819–4826. https://doi.org/10.1242/dev.124.23.4819
Eisner, A., Pazyra-Murphy, M.F., Durresi, E., Zhou, P., Zhao, X., Chadwick, E.C. et al. (2015) The Eya1 phosphatase promotes Shh signaling during retinal development. Dev. Cell 36, 267–276.

Jusiak, B., Abulimiti, A., Haelttman, N., Chen, R. and Mardon, G. (2012) MAPK target sites of eyes absent are not required for eye development or survival in Drosophila. PLoS One 7, e50776. https://doi.org/10.1371/journal.pone.0050776

Jin, M., Jusiak, B., Bai, Z. and Mardon, G. (2013) Eyes absent tyrosine phosphatase activity is not required for Drosophila development or survival. PLoS One 8, e58818. https://doi.org/10.1371/journal.pone.0058818

Davis, T.L., Hoi, C.S.L. and Rebay, I. (2017) Mutations that impair Eyes absent tyrosine phosphatase activity in vitro reduce robustness of retinal development process. Biochemical Society Transactions 49, 1397–1408. https://doi.org/10.1042/BST20130302
Dardente, H., Wyse, C.A., Birmie, M.J., Dupre, S.M., Loudon, A.S., Lincoln, G.A. et al. (2010) A molecular switch for photoperiod responsiveness in mammals. *Curr. Biol.* 20, 2193–2198. https://doi.org/10.1016/j.cub.2010.10.048

Ebenhöh, O. and Hazlerigg, D. (2013) Modelling a molecular calendar: the seasonal photoperiodic response in mammals. *Chaos Solitons Fractals* 50, 39–47. https://doi.org/10.1016/j.chaos.2012.11.007

Abreux, A., Xue, Y., Cai, Y., Lewald, K.M., Nguyen, H.N., Zhang, Y. et al. (2020) EYES ABSENT and TIMELESS integrate photoperiodic and temperature cues to regulate seasonal physiology in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 117, 15293–15304. https://doi.org/10.1073/pnas.2004262117

Orten, D.J., Fischer, S.M., Sorensen, J.L., Rashadkarisma, U., Cremers, C.W., Mares, H.A. et al. (2008) Branchio-oto-renal syndrome (BOR): novel mutations in the EYA1 gene, and a review of the mutational genetics of BOR. *Hum. Mutat.* 29, 537–544. https://doi.org/10.1002/humu.20691

Unzoki, A., Morisada, N., Nozu, K., Ye, M.J., Ito, S., Matsunaga, T. et al. (2018) Clinically diverse phenotypes and genotypes of patients with branchio-oto-renal syndrome. *Hum. Genet.* 63, 647–656. https://doi.org/10.1007/s00439-018-0429-8

Agez, L., Laurent, V., Guerrero, H.Y., Pevet, P., Masson-Pevet, M. and Gauer, F. (2009) Endogenous melatonin provides an effective circadian message to both the suprachiasmatic nuclei and the pars tuberalis of the rat. *J. Pineal Res.* 46, 95–105. https://doi.org/10.1111/j.1600-079X.2008.00636.x

Trends Genet. 21, 163–171. https://doi.org/10.1016/j.tig.2005.01.005

Orten, D.J., Fischer, S.M., Sorensen, J.L., Rashadkarisma, U., Cremers, C.W., Mares, H.A. et al. (2008) Branchio-oto-renal syndrome (BOR): novel mutations in the EYA1 gene, and a review of the mutational genetics of BOR. *Hum. Mutat.* 29, 537–544. https://doi.org/10.1002/humu.20691

Rayapureddi, J.P. and Hegde, R.S. (2006) Branchio-oto-renal syndrome associated mutations in Eyes Absent 1 result in loss of phosphatase activity. *Biochemical Society Transactions* 34, 1397–1408. https://doi.org/10.1042/BS02013002

Buller, C., Xu, X., Marquis, V., Schwanke, R. and Xu, P.X. (2001) Molecular effects of Eya1 domain mutations causing organ defects in BOR syndrome. *Hum. Mol. Genet.* 10, 733–741. https://doi.org/10.1093/hmg/10.3.195

Taylor, M.H. and Wilton, N.C. (2007) Bradycardia with sevoflurane in siblings with Branchio-oto-renal syndrome. *Paediatr. Anaesth.* 17, 80–83. https://doi.org/10.1111/j.1460-9592.2006.02024.x

Taylor, M.H. and Wilton, N.C. (2007) Bradycardia with sevoflurane in siblings with Branchio-oto-renal syndrome. *Paediatr. Anaesth.* 17, 80–83. https://doi.org/10.1111/j.1460-9592.2006.02024.x

Makishima, T., Madeo, A.C., Brewer, C.C., Zalewski, C.K., Butman, J.A., Sachdev, V. et al. (2007) Nonsyndromic hearing loss DFNA10 and a novel mutation in the transcriptional activator EYA4 cause dilated cardiomyopathy and sensorineural hearing loss. *Hum. Mol. Genet.* 16, 2775–2781. https://doi.org/10.1093/hmg/ddm237

Shinmasa, N., Watanabe, K., Hara, M. and Kosaki, K. (2004) EYA1 mutation in a newborn female presenting with cardiacofacial syndrome. *Pediatr. Cardiol.* 25, 411–413. https://doi.org/10.1007/s00246-003-0271-3

Taylor, M.H. and Wilton, N.C. (2007) Bradycardia with sevoflurane in siblings with Branchio-oto-renal syndrome. *Paediatr. Anaesth.* 17, 80–83. https://doi.org/10.1111/j.1460-9592.2006.02024.x

Schönberger, J., Wang, L., Shih, J.T., Kim, S.D., Depreux, F.F., Zhu, H. et al. (2005) Mutation in the transcriptional coactivator EYA4 causes dilated cardiomyopathy and sensorineural hearing loss. *Nat. Genet.* 37, 418–422. https://doi.org/10.1038/ng1527

De Leenheer, E.M., Huygen, P.L., Wayne, S., Verstreken, M., Declau, F., Van Camp, G. et al. (2002) DFNA10/EYA4—the clinical picture. *Adv. Otorhinolaryngol.* 61, 73–78. https://doi.org/10.1006/loru.2002.006007

Wayne, S., Robertson, N.G., Declau, F., Chen, N., Verstreken, M., Prasad, S. et al. (2001) Mutations in the transcriptional activator EYA4 cause late-onset deafness at the DFNA10 locus. *Hum. Mol. Genet.* 10, 195–200. https://doi.org/10.1093/hmg/10.3.195

Makishima, T., Madeo, A.C., Brewer, C.C., Zalewski, C.K., Butman, J.A., Sachdev, V. et al. (2007) Nonsyndromic hearing loss DFNA10 and a novel mutation of EYA4: evidence for correlation of normal cardiac phenotype with truncating mutations of the Eya domain. *Am. J. Med. Genet. A* 143A, 1592–1598. https://doi.org/10.1002/ajmg.a.31793

Hildebrand, M.S., Coman, D., Yang, T., Gardner, R.J., Rose, E., Smith, R.J. et al. (2007) A novel splice site mutation in EYA4 causes DFNA10 hearing loss. *Am. J. Med. Genet. A* 143A, 1599–1604. https://doi.org/10.1002/ajmg.a.31860

Ahmadmehrabi, S., Li, B., Park, J., Devkota, B., Vukovic, M., Ko, Y.A. et al. (2021) Genome-first approach to rare EYA4 variants and cardio-auditory phenotypes in adults. *Hum. Genot.* 140, 957–967. https://doi.org/10.1002/humu.20691

Tingaud-Sequeira, A., Trinouille, A., Salaria, M., Stapleton, R., Claverol, S., Plaisant, C. et al. (2021) A recurrent missense variant in EYA3 gene is associated with oculo-auriculo-vertebral spectrum. *Hum. Genot.* 140, 933–944. https://doi.org/10.1002/humu.20225-6

Wang, Y., Pandey, R.N., Riffle, S., Chintala, H., Wikenheiser-Brokamp, K.A. and Hegde, R.S. (2018) The protein tyrosine phosphatase activity of eyes absent contributes to tumor angiogenesis and tumor growth. *Mol. Cancer Ther.* 17, 1659–1669. https://doi.org/10.1158/1535-7163.MCT-18-0057

Wang, Y., Pandey, R.N., Roychoudhury, K., Miliewski, D., Kalin, T.V., Szabo, S. et al. (2021) Targeting Eya3 in Ewing Sarcoma Retards tumor growth and angiogenesis. *Mol. Cancer Ther.* 20, 803–815. https://doi.org/10.1158/1535-7163.MCT-20-0479

Ananthanarayan, J., Zhou, H., Zhang, L., Hetz, T., Vincent, M.Y., Bleins, M.A. et al. (2019) Structural and functional analyses of an allosteric EYA2 phosphatase inhibitor that has on-target effects in human lung cancer cells. *Mol. Cancer Ther.* 18, 1484–1496. https://doi.org/10.1158/1535-7163.MCT-18-1239

Wang, Y., Pandey, R.N., York, A.J., Maltesa, J., Nichols, W.C., Hu, Y.C. et al. (2019) The EYA3 tyrosine phosphatase activity promotes pulmonary vascular remodeling in pulmonary arterial hypertension. *Nat. Commun.* 10, 4143. https://doi.org/10.1038/s41467-019-12226-1

Rebay, I., Silver, S.J. and Tootle, T.L. (2005) New vision from Eyes absent: transcription factors as enzymes. *Trends Genet.* 21, 163–171. https://doi.org/10.1016/j.tig.2005.01.005
Khersonsky, O. and Tawfik, D.S. (2010) Enzyme promiscuity: a mechanistic and evolutionary perspective. *Annu. Rev. Biochem.* **79**, 471–505 https://doi.org/10.1146/annurev-biochem-030409-143718

Khersonsky, O., Roodveldt, C. and Tawfik, D.S. (2006) Enzyme promiscuity: evolutionary and mechanistic aspects. *Curr. Opin. Chem. Biol.* **10**, 498–508 https://doi.org/10.1016/j.cbpa.2006.08.011

Copley, S.D. (2017) Shining a light on enzyme promiscuity. *Curr. Opin. Struct. Biol.* **47**, 167–175 https://doi.org/10.1016/j.sbi.2017.11.001

Kim, S.S., Zhang, R., Braunstein, S.E., Joachimiak, A., Cvekl, A. and Hegde, R.S. (2002) Structure of the retinal determination protein dachshund reveals a DNA binding motif. *Structure (Camb)* **10**, 787–795 https://doi.org/10.1016/S0969-2126(02)00769-4

Wood, S.H., Hindle, M.M., Mizono, Y., Cheng, Y., Saer, B.R.C., Miedzinska, K. et al. (2020) Circadian clock mechanism driving mammalian photoperiodism. *Nat. Commun.* **11**, 4291 https://doi.org/10.1038/s41467-020-18061-z