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The Eya Phosphatase: its unique role in cancer

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Abstract

The Eya proteins were originally identified as essential transcriptional co-activators of the Six family of homeoproteins. Subsequently, the highly conserved C-terminal domains of the Eya proteins were discovered to act as a Mg^{2+} -dependent Tyr phosphatases, making Eyas the first transcriptional activators to harbor intrinsic phosphatase activity. Only two direct targets of the Eya Tyr phosphatase have been identified: H2AX, whose dephosphorylation directs cells to the DNA repair instead of the apoptotic pathway upon DNA damage, and ER β , whose dephosphorylation inhibits its anti-tumor transcriptional activity. The Eya Tyr phosphatase mediates breast cancer cell transformation, migration, invasion, as well as metastasis, through targets not yet identified. Intriguingly, the N-terminal domain of Eya contains a separate Ser/Thr phosphatase activity implicated in innate immunity and in regulating c-Myc stability. Thus, Eya proteins are highly complex, containing two separable phosphatase domains and a transcriptional activation domain, thereby influencing tumor progression through multiple mechanisms.

Keywords

Phosphatase; Cancer; Eya; Inhibitor; Biochemistry

Eya proteins were first identified as essential co-activators of the Six family of homeoproteins, which are expressed during early embryogenesis and essential for the development of numerous organs^{1–5}. Since some Six family members, such as Six1, do not have intrinsic activation or repression domains, they require the Eya co-activators to regulate transcription, both in normal development^{6–10} and in various diseases^{10–19}. The mammalian Eya family consists of four members (Eya1–4), all of which contain a highly conserved C-terminal Eya Domain (CTD or ED, sequence identity between 83–89% among human Eya

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family members) that interacts with the Six domain (SD) of most Six family members, and a second less conserved Eya domain/N-terminal activation domain that is Pro/Ser/Thr (P/S/T)-rich (ED2/NTD)²⁰ (Fig. 1). Cellular compartmentalization of Eya is achieved via interaction of ED with the Six family members, which translocate Eya into the nucleus. Once in the nucleus, the Six/Eya complex functions as a bipartite transcription factor, with the Six family member providing DNA-binding specificity, and the Eya family member providing transactivation activity^{7,21}. In contrast, the Abl kinase directs Eya to the cytoplasm by directly phosphorylating its P/S/T-rich region, where it can carry out additional functions unrelated to its action as a transcriptional cofactor²².

Like Six family members, Eya proteins are critical for the development of multiple organs, in part by acting with Six proteins to promote proliferation and survival of progenitor cell populations²⁰. Mutations in Six1 and/or Eya lead to various genetic disorders^{10–19}. The crystal structure of maltose binding protein (MBP) fused Six1 in complex with Eya2 ED has been determined, revealing the molecular details of the Eya2 ED-Six1 interaction, as well as the structural basis of mutations causing branchio-oto-renal (BOR) syndrome¹⁵ (Fig. 2). Six and Eya family members are typically down regulated after organ development is complete, but various Six and Eya family members (particularly Six1, 2 and Eya1, 2) are re-expressed in multiple tumor types, including ovarian^{23,24}, breast^{25,26}, glioblastomas²⁷, leukemia²⁸, and Wilms' tumor^{29–31}. Overexpression of either Six1 or Eya (or both together) correlates with recurrence, metastasis, and decreased overall survival in a variety of tumor types^{24,31–37}. Disrupting the interaction between Six1 and Eya2 significantly reduces Six1-mediated metastasis and increases survival in a xenograft breast cancer model¹⁵, underscoring the importance of the Six1-Eya interaction in tumor progression.

The Tyr phosphatase activity of Eya

In addition to interacting with Six1, the C-terminal ED of Eya proteins also contains signature motifs of the haloacid dehalogenase (HAD) hydrolases, a diverse collection of enzymes including phosphatases^{2,41,42}. HAD family phosphatases, including Eya, use an Asp as their active site residue instead of the more commonly used Cys residue in most cellular protein Tyr phosphatases⁴³. The crystal structure of Eya2 ED supports the structural and mechanistic similarities between Eya and other HAD family phosphatases⁴⁴ (Fig. 2). A few other HAD phosphatases (for example, Scp1 and Chronophin) target proteins, however, most HAD phosphatases do not have protein phosphatase activity⁴⁵. All other known HAD protein phosphatases dephosphorylate Ser/Thr residues, while Eya targets phosphorylated Tyr⁴⁶. Schor and colleagues recently demonstrated that methylation of the highly conserved residues R304 and R306 in Eya1 is essential for its Tyr phosphatase activity, revealing a possible mechanism through which the Tyr phosphatase activity of Eya can be regulated⁴⁷.

A possible relationship between the Tyr phosphatase activity of Eya and its transcriptional activity was illustrated by the observation that mouse Eya proteins can utilize their intrinsic phosphatase activity to switch the Six1 transcriptional complex from a repressor to an activator complex for Six1-induced genes², although the mechanism of this switch remains unclear. In *Drosophila*, while the phosphatase activity of Eya is not globally required for *Sine oculis* (the *Drosophila* homolog of Six)-mediated transcription, it is required to enhance

transcription of a subset of genes regulated by *Sine oculis*⁴⁸. The Eya proteins therefore represent the first transcription factors with intrinsic phosphatase activity that modulate transcriptional complexes^{2,41,42}. Interestingly, despite the finding that the Eya Tyr phosphatase activity may regulate Six-mediated transcription, whether this activity is required for *Drosophila* eye development remains controversial. Early studies from the Hegde, Mardon, and Rebay groups strongly indicate that the Eya Tyr phosphatase plays a crucial role in normal *Drosophila* eye development^{41,42}. However, this notion was challenged by two recent studies from Mardon and colleagues, who demonstrated that Eya mainly functions as a transcriptional activator during *Drosophila* eye development, whereas its Tyr phosphatase activity is dispensable^{49,50}.

The Tyr phosphatase activity of Eyas has been shown to regulate multiple cellular phenotypes, many of which are associated with tumor progression. In normal mouse myoblasts, the Eya3 Tyr phosphatase is critical for cellular proliferation². Eyas have further been implicated in polarity of lung epithelium, where the Eya1 Tyr phosphatase activity regulates tight junction assembly through regulation of α PKC-zeta-Notch1-Cdc42 signaling⁵¹. In breast cancer cells, Eya1 also promotes cellular proliferation, by upregulating Cyclin D1 in a manner dependent on its Tyr phosphatase activity⁵². Using two different breast cancer cell lines and both overexpression and knockdown systems, and examining multiple different Eya family members, Hegde and colleagues demonstrated that Eya proteins, and in particular their Tyr phosphatase activity, are critical for transformation, migration, invasion, and metastasis in breast cancer, even though an effect on cellular proliferation was not observed in this model⁴⁰. The effects of the Tyr phosphatase activity of Eya on these tumor-associated phenotypes were proposed to be via a cytoplasmic rather than nuclear role of Eya. Given that the identified targets of Eya to date are all nuclear^{53–55}, it is unclear how the Eya Tyr phosphatase promotes tumorigenic phenotypes in the cytoplasm, although Hegde and colleagues provide evidence that Eya may utilize its Tyr phosphatase activity to regulate motility through altering Rho and Rac/cdc42 activities⁴⁰. Intriguingly, the Tyr phosphatase activity of Eya1 was also shown to be important for Six1/Eya1-mediated activation of Sonic hedgehog (Shh) signaling by up-regulating Nrp 1/2, thereby contributing to the growth of medulloblastoma, subtypes of which are dependent on Shh³⁸. While the precise mechanism by which Eya's phosphatase enhances tumor progression remains unknown, these data suggest that targeting the Tyr phosphatase of Eya may inhibit the growth and progression of multiple tumor types.

To date, only two physiological substrates of the Eya Tyr phosphatase have been identified. Eya1, 2, and 3 have all been shown to dephosphorylate the C-terminal pY142 on histone variant H2AX in human and mouse embryonic cell lines^{53,54}. This dephosphorylation is critical for directing cells to the DNA repair (by recruiting the MDC1/MRN complex) instead of apoptotic pathway under DNA damaging conditions^{53,54}. Thus, knockdown of Eya leads to a significant increase in apoptotic cells in response to hypoxia or ionizing radiation^{53,54}. Consistent with this notion, Eya3 knockdown results in decreased DNA repair in response to DNA damage in Ewing sarcoma cells, sensitizing these cells to DNA-damaging chemotherapeutics used to treat the disease⁵⁶. Currently, about half of all people with cancer are treated with radiation therapy, either alone or together with other cancer treatments, to kill cancer cells and reduce tumor burden⁵⁷. Selectively sensitizing tumor

tissue by engaging the apoptotic program of a cell is of great interest to the field of radiation oncology⁵⁸. It is foreseeable that inhibitors of Eya's Tyr phosphatase activity may increase the efficiency of radiation or other DNA damaging related therapy in cancers that are known to overexpress Eya, including breast cancers^{59,60}, Wilms' tumor²⁹, ovarian carcinomas²⁴, and Ewings Sarcomas (EWS)⁵⁶.

The second physiological substrate identified for the Eya Tyr phosphatase is estrogen receptor β (ER β)⁵⁵. ER β , when phosphorylated by Abl kinase on its Y36 residue, is able to recruit transcriptional co-activators, leading to activation of downstream ER β targets, and inhibition of cancer cell growth in culture and mouse xenografts. Eya2 can dephosphorylate pY36 on ER β , thus inhibiting its transactivation ability and negating its anti-tumor activity. In human breast cancer, high levels of pY36 on ER β correlate with elevated levels of Abl, but reduced levels of Eya2, and the presence of pY36-specific ER β strongly correlates with disease-free and overall survival in patients with stage II and III disease. Interestingly, Abl also directs Eya to the cytoplasm by phosphorylating Eya's P/S/T-rich region²², therefore, Abl may maintain the tumor suppressive role of ER β via both phosphorylating Y36 in ER β and sequestering Eya. Considering that ER β , in contrast to ER α , is present in approximately half of triple negative breast cancers⁵⁵, an aggressive subtype of breast cancer that does not have targeted therapeutic options, inhibiting the Eya2 phosphatase activity may potentiate the anti-tumor activity of ER β in these tumors.

Eya's Ser/Thr phosphatase activity

Surprisingly, the NTD of Eya contains Ser/Thr phosphatase activity in addition to its transactivation activity⁶¹. Thus, the Eya proteins may be the only known dual phosphatases in which the phosphatase activities reside in two separable domains. While the NTD is less conserved than the ED between different Eya family members (29–37% identity), conservation is high for a particular Eya family member between species (*e.g.*, identity in the NTD of Eya3 amongst mammals is 97–100%)²⁰. Intriguingly, this domain shows no sequence homology to any known phosphatase motifs^{61,62}, although a number of residues (C56, Y77, H79, and Y90) in Eya3, conserved among different species, have been shown to be important for its Ser/Thr phosphatase activity⁶². The lack of conservation to known Ser/Thr phosphatases in the NTD of Eya leaves a critical unanswered question as to how this domain acts as a Ser/Thr phosphatase.

Recently an entirely novel function of Eya's Ser/Thr phosphatase activity in innate immunity was identified. In *Drosophila*, Eya was shown to be critical in the initiation of an innate immune response against foreign DNA/RNA, in a manner dependent on its Ser/Thr phosphatase activity^{61,63}. Similarly, the Thr phosphatase activity of mammalian Eya4 enhances innate immune responses against viruses in mouse embryo fibroblasts (MEFs), in part by promoting CXCL10 and IFN- β expression⁶¹. Despite identification of binding partners of Eya within the NF- κ B pathway^{10,11}, its direct Ser/Thr phosphatase targets that influence innate immunity are unknown. In cancer, immune cells play dual, paradoxical roles in both tumor regression/stasis and in tumor progression. Infiltration of CD8+ cytotoxic T-cells, classical macrophages, CD4+ Th1 cells and other immune cells can promote cell lysis, angiostasis, and the death of cancer cells leading to stasis or regression of

tumors⁶⁴. In contrast, infiltrating CD4⁺ Th2 cells, CD4⁺ Treg cells, and tumor-associated macrophages (TAMs) can lead to remodeling of the ECM and to the secretion of MMPs, which support secretion of growth/survival factors, genomic instability of cancer cells, fibroblast activation, angiogenesis and lymphangiogenesis, all factors that can be pro-tumorigenic and/or pro-metastatic^{65–73}. Many chemokines, which affect immune cell infiltration, are altered in cancers. CXCL10 has historically been associated with anti-tumor properties, such as inhibition of angiogenesis⁷⁴. However, recently, CXCL10 has been implicated in pro-tumorigenic and metastatic activities, likely due to its effects on macrophage migration and polarization and its ability to attract subsets of CD4⁺ T-cells^{75–78}. For example, increased CXCL10 is associated with increased lymphocyte infiltration and poor prognosis⁷⁸ in breast cancer. Furthermore, in experimental models of breast cancer, CXCL10 has been shown to promote tumor progression and metastasis^{79–81}. These observations suggest that the Ser/Thr phosphatase activity of Eya can potentially promote tumor progression and metastasis through altering the immune response to tumors, although the role of Eya in tumor immunity has not yet been examined.

The only potential target identified for the Ser/Thr phosphatase activity of Eya is c-Myc. Eya1 was shown to contribute to the maintenance of nephron progenitor pool by dephosphorylating pT58 and stabilizing c-Myc via its Ser/Thr phosphatase activity^{82,83}. Whether Eya regulates c-Myc in cancers has not been directly investigated, but given the key transcriptional and oncogenic role of c-Myc, this aspect of Eya biology will certainly be a topic worthy of further investigation. Of note, ~15% of all human genes as well as multiple cellular processes such as cell cycle progression, transformation, and apoptosis are regulated by c-Myc⁸⁴. Accordingly, overexpression or hyperactivation of c-Myc has been long reported as a key driver of cancer, and poor prognosis is linked to c-Myc amplification in multiple tumor types^{85–88}. Due to its critical roles in tumor progression, targeting c-Myc is a highly sought after therapeutic approach, however, no druggable domains exist in its helix-loop-helix structure. Since Eya is involved in the regulation of c-Myc, c-Myc may be alternatively targeted by inhibiting the Ser/Thr phosphatase activity of Eya, circumventing the difficulty in direct targeting the c-Myc transcription factor.

Targeting the Eya phosphatase for potential cancer therapy

Given the role of Eya's Tyr phosphatase in cancer, targeting this activity may be an attractive approach for cancer therapy. Indeed, multiple labs have developed preliminary inhibitors targeting the Tyr phosphatase activity of Eya, which has a well-defined active site that is significantly different from most cellular phosphatases and thus offers the potential for inhibitor specificity. This is in marked contrast to most phosphatase inhibitors, where specificity is difficult to achieve, leading to substantial toxicities⁸⁹. Using high throughput screening, Hegde and colleagues identified benzobromarone-related compounds which target the Tyr phosphatase activity of Eya2 and Eya3^{39,90}. The identified lead compounds are proposed to bind immediately adjacent to the active site and can inhibit cell proliferation, motility, and angiogenic tubulogenesis and sprouting. Benzobromarone and related benzarone compounds significantly reduced retinal neovascularization compared to vehicle controls, demonstrating promising *in vivo* activity⁹¹. Using virtual screening methods, Park and colleagues identified additional classes of Eya active site inhibitors carrying various Mg²⁺

chelating groups, although the cellular effects of these compounds have not been evaluated⁹². We have identified a series of N-arylidenebenzohydrazide-containing compounds through high throughput screening that specifically inhibit Eya2 (but not other Eya family members) through an allosteric mechanism^{93,94}. These compounds can reverse EYA2 phosphatase-dependent cell migration in MCF10A cells, suggesting that these compounds could ultimately inhibit metastasis⁹⁴.

Further optimization of the above small molecule compounds to improve their potency and pharmacological properties followed by thorough preclinical evaluation of these compounds will be the next step if these compounds are to be developed into better research tools or potential therapeutic agents to inhibit tumor growth and metastasis. Furthermore, these novel Eya Tyr phosphatase inhibitors should be tested in combination with other therapeutic agents, particularly those that act through inducing DNA damage, to evaluate the potential of these compounds to be used in combination with other drugs. Finally, because the active site and molecular mechanism of Eya's Ser/Thr phosphatase activity is not yet well characterized, and the role of this activity in tumor onset and progression remains unknown, inhibitor development against the Ser/Thr phosphatase of Eya has not yet begun. Thus, understanding the molecular mechanism of the Eya Ser/Thr phosphatase activity remains a critical area of research, particularly if the two phosphatase activities of Eya cooperatively mediate tumor progression.

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References

1. Ozaki H, et al. Six1 controls patterning of the mouse otic vesicle. *Development*. 2004; 131:551–62. [PubMed: 14695375]
2. Li X, et al. Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. *Nature*. 2003; 426:247–54. [PubMed: 14628042]
3. Grifone R, et al. Six1 and Six4 homeoproteins are required for Pax3 and Mrf expression during myogenesis in the mouse embryo. *Development*. 2005; 132:2235–49. [PubMed: 15788460]
4. Ikeda K, et al. Six1 is essential for early neurogenesis in the development of olfactory epithelium. *Dev Biol*. 2007; 311:53–68. [PubMed: 17880938]
5. Zheng W, et al. The role of Six1 in mammalian auditory system development. *Development*. 2003; 130:3989–4000. [PubMed: 12874121]
6. Christensen KL, Patrick AN, McCoy EL, Ford HL. The six family of homeobox genes in development and cancer. *Adv Cancer Res*. 2008; 101:93–126. [PubMed: 19055944]
7. Ohto H, et al. Cooperation of six and eya in activation of their target genes through nuclear translocation of Eya. *Mol Cell Biol*. 1999; 19:6815–24. [PubMed: 10490620]
8. Grifone R, et al. Six1 and Eya1 expression can reprogram adult muscle from the slow-twitch phenotype into the fast-twitch phenotype. *Mol Cell Biol*. 2004; 24:6253–67. [PubMed: 15226428]
9. Zou D, et al. Patterning of the third pharyngeal pouch into thymus/parathyroid by Six and Eya1. *Dev Biol*. 2006; 293:499–512. [PubMed: 16530750]
10. Blevins MA, Towers CG, Patrick AN, Zhao R, Ford HL. The SIX1-EYA transcriptional complex as a therapeutic target in cancer. *Expert Opin Ther Targets*. 2015; 19:213–25. [PubMed: 25555392]

11. Abdelhak S, et al. Clustering of mutations responsible for branchio-oto-renal (BOR) syndrome in the eyes absent homologous region (eyaHR) of EYA1. *Hum Mol Genet.* 1997; 6:2247–55. [PubMed: 9361030]
12. Ruf RG, et al. SIX1 mutations cause branchio-oto-renal syndrome by disruption of EYA1-SIX1-DNA complexes. *Proc Natl Acad Sci U S A.* 2004; 101:8090–5. [PubMed: 15141091]
13. Zhang Y, Knosp BM, Maconochie M, Friedman RA, Smith RJ. A comparative study of Eya1 and Eya4 protein function and its implication in branchio-oto-renal syndrome and DFNA10. *J Assoc Res Otolaryngol.* 2004; 5:295–304. [PubMed: 15492887]
14. Patrick AN, Schiemann BJ, Yang K, Zhao R, Ford H. Biochemical and functional characterization of 6 Six1 branchio-Oto-renal syndrome mutations. *J Biol Chem.* 2009
15. Patrick AN, et al. Structure-function analyses of the human SIX1-EYA2 complex reveal insights into metastasis and BOR syndrome. *Nat Struct Mol Biol.* 2013; 20:447–53. [PubMed: 23435380]
16. Kong D, et al. The retinal determination gene network: from developmental regulator to cancer therapeutic target. *Oncotarget.* 2016; 7:50755–50765. [PubMed: 27203207]
17. Liu Y, et al. The DACH/EYA/SIX gene network and its role in tumor initiation and progression. *Int J Cancer.* 2016; 138:1067–75. [PubMed: 26096807]
18. Tadjuidje E, Hegde RS. The Eyes Absent proteins in development and disease. *Cell Mol Life Sci.* 2013; 70:1897–913. [PubMed: 22971774]
19. Xu PX. The EYA-SO/SIX complex in development and disease. *Pediatr Nephrol.* 2013; 28:843–54. [PubMed: 22806561]
20. Tadjuidje E, Hegde RS. The Eyes Absent proteins in development and disease. *Cell Mol Life Sci.* 2012
21. Xu PX, Cheng J, Epstein JA, Maas RL. Mouse Eya genes are expressed during limb tendon development and encode a transcriptional activation function. *Proc Natl Acad Sci U S A.* 1997; 94:11974–9. [PubMed: 9342347]
22. Xiong W, Dabbouseh NM, Rebay I. Interactions with the Abelson tyrosine kinase reveal compartmentalization of eyes absent function between nucleus and cytoplasm. *Dev Cell.* 2009; 16:271–9. [PubMed: 19217428]
23. Behbakht K, et al. Six1 overexpression in ovarian carcinoma causes resistance to TRAIL-mediated apoptosis and is associated with poor survival. *Cancer Res.* 2007; 67:3036–42. [PubMed: 17409410]
24. Zhang L, et al. Transcriptional coactivator Drosophila eyes absent homologue 2 is upregulated in epithelial ovarian cancer and promotes tumor growth. *Cancer Res.* 2005; 65:925–32. [PubMed: 15705892]
25. Farabaugh SM, Micalizzi DS, Jedlicka P, Zhao R, Ford HL. Eya2 is required to mediate the pro-metastatic functions of Six1 via the induction of TGF-beta signaling, epithelial-mesenchymal transition, and cancer stem cell properties. *Oncogene.* 31:552–62.
26. Wang CA, et al. Homeoprotein Six2 promotes breast cancer metastasis via transcriptional and epigenetic control of E-cadherin expression. *Cancer Res.* 2014; 74:7357–70. [PubMed: 25348955]
27. Auvergne RM, et al. Transcriptional differences between normal and glioma-derived glial progenitor cells identify a core set of dysregulated genes. *Cell Rep.* 3:2127–41.
28. Wang QF, et al. MLL fusion proteins preferentially regulate a subset of wild-type MLL target genes in the leukemic genome. *Blood.* 117:6895–905.
29. Li CM, et al. Gene expression in Wilms' tumor mimics the earliest committed stage in the metanephric mesenchymal-epithelial transition. *Am J Pathol.* 2002; 160:2181–90. [PubMed: 12057921]
30. Wegert J, et al. Mutations in the SIX1/2 pathway and the DROSHA/DGCR8 miRNA microprocessor complex underlie high-risk blastemal type Wilms tumors. *Cancer Cell.* 2015; 27:298–311. [PubMed: 25670083]
31. Walz AL, et al. Recurrent DGCR8, DROSHA, and SIX homeodomain mutations in favorable histology Wilms tumors. *Cancer Cell.* 2015; 27:286–97. [PubMed: 25670082]
32. Farabaugh SM, Micalizzi DS, Jedlicka P, Zhao R, Ford HL. Eya2 is required to mediate the pro-metastatic functions of Six1 via the induction of TGF-beta signaling, epithelial-mesenchymal transition, and cancer stem cell properties. *Oncogene.* 2012; 31:552–62. [PubMed: 21706047]

33. Ng KT, et al. Clinicopathological significance of homeoprotein Six1 in hepatocellular carcinoma. *Br J Cancer*. 2006; 95:1050–5. [PubMed: 17008870]
34. Tan J, Zhang C, Qian J. Expression and significance of Six1 and Ezrin in cervical cancer tissue. *Tumour Biol*. 2011; 32:1241–7. [PubMed: 21874375]
35. Iwanaga R, et al. Expression of Six1 in luminal breast cancers predicts poor prognosis and promotes increases in tumor initiating cells by activation of extracellular signal-regulated kinase and transforming growth factor-beta signaling pathways. *Breast Cancer Res*. 2012; 14:R100. [PubMed: 22765220]
36. Micalizzi DS, et al. The Six1 homeoprotein induces human mammary carcinoma cells to undergo epithelial-mesenchymal transition and metastasis in mice through increasing TGF-beta signaling. *J Clin Invest*. 2009; 119:2678–90. [PubMed: 19726885]
37. Qamar L, et al. Specificity and prognostic validation of a polyclonal antibody to detect Six1 homeoprotein in ovarian cancer. *Gynecol Oncol*. 2012
38. Eisner A, et al. The Eya1 phosphatase promotes Shh signaling during hindbrain development and oncogenesis. *Dev Cell*. 2015; 33:22–35. [PubMed: 25816987]
39. Tadjuidje E, et al. The EYA tyrosine phosphatase activity is pro-angiogenic and is inhibited by benzobromarone. *PLoS One*. 2012; 7:e34806. [PubMed: 22545090]
40. Pandey RN, et al. The Eyes Absent phosphatase-transactivator proteins promote proliferation, transformation, migration, and invasion of tumor cells. *Oncogene*. 2010; 29:3715, 22. [PubMed: 20418914]
41. Rayapureddi JP, et al. Eyes absent represents a class of protein tyrosine phosphatases. *Nature*. 2003; 426:295–8. [PubMed: 14628052]
42. Tootle TL, et al. The transcription factor Eyes absent is a protein tyrosine phosphatase. *Nature*. 2003; 426:299–302. [PubMed: 14628053]
43. Tonks NK. Protein tyrosine phosphatases: from genes, to function, to disease. *Nat Rev Mol Cell Biol*. 2006; 7:833–46. [PubMed: 17057753]
44. Jung SK, et al. Crystal structure of ED-Eya2: insight into dual roles as a protein tyrosine phosphatase and a transcription factor. *FASEB J*. 2010; 24:560–9. [PubMed: 19858093]
45. Seifried A, Schultz J, Gohla A. Human HAD phosphatases: structure, mechanism, and roles in health and disease. *FEBS J*. 2013; 280:549–71. [PubMed: 22607316]
46. Krishnan N, et al. Dephosphorylation of the C-terminal tyrosyl residue of the DNA damage-related histone H2A.X is mediated by the protein phosphatase eyes absent. *J Biol Chem*. 2009; 284:16066–70. [PubMed: 19351884]
47. Li X, et al. Methylation of the phosphatase-transcription activator EYA1 by protein arginine methyltransferase 1: mechanistic, functional, and structural studies. *FASEB J*. 2017
48. Jemc J, Rebay I. Identification of transcriptional targets of the dual-function transcription factor/phosphatase eyes absent. *Dev Biol*. 2007; 310:416–29. [PubMed: 17714699]
49. Jin M, Jusiak B, Bai Z, Mardon G. Eyes absent tyrosine phosphatase activity is not required for Drosophila development or survival. *PLoS One*. 2013; 8:e58818. [PubMed: 23554934]
50. Jin M, Mardon G. Distinct Biochemical Activities of Eyes absent During Drosophila Eye Development. *Sci Rep*. 2016; 6:23228. [PubMed: 26980695]
51. El-Hashash AH, et al. Eya1 protein phosphatase regulates tight junction formation in lung distal epithelium. *J Cell Sci*. 2012; 125:4036–48. [PubMed: 22685326]
52. Wu K, et al. EYA1 phosphatase function is essential to drive breast cancer cell proliferation through cyclin D1. *Cancer Res*. 73:4488–99. [PubMed: 23636126]
53. Cook PJ, et al. Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. *Nature*. 2009; 458:591–6. [PubMed: 19234442]
54. Krishnan N, et al. Dephosphorylation of the C-terminal tyrosyl residue of the DNA damage-related histone H2A.X is mediated by the protein phosphatase eyes absent. *J Biol Chem*. 2009
55. Yuan B, et al. A phosphotyrosine switch determines the antitumor activity of ERbeta. *J Clin Invest*. 2014; 124:3378–90. [PubMed: 24960160]

56. Robin TP, et al. EWS/FLI1 regulates EYA3 in Ewing sarcoma via modulation of miRNA-708, resulting in increased cell survival and chemoresistance. *Mol Cancer Res.* 2012; 10:1098–108. [PubMed: 22723308]
57. Baskar R, Lee KA, Yeo R, Yeoh KW. Cancer and radiation therapy: current advances and future directions. *Int J Med Sci.* 2012; 9:193–9. [PubMed: 22408567]
58. Bernier J, Hall EJ, Giaccia A. Radiation oncology: a century of achievements. *Nat Rev Cancer.* 2004; 4:737–47. [PubMed: 15343280]
59. Wang Y, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet.* 2005; 365:671–9. [PubMed: 15721472]
60. van de Vijver MJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med.* 2002; 347:1999–2009. [PubMed: 12490681]
61. Okabe Y, Sano T, Nagata S. Regulation of the innate immune response by threonine-phosphatase of Eyes absent. *Nature.* 2009; 460:520–4. [PubMed: 19561593]
62. Sano T, Nagata S. Characterization of the threonine-phosphatase of mouse eyes absent 3. *FEBS Lett.* 2011; 585:2714–9. [PubMed: 21821028]
63. Liu X, et al. Drosophila EYA regulates the immune response against DNA through an evolutionarily conserved threonine phosphatase motif. *PLoS One.* 2012; 7:e42725. [PubMed: 22916150]
64. Coussens LM, Werb Z. Inflammation and cancer. *Nature.* 2002; 420:860–7. [PubMed: 12490959]
65. Chow MT, Moller A, Smyth MJ. Inflammation and immune surveillance in cancer. *Semin Cancer Biol.* 2012; 22:23–32. [PubMed: 22210181]
66. DeNardo DG, Coussens LM. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. *Breast Cancer Res.* 2007; 9:212. [PubMed: 17705880]
67. Beyer M, Schultze JL. Regulatory T cells in cancer. *Blood.* 2006; 108:804–11. [PubMed: 16861339]
68. Pardoll D. Metastasis-promoting immunity: when T cells turn to the dark side. *Cancer Cell.* 2009; 16:81–2. [PubMed: 19647215]
69. DeNardo DG, et al. CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell.* 2009; 16:91–102. [PubMed: 19647220]
70. Heys SD, et al. Characterisation of tumour-infiltrating macrophages: impact on response and survival in patients receiving primary chemotherapy for breast cancer. *Breast Cancer Res Treat.* 2012; 135:539–48. [PubMed: 22886449]
71. Eichbaum C, et al. Breast cancer cell-derived cytokines, macrophages and cell adhesion: implications for metastasis. *Anticancer Res.* 2011; 31:3219–27. [PubMed: 21965729]
72. Aranda V. Cancer: Metastasis with a little help from MDSCs. *Nat Med.* 2011; 17
73. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer.* 2006; 6:24–37. [PubMed: 16397525]
74. Palacios-Arreola MI, et al. The role of chemokines in breast cancer pathology and its possible use as therapeutic targets. *J Immunol Res.* 2014; 2014:849720. [PubMed: 25165728]
75. Man K, et al. Molecular signature linked to acute phase injury and tumor invasiveness in small-for-size liver grafts. *Ann Surg.* 2010; 251:1154–61. [PubMed: 20485145]
76. Raitman, I. University of Toronto. 2010.
77. Koch MA, et al. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol.* 2009; 10:595–602. [PubMed: 19412181]
78. Mulligan AM, et al. Tumoral lymphocytic infiltration and expression of the chemokine CXCL10 in breast cancers from the Ontario Familial Breast Cancer Registry. *Clin Cancer Res.* 2013; 19:336–46. [PubMed: 23213058]
79. Datta D, et al. Ras-induced modulation of CXCL10 and its receptor splice variant CXCR3-B in MDA-MB-435 and MCF-7 cells: relevance for the development of human breast cancer. *Cancer Res.* 2006; 66:9509–18. [PubMed: 17018607]

80. Walser TC, et al. Antagonism of CXCR3 inhibits lung metastasis in a murine model of metastatic breast cancer. *Cancer Res.* 2006; 66:7701–7. [PubMed: 16885372]
81. Ma X, et al. CXCR3 expression is associated with poor survival in breast cancer and promotes metastasis in a murine model. *Mol Cancer Ther.* 2009; 8:490–8. [PubMed: 19276169]
82. Li J, et al. EYA1's conformation-specificity in dephosphorylating phosphothreonine in Myc and its activity on Myc stabilization in breast cancer. *Mol Cell Biol.* 2016
83. Xu J, et al. Eya1 interacts with Six2 and Myc to regulate expansion of the nephron progenitor pool during nephrogenesis. *Dev Cell.* 2014; 31:434–47. [PubMed: 25458011]
84. Poole CJ, van Riggelen J. MYC-Master Regulator of the Cancer Epigenome and Transcriptome. *Genes (Basel).* 2017; 8
85. Blancato J, Singh B, Liu A, Liao DJ, Dickson RB. Correlation of amplification and overexpression of the c-myc oncogene in high-grade breast cancer: FISH, in situ hybridisation and immunohistochemical analyses. *Br J Cancer.* 2004; 90:1612–9. [PubMed: 15083194]
86. Sato H, Minei S, Hachiya T, Yoshida T, Takimoto Y. Fluorescence in situ hybridization analysis of c-myc amplification in stage TNM prostate cancer in Japanese patients. *Int J Urol.* 2006; 13:761–6. [PubMed: 16834657]
87. Kozma L, Kiss I, Szakall S, Ember I. Investigation of c-myc oncogene amplification in colorectal cancer. *Cancer Lett.* 1994; 81:165–9. [PubMed: 8012933]
88. Park JR, Eggert A, Caron H. Neuroblastoma: biology, prognosis, and treatment. *Pediatr Clin North Am.* 2008; 55:97–120. x. [PubMed: 18242317]
89. Barr AJ. Protein tyrosine phosphatases as drug targets: strategies and challenges of inhibitor development. *Future Med Chem.* 2010; 2:1563–76. [PubMed: 21426149]
90. Pandey RN, et al. Structure-activity relationships of benzobromarone metabolites and derivatives as EYA inhibitory anti-angiogenic agents. *PLoS One.* 2013; 8:e84582. [PubMed: 24367676]
91. Wang Y, et al. The Eyes Absent Proteins in Developmental and Pathological Angiogenesis. *Am J Pathol.* 2016; 186:568–78. [PubMed: 26765957]
92. Park H, et al. Structure-based virtual screening approach to the discovery of novel inhibitors of eyes absent 2 phosphatase with various metal chelating moieties. *Chem Biol Drug Des.* 78:642–50. [PubMed: 21777393]
93. Krueger AB, et al. Identification of a selective small-molecule inhibitor series targeting the eyes absent 2 (Eya2) phosphatase activity. *J Biomol Screen.* 18:85–96.
94. Krueger AB, et al. Allosteric Inhibitors of the Eya2 Phosphatase Are Selective and Inhibit Eya2-mediated Migration. *J Biol Chem.*

Fig. 1.
The many functions of the phosphatase activities of Eya proteins using the 526 amino acid-mEya3 as an example. Please see the main text for a more detailed description of each function.

Fig. 2.

Crystal structures of Eya2 ED and MBP-Six1 + Eya2 ED (PDB IDs are included in parentheses) offer insights into the catalytic mechanism of Eya2's Tyr phosphatase activity and its interaction with Six1.