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The SIX1-EYA transcriptional complex as a therapeutic target in cancer

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Abstract

Introduction—The SIX homeodomain proteins and the EYA family of co-activators form a bipartite transcription factor complex that promotes the proliferation and survival of progenitor cells during organogenesis and is down-regulated in most adult tissues. Abnormal over-expression of SIX1 and EYA in adult tissue is associated with the initiation and progression of diverse tumor types. Importantly, SIX1 and EYA are often co-overexpressed in tumors, and the SIX1-EYA2 interaction has been shown to be critical for metastasis in a breast cancer model. The EYA proteins also contain protein tyrosine phosphatase activity, which plays an important role in breast cancer growth and metastasis as well as directing cells to the repair pathway upon DNA damage.

Areas covered—This review provides a summary of the SIX1/EYA complex as it relates to development and disease and the current efforts to therapeutically target this complex.

Expert opinion—Recently, there have been an increasing number of studies suggesting that targeting the SIX1/EYA transcriptional complex will potently inhibit tumor progression. Although current attempts to develop inhibitors targeting this complex are still in the early stages, continued efforts towards developing better compounds may ultimately result in effective anti-cancer therapies.

1. Introduction

The sine oculis homeodomain (SIX) proteins are a family of transcription factors evolutionarily conserved from lower invertebrates to humans¹. The SIX proteins were originally identified in *Drosophila* as vital regulators of eye development, and have since been shown to participate in the expansion and differentiation of cell populations in

numerous organs including the retina, craniofacial structures, auditory system, brain, lung, muscle, kidney, and gonads, amongst others². SIX proteins promote cell proliferation through the regulation of multiple cell cycle genes known to affect the G1/S and G2/M transitions, as well as progression through S-phase³⁻⁷. Loss and gain-of-function studies have implicated SIX1 as a regulator of multiple tissue-specific differentiation factors that control cell fate⁸⁻¹⁰. Moreover, mutations within the SIX family, both naturally occurring and genetically engineered, have adverse consequences on the development of organs/tissues¹¹⁻¹⁶, confirming its role as a critical regulator of organogenesis.

In humans, there are six family members (*SIX1-6*) that are homologous to the *Drosophila* sine oculis (so) gene¹⁷. All SIX proteins are composed of two conserved domains, the nucleic acid recognition homeodomain (HD) and the SIX domain (SD)¹⁸. DNA binding is primarily mediated through the HD, while protein-protein interactions are mediated through the SD. However, there is strong evidence suggesting that the SD also contributes to the SIX1-DNA interaction, though this has yet to be directly confirmed by structural analyses¹⁹. In addition to the SD and HD, SIX2, SIX4, and SIX5 also have intrinsic transcriptional activation domains (AD), and thus may not require co-activator proteins, such as the EYAs described below, to promote transcription under all circumstances^{20, 21}.

The remaining SIX proteins, including SIX1, have no intrinsic activation domains and require a family of co-activators, EYA1-4, to mediate transcriptional activation. The human EYA proteins are homologs of the *Drosophila* eyes absent (*eya*) proteins that contain a highly conserved Eya domain (ED), responsible for interacting with the SD²². The SIX proteins actively translocate EYAs into the nucleus, where EYAs stimulate SIX-mediated transcription through their proline/serine/threonine (P/S/T) rich transactivation domains^{23, 24}. However, in cases in which EYA co-activators are absent, SIX1 can bind to promoters in conjunction with the co-repressor DACH1 to block transcription of target genes⁴. Embedded within the P/S/T domain of the EYA proteins is a separate ED, known as Eya domain 2 (ED2). This domain possesses threonine phosphatase activity, which has been shown to play a role in innate immunity^{25, 26}. Similar to SIX1, EYA proteins are also critical for the development of multiple organs in part by promoting proliferation and survival of progenitor cell populations²⁷.

Interestingly, not only is the ED able to interact with SIX proteins to stimulate transcription, the ED also possesses Mg²⁺-dependent tyrosine phosphatase activity^{28, 29}. The EYA proteins use a nucleophilic aspartic acid residue and a divalent metal ion to catalyze tyrosine dephosphorylation. Using SIX1-dependent reporter assays, the phosphatase activity of EYA3 has been shown to be required for the transcription of a subset of SIX1-induced genes⁴. However, a detailed understanding of the exact mechanism by which this occurs is lacking. To date, there are only two validated *in vivo* targets for EYA's tyrosine phosphatase activity. The first target is the histone variant, H2AX^{30, 31}. Upon DNA damage, EYAs dephosphorylate H2AX, directing cells to DNA repair rather than apoptotic pathways. More recently, EYA2 has been shown to inhibit the transcriptional activity of the estrogen receptor β (ER β), through the dephosphorylation of the Y36 residue³². The phosphorylated state of Y36 promotes the interaction between ER β and its co-activators, stimulating

transcriptional activation and subsequent inhibition of cell proliferation and differentiation³³.

Loss- and gain-of-function analyses in fruit flies, fish, frogs, and mice have been used to confirm the importance of both *SIX* and *EYA* genes in development, which is underscored by the existence of naturally occurring mutations of *SIX* and *EYA* in various human genetic syndromes^{14, 16, 34–41}. Proteins from both families are co-expressed in intricate spatio-temporal patterns in embryonic tissues to direct complex developmental processes². There is much evidence that both *EYA* and *SIX1* are required not only for cell proliferation but also for cell survival. For instance, the *Eyal* knockout phenocopies the *Six1* knockout in mice, and organ defects in both are attributed to a decrease in progenitor cell proliferation and an increase in cellular apoptosis, thus leading to reduced size or absence of organ development^{36, 42}. In both *Six1* and *Eyal* knockout mice, embryonic lung epithelial cells show increased expression of differentiation markers and a loss of progenitor cell markers, supporting their role as critical regulators of epithelial progenitor cells⁸. Additionally, in kidney development, knockout studies have shown that *Six1*, *Six2*, *Six4*, and *Eyal* each regulate critical genes required during development, including *GDNF*, which is important for the maintenance of the mesenchymal progenitor pool^{2, 9, 10, 35}. The loss of any of these Six family members leads to premature differentiation and depletion of the progenitor cell population. The studies highlighted above reveal the numerous developmental roles of the *SIX1/EYA* transcriptional complex, such as maintaining progenitor pools, cellular proliferation, and directing differentiation programs, confirming that the complex is a critical regulator of tissue growth and patterning.

2. The role of *SIX1* and *EYA* in genetic syndromes

Naturally occurring mutations in both *SIX* and *EYA* are implicated in multiple developmental disorders confirming their critical roles in organogenesis². In particular, mutations in *SIX1*, *SIX5*, or *EYA1* can lead to branchio-oto-renal (BOR) syndrome, a developmental disorder defined by hearing loss, bronchial fistulae, and renal anomalies⁴³. *EYA1* is the most commonly mutated gene in BOR syndrome with currently 14 known mutations located within the ED^{11, 44, 45}, which contains both the *SIX1* interaction site, as well as its tyrosine phosphatase activity⁴⁶. Structural and biochemical analysis of *EYA1/2* have provided insights into the molecular mechanisms of several BOR mutations¹⁹. Three of the BOR mutations (V496E, delV499, and L529P) are located in the *SIX1* interface and are predicted to abrogate *SIX1-EYA2* binding. Another mutant, N433P, resides in the middle of the catalytic domain and is shown to disrupt phosphatase activity; however it is still capable of binding *SIX1* in cells^{47, 48}. In addition to *EYA1*, mutations in *SIX1* have also been reported in BOR patients. Biochemical analyses of 6 of these mutations reveal that five act by disrupting the *SIX1*-DNA interaction^{38, 46}. The remaining *SIX1* BOR mutation, V17E, is located at the *SIX1-EYA* interface and disrupts the *SIX1-EYA* interaction⁴⁶. These natural mutations confirm that the *SIX1/EYA* transcriptional complex, including *EYA*'s phosphatase activity, is critical for normal organ development.

3. Oncogenic roles of the SIX1 transcription factor

The ability of the SIX transcriptional complex to direct multiple developmental programs necessary for the maintenance and expansion of progenitor populations, suggests that the abnormal overexpression of SIX transcriptional complex proteins in adult tissues could contribute to both tumorigenesis and tumor progression. Tumorigenic cells often resemble a more embryonic state, having been reprogrammed to maintain proliferation, survival, and additional cancer hallmark pathways (Figure 1), suggesting that correcting aberrant developmental transcriptional pathways in malignant cells may be a useful therapeutic approach⁴⁹. To date, SIX1 is the most heavily implicated SIX family member in cancer. Therefore, we will focus the remainder of this article on SIX1 and its known oncogenic roles.

SIX1 overexpression in cancers was first observed in 1998, where high levels of SIX1 expression in primary breast cancers and metastatic lesions were argued to influence tumor progression, at least in part, through its ability to attenuate the G2 cell cycle checkpoint in response to DNA damage³. Subsequently, multiple studies have confirmed that overexpression of SIX1 is tied to the occurrence and progression of breast cancer as well as other cancer types, in part through its ability to influence cell cycle control^{50–55}.

Over the past decade SIX1 has been implicated in multiple “hallmarks of cancer” including sustained proliferative signaling^{5, 56, 57}, invasion and metastasis^{51, 57–61}, evasion of growth suppressors⁶², induction of genomic instability⁶³, and resistance of cell death^{53, 64–66} (Figure 1). SIX1 is able to maintain cellular proliferation through the transcriptional regulation of numerous genes involved in cell cycle progression. Cyclin D1 has been shown to be a direct transcriptional target of SIX1 in pancreatic cancer⁵⁶ and rhabdomyosarcoma (RMS)⁵⁷, while Cyclin A1 is transcriptionally activated by SIX1 in breast cancer⁵ and c-Myc is transcriptionally regulated by SIX1 in rhabdomyosarcoma (RMS)⁵⁷.

The second cancer hallmark in which SIX1 has been implicated is activating invasion and metastasis. Indeed, SIX1 has been shown to be both pro-oncogenic and metastatic. Mammary specific Six1 overexpression in transgenic mice promotes highly aggressive tumors that undergo an epithelial to mesenchymal transition (EMT) and exhibit stem cell phenotypes⁵⁹. Six1-induced EMT has been shown to be through its ability to stimulate TGF- β signaling, which is also critical for Six1-mediated metastasis in experimental metastasis mouse models of mammary cancer^{51, 58}. In addition, SIX1 stimulates lymphangiogenesis in breast cancer through transcriptionally regulating VEGF-C, which then further contributes to its ability to mediate metastasis⁶⁰. Finally, in RMS, SIX1 directly promotes the expression of Ezrin, a protein known to play an important role in cell surface adhesion, cellular migration and domain organization within the plasma membrane⁵⁷. SIX1 has also been implicated in metastasis of other tumor types, including hepatocellular carcinoma, where it is associated with both proliferation and invasion⁶¹. Together, these studies demonstrate a critical role for SIX1 in tumor progression.

Another hallmark of cancer with which SIX1 is associated is the evasion of growth suppression. Indeed, we have shown in an experimental metastasis model that Six1 can

induce a switch in TGF- β signaling from tumor suppressive to tumor promotional, overcoming the growth suppressive function of TGF- β to allow its pro-migratory and invasive functions to be dominant⁶². Not only is SIX1 involved in overcoming growth suppression, but it has also been implicated in resistance to cell death. Studies have shown that SIX1 overexpression induces resistance to apoptosis and more specifically to tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-mediated apoptosis^{53, 64, 65}. Furthermore, SIX1 overexpression can circumvent paclitaxel-induced apoptosis in breast cancer cells⁶⁶. In addition to its documented effect on these original hallmarks of cancer, SIX1 has been shown to affect a cancer enabling characteristic, genome instability, where its overexpression has been shown to increase double strand breaks in breast cells⁶³.

Importantly, the ability of SIX1 to mediate these hallmarks of cancer is not restricted to one tumor type. Instead, SIX1 overexpression has been correlated with poor prognosis in numerous cancers including breast^{51, 59}, lung⁵⁴, pancreatic⁵⁶, cervical^{55, 67}, colorectal^{68, 69}, and ovarian cancers⁵³, amongst others. Furthermore, knockdown of SIX1 in multiple tumor types, including breast⁶⁰ and colorectal cancers⁷⁰, hepatocellular carcinomas⁶¹, and rhabdomyosarcomas⁵⁰, dramatically decreases tumor size and metastasis in animal models. Currently, few studies have examined the mechanisms leading to *Six1* re-expression in cancer and thus this remains an important topic to be investigated. There is evidence that *SIX1* can be amplified in a small percentage of breast cancers⁷¹, and that it can be regulated by microRNA-185, which is lost in cancers thereby allowing *Six1* to be re-expressed⁶⁴.

4. Oncogenic roles of the EYA family

EYAs are overexpressed in many cancers including Wilms' tumor⁷², Ewing sarcoma⁷³, breast⁷⁴, ovarian⁷⁵, and lung cancers⁷⁶. In general, overexpression of EYAs has been shown to be pro-tumorigenic²⁷, but in some cases the EYA proteins may have tumor suppressive functions. Thus, understanding the biology of EYAs in cancer may be more complex than that of SIX1, and this complexity may in part be attributable to its dual activities as both a transcriptional cofactor and phosphatase. For example, while EYA2 amplification and/or overexpression has been observed in numerous cancers^{19, 75, 77}, the *EYA2* gene is hypermethylated, resulting in decreased expression, in both colorectal⁷⁸ and pancreatic cancers⁷⁹, when compared to normal tissues. Similarly, EYA3, which has been shown to have a pro-oncogenic role in Ewing Sarcoma⁷³, is deleted in pancreatic adenocarcinomas⁸⁰. While EYA1 is increased downstream of the MLL-ENL fusion protein in acute leukemia and its expression in murine hematopoietic progenitor cells leads to their immortalization⁸¹ and transformation in cooperation with *Six1*, *Eya* overexpression has also been shown to activate an apoptotic program in a murine myeloid cell line⁸². Together, these data suggest that the EYA proteins may have multiple roles in cancer, and that they act in a highly context-dependent manner. Nonetheless, in the numerous cases where EYA overexpression is pro-tumorigenic, its overexpression has been implicated in a number of cancer hallmarks, including sustained proliferative signaling, resistance to cell death, angiogenesis, invasion and metastasis, and replicative immortality^{4, 19, 30, 32, 81, 83–85}. Due to the multiple biochemical activities of the EYA proteins, it is likely that EYAs contribute to these hallmarks through various mechanisms of action. Below we address how the overexpression of *Eya* proteins in selected tissues may mediate these hallmarks, either through their role as

transcriptional activators along with SIX1, or their role as tyrosine phosphatases. To date, the role of the threonine activity of EYA in cancer has not been explored, and remains an interesting avenue of investigation.

5. The role of the SIX1/EYA interaction in tumorigenesis and metastasis

Both SIX1 and EYA are overexpressed in multiple cancer types including ovarian^{53, 75}, breast⁷⁷, glioblastomas⁸⁶, leukemia⁸¹, and Wilms' tumor⁷². Moreover, we found that overexpression of either SIX1 or EYA commonly correlated with recurrence, metastasis, and a decreased overall survival in a variety of tumor types, using the Oncomine cancer microarray database¹⁹. Examination of SIX1 and EYA2 expression in the van de Vijver and Wang breast cancer gene expression datasets led to the observation that only when both proteins are overexpressed together does one observe a shortened time to metastasis and relapse, as well as shortened overall survival⁷⁷. Furthermore, in breast cancer cell lines, EYA2 is required for many of the SIX1-induced pro-metastatic phenotypes, including enhanced TGF- β signaling and EMT^{19, 77}. These data imply a coordinated action is required between the two proteins in breast and other cancer types and reinforce the hypothesis that EYA2 is required for the ability of SIX1 to mediate tumor progression.

Replicative immortality is a widely accepted hallmark of cancer. As outlined above, Eya1 has been shown to transform and immortalize hematopoietic progenitor cells (HPCs) *in vitro*⁸¹. Although Six1 alone was unable to immortalize HPCs, co-transduction of Six1 and Eya1 resulted in an increase in the number of immortalized cells over Eya1 alone, indicating that Six1 is able to enhance the transforming capacity of Eya1⁸¹.

Our group recently demonstrated the importance of a direct protein interaction between SIX1 and EYA to mediate breast cancer associated metastasis, an additional hallmark of cancer, using the single amino acid BOR mutation, V17E, which disrupts the SIX1-EYA interaction¹⁹. In contrast to wildtype SIX1, SIX1 V17E is unable to activate TGF- β signaling or induce EMT-like characteristics in MCF7 breast cancer cells. In a mouse model of late stage metastasis, mice injected with MCF7 cells carrying the *SIX1-V17E* mutation did not display enhanced metastasis or shortened survival above controls cells, in contrast to MCF7 cells overexpressing wildtype SIX1¹⁹. These data suggest that the SIX1-EYA interaction is critical for SIX1-mediated tumor progression and that inhibition of this protein complex could prevent tumor progression.

6. The role of EYA's phosphatase activity in tumorigenesis and metastasis

In addition to their role as co-activators of the SIX1 transcriptional complex, the EYA proteins are protein tyrosine phosphatases belonging to the haloacid dehalogenase (HAD) family of enzymes. The EYA phosphatase activity has been shown to play a role in chronic cellular proliferation, again, a hallmark of cancers. However, this role has been controversial. Although Hegde and colleagues detected no changes in cellular proliferation using phosphatase-deficient EYA2/3⁸⁴, Wu and colleagues, using a different breast cancer model, have demonstrated that EYA1 promotes cellular proliferation in a manner dependent on its phosphatase activity, through up-regulation of Cyclin D1⁸³. In support of the latter study, Rosenfeld and colleagues had earlier demonstrated that the Eya3 phosphatase activity

plays an important role in cellular proliferation in mouse myoblasts⁴. More recently, work by Yuan and colleagues identified a second *in vivo* target for EYA2 tyrosine phosphatase activity that is an important mediator of cellular proliferation³². The dephosphorylation of residue Y36 on ER β by EYA2 ablates ER β transcriptional activation and thus reduces its ability to inhibit tumor cell growth³².

Overexpression experiments using either wildtype or phosphatase-dead EYA mutants in breast cancer cells demonstrate that the EYA tyrosine phosphatase activity is critical for EYA-dependent transformation, migration, invasion, and metastasis⁸⁴. Of interest, the EYA3 tyrosine phosphatase activity has recently been shown to influence another cancer hallmark, angiogenesis, both *in vitro* and *in vivo* models using small molecule inhibitors and genetic approaches⁸⁵.

The final cancer hallmark in which EYA's phosphatase activity has been implicated is resisting cell death. EYA plays a role in the DNA damage response through its ability to dephosphorylate the minor histone protein, H2AX, upon DNA damage^{30, 31}. EYA1, 2 and 3 dephosphorylate H2AX at the C-terminal tyrosine 142 residue in mouse and human embryonic cell lines^{30, 31}. Dephosphorylation of H2AX by EYA leads to the recruitment of the MDC1/MRN repair complex and directs cells away from apoptotic pathways and towards DNA repair³⁰. Moreover, knockdown of EYA increases the number of apoptotic cells in response to hypoxia or ionizing radiation³⁰, and knockdown of EYA3 has been shown to sensitize Ewing sarcoma cells to DNA damaging chemotherapeutics⁷³. While in the majority of circumstances EYA is shown to resist cell death, as outlined above, in some studies EYA has actually been shown to promote cell death⁸². Nonetheless, the bulk of studies suggest that therapeutically targeting the phosphatase activity of EYA may be beneficial in two ways, through the prevention of tumor progression (presumably in part through its role as a transcription factor with SIX1), as well as through sensitizing cells to DNA-damaging chemotherapeutics.

7. Opportunities for Targeting the SIX1/EYA Transcriptional Complex

As a global regulator of tumor progression, inhibition of the SIX1 transcriptional complex could be a powerful approach that is therapeutically beneficial for many different cancers. Although traditionally considered a difficult target, recently significant progress has been made in targeting various aspects of transcriptional regulation including enzymatic activity, protein-protein interactions, epigenetic alterations, and DNA binding⁸⁷. A large body of work has been performed to validate the SIX1 transcriptional complex as a therapeutic target. The SIX1 transcriptional complex is an attractive target due to its decreased presence in normal adult tissues and its ability to influence a broad range of developmental programs critical for tumorigenesis/metastasis when aberrantly re-expressed⁸⁸. Since it is traditionally difficult to target transcription factor-DNA interactions, we will focus on the importance and implications of targeting the SIX1-EYA protein-protein interaction and EYA phosphatase activity in SIX1-mediated tumor progression.

7.1. Disrupting the SIX1/EYA interaction

Since the SIX1-EYA developmental transcriptional complex is down-regulated in most adult tissues, inhibiting the SIX1-EYA interaction may be a unique and specific approach that can disrupt multiple stages of the tumorigenic process in many tumor types with limited side effects. Although developing therapeutics targeting protein interactions is considered a difficult endeavor, major strides have been made recently with more than 12 small-molecule protein-protein interaction inhibitors currently in clinical development⁸⁹.

The co-crystal of the SIX1-EYA2 complex reveals that SIX1 predominately uses a single amphipathic α -helix, located in the SD, to bind into a groove on EYA2 ED through hydrophobic interactions, hydrogen bonds, and salt bridges (Figure 2)¹⁹. Sequence alignment of the EYA family members reveals at least 93% sequence identity among EYA1, 2, and 4 at the SIX1 interface, and a 67% sequence identity (93% similarity) to EYA3. In addition, the SIX1-EYA2 interface is only about 800 \AA^2 , which is smaller than the average globular protein interface of 1,200–2,000 \AA^2 ^{19, 89}. Often, these helix-grooves can form smaller pockets more comparable to classic enzyme active sites than the flat planar surfaces of globular protein interfaces, due to their structured and well-defined clefts⁸⁹. This suggests that a small molecule inhibitor could be designed to bind all EYA family members in this binding cleft to perturb SIX1 mediated transcription. Finally, the SD, specific to the SIX family, does not display structural homology to any protein currently deposited in the protein data bank, including other homeoproteins¹⁹. This suggests that the development of a small molecule capable of disrupting the SIX1/EYA2 interaction would display high selectivity.

To date, the greatest successes in targeting protein-protein interactions with small molecules have been through targeting this type of protein-protein interaction. For example, the α -helix of the BAX BH3 domain interacting with BCL-X_L is successfully disrupted by ABT-263 (Figure 2), which is currently in clinical trials for the treatment of multiple cancers⁹⁰. ABT-263 is one of the first inhibitors to originate from fragment screening, which was ultimately crafted into a potent and selective inhibitor with a K_i of 0.5 nM⁹¹. Additionally, there are two mid-nanomolar inhibitors in clinical trials that inhibit the P53-MDM2 interaction (Figure 2), which are referred to as the Nutlins^{92, 93}. The Nutlin family binds in the cleft on MDM2 in which a single helix of P53 binds, and was developed from compounds originally discovered through screening efforts by surface plasmon resonance^{94, 95}.

The pioneering work of Wells and colleagues, using alanine scanning approaches, revealed the disparate contributions of residues at protein interfaces to the total binding energy⁹⁶. This experiment confirmed that smaller patches at the protein interface largely contribute to the overall interaction such that, small molecules able to disrupt these “hot spots”, could effectively disrupt protein-protein interactions. This work, in combination with the recognized importance of the SIX1-EYA2 protein interaction (through the disruption of the interaction by mutating one residue in the SIX1 interface) in TGF- β signaling, EMT and metastasis¹⁹, suggest a small molecule inhibitor targeting this protein interaction may be an effective therapeutic approach.

One of the largest advantages to targeting the SIX1/EYA interface is that SIX1 is not expressed in most adult tissues with the exception of skeletal muscle, and thus side effects in response to such a therapy would likely be limited. In skeletal muscle, Six1 and Eya1 have been shown to reprogram adult slow-twitch oxidative fibers towards a fast twitch glycolytic⁹⁷ and their expression is increased in response to muscle overload⁹⁸. Of note, Six1 is specifically expressed in the satellite cells in skeletal muscle, which are the stem cells within muscle that are important for establishment and regeneration of the tissue^{99–101}. In adult mice, a conditional knockout of Six1 in this population of cells demonstrated that Six1 was necessary for proper tissue regeneration and replenishment of the stem cell pool following muscle trauma⁹⁹. However, loss of Six1 had no effect on muscle weight or histology in the adult mice, and importantly, did not affect the *maintenance* of the stem cell population in muscle¹⁰². Thus, although Six1 does not appear to be critical for normal adult muscle homeostasis, it is important in the muscle if trauma occurs. Because muscle wasting is often observed in cancer patients¹⁰³, one would want to carefully monitor skeletal muscle enzymes in response to any inhibitors developed that target the Six1/Eya complex. Finally, it should be noted that Six1 is repressed by Ezh2 in the postnatal heart, and its inappropriate re-expression leads to cardiac hypertrophy¹⁰². Thus, the targeting and subsequent inactivation of Six1 is unlikely to have any adverse consequences in the heart. Overall, because we do not expect that inhibition of Six1/Eya will have an effect in most tissues (due to the paucity of Six1 expression in adult tissue), chronic use of inhibitors targeting the Six1 complex is expected to be well tolerated.

7.2. Inhibition of EYA phosphatase activity

A second approach to targeting the SIX1/EYA transcription complex is through targeting EYA's phosphatase activity. Structurally, the EYA proteins have a well-defined and unique active site that should be capable of accommodating a small molecule inhibitor¹⁰⁴. Traditionally, the identification of phosphatase inhibitors specific to their intended target is difficult, since many phosphatases have structurally similar active sites¹⁰⁵. However, EYA is distinct in that it contains a unique catalytic core and helical cap¹⁰⁴. EYA phosphatases utilize an aspartic acid as their catalytic residue, in contrast to the catalytic cysteine residue used in most cellular phosphatases. EYAs also distinguish themselves within the HAD family by targeting phosphorylated tyrosine residues, while substrates of most other HAD members are small molecules or phosphorylated serine/threonines²⁹. The unique structure of EYA's active site suggests that the identification of a selective EYA phosphatase inhibitor is possible. Indeed, both small molecule and virtual screening efforts have facilitated the discovery of specific EYA phosphatase inhibitors^{85, 106–109}.

To date, benzbromarone (Figure 3A), and a few derivatives and metabolites, are the most thoroughly investigated active site inhibitors of the EYA family^{85, 106}. These inhibitors were originally identified through enzymatic screening of the NCI diversity library and were further validated in cell culture models⁸⁵. These inhibitors display moderate IC₅₀ values (> 8 μM) in enzymatic assays with either EYA2 or 3, and are effective in cellular assays, inhibiting cell motility, and angiogenic tubulogenesis as well as sprouting⁸⁵. Additionally, the inhibitors were able to reduce cell proliferation after a 72 hour treatment, supporting the role of EYA phosphatase activity in proliferation¹⁰⁶. Using docking studies and kinetic

analysis, Hegde and colleagues proposed that benzbromarone binds immediately adjacent to the phosphotyrosine binding site and is an uncompetitive inhibitor of EYA2 and EYA3⁸⁵. In the clinic, these compounds hold promise, since benzbromarone has been used for many years in the treatment of gout, and its pharmacological profile is well known. However, due to reports of unwanted hepatotoxicity, this compound will require further chemical optimization before it can be re-purposed in the clinic as an EYA inhibitor.

In addition, other classes of EYA active site inhibitors have been discovered through virtual screening efforts (Figure 3B)¹⁰⁷. These compounds are proposed to bind in the active site of EYA2 and chelate the essential Mg^{2+} ion. Park and colleagues further optimized the compounds through the creation of multiple hydrogen bonds and van der Waals contacts with nearby residues to achieve IC_{50} values ranging from 6–50 μM ¹⁰⁸. However, the selectivity and efficacy of these inhibitors in biochemical and cellular assays has not yet been validated, and thus require further study before their clinical potential can be determined.

Using quantitative high-throughput screening approaches our group has identified an additional class of EYA2 ED specific inhibitors, which appear to act through an allosteric mechanism^{109, 110}. In this work, we used a fluorescent phosphatase assay to screen the NIH Molecular Libraries Probe Production Centers Network (MLPCN) library of over 330,000 compounds, which led to the identification of a series of N-arylidenebenzohydrazide-containing compounds that inhibit EYA2 (Figure 3C)¹⁰⁹. The top molecules within this series have low micromolar IC_{50} values and were validated in a secondary assay monitoring the dephosphorylation of the pH2AX peptide substrate by EYA2. These inhibitors display specificity towards EYA2 over other cellular phosphatases, including PTP1B (a prototypic tyrosine phosphatase), PPM1A (a Mg^{2+} dependent Ser/Thr phosphatase), and Scp1 (a HAD family serine phosphatase). Importantly, these compounds are able to reverse EYA2 phosphatase-dependent cell migration in MCF10A cells, suggesting that these compounds could ultimately inhibit metastasis¹¹⁰.

Extensive mutational and kinetic analyses indicate that these N-arylidenebenzohydrazide compounds are reversible inhibitors with a long occupation time and interact with EYA2 at an allosteric site opposite of the enzymatic active site¹¹⁰. The allosteric nature of these inhibitors provides additional selectivity for EYA2 over another EYA family member, EYA3¹¹⁰. Allosteric modulators can offer an advantage over orthosteric ones because of their ability to increase selectivity and modulatory control¹¹¹. These molecules are promising leads that can potentially be developed into highly specific therapeutic agents targeting tumors overexpressing EYA2.

8. Expert Opinion

Many of the most successful cancer drugs developed to date have molecular targets that drive a known hallmark of cancer including evasion of growth suppressors, sustained proliferative signaling, enabling replicative immortality, resisting cell death, promoting invasion and metastasis, and inducing angiogenesis. Drugs with this directed mechanism of action, in theory, should have fewer off-target effects and subsequent nonspecific toxicity.

Indeed, many anticancer therapeutics have been specifically designed to target pathways critical for tumor progression that are reduced or absent in normal adult tissues, thereby limiting harmful side effects. Unfortunately, therapies that target only one key hallmark pathway often lose potency over time as the cancer cells develop resistance, resulting in clinical relapse. Therefore, the development of therapies co-targeting multiple cancer hallmarks will result in a more effective and durable cancer treatment. Aberrant transcription factor expression can result in altered gene expression profiles, and is known to be fundamental to the acquisition of hallmark capabilities¹¹². Indeed, many signaling pathways that are altered in cancer are believed to act through certain key transcription factors, and thus targeting transcriptional complexes may be a means of targeting a central node of the disease. Direct intervention of the transcriptional process is thus an exciting strategy to correct gene expression in cancer cells. In particular, the SIX1/EYA transcriptional complex is an ideal target for anti-cancer therapy, since it is largely a developmental complex that is abnormally overexpressed in many cancer types and regulates multiple pathways critical for tumorigenesis.

The combination of both structural and functional data strongly suggest that the perturbation of the SIX1 transcriptional complex using small molecules may be a feasible approach in developing novel anti-cancer agents. Two of the more attractive approaches towards achieving this goal are the inhibition of either the interaction between SIX1 and EYA or EYA's enzymatic activity. Both approaches hold great promise, but are not without their own risks.

Protein-protein interactions are integral for most cellular functions, and they represent a substantial opportunity for drug development. Despite the evidence that protein interfaces have significant potential for pharmacological intervention; relatively few examples exist in the discovery and development of potent and selective protein-protein interaction inhibitors. Therefore, the disruption of protein interactions using small molecules is still considered a challenging task, owing to the often large planar surfaces of globular protein interactions and the relatively small footprints of a small molecule. Nevertheless, potent small molecule inhibitors of protein-protein interactions have been discovered and are currently in clinical trials. The SIX1-EYA interaction, in particular, has some characteristics that suggest it too is amenable to small molecule intervention. The high-resolution crystal structure of the SIX1/EYA2 complex and mutational analysis have provided key information to support this statement. SIX1 interacts with EYA through its unique SD, using predominantly a single α -helix. The interface is relatively small compared to a typical protein-protein interface and a single amino acid mutation on the SIX1 helix is able to disrupt the SIX1-EYA interaction. The molecular details of the topology of SIX1-EYA interface provided by the crystal structure will help facilitate any future high-throughput screening and virtual-based discovery efforts to identify and optimize small molecule inhibitors of the SIX1/EYA2 interaction.

Tyrosine phosphatases play critical roles in cell signaling and physiological processes. When mis-regulated, they can function as oncoproteins, modulating several key pathways contributing to the hallmarks of cancer¹¹³. The EYA family of atypical tyrosine phosphatases appears to be no exception, promoting proliferation, motility, and

angiogenesis. In particular, EYA's enzymatic activity is an attractive target for future anti-cancer therapies because of the uniqueness of its catalytic domain. EYA phosphatases utilize an aspartic acid, rather than the more common cysteine, as their catalytic residue and are unique within the HAD family, targeting phosphorylated tyrosine residues. Historically, small molecule inhibitors of tyrosine phosphatases have been easy to identify, including both natural products and synthetic compounds. Unfortunately, the close sequence identity between the different phosphatase family members has made the identification of specific inhibitors challenging. However, the unique structure and catalytic mechanism of EYA's active site suggests that the identification of a selective EYA phosphatase inhibitor is possible.

To date, there are three classes of inhibitors that have been published targeting the enzymatic activity of EYA2, each class having their own advantages and limitations. Individually, these inhibitors all display moderate IC₅₀ values, in the low micromolar range. Therefore, additional medicinal chemistry efforts to improve their potency could prove valuable. Benzbromarone is already well understood pharmacologically, however, due to unwanted drug-drug interactions with CYP2C9 substrates and reports of subsequent hepatotoxicity, benzbromarone is no longer marketed in the U.S. Additional chemical optimization of benzbromarone to remove this unwanted toxicity while retaining its activity may be necessary before it can be re-purposed in the clinic. The Mg²⁺-chelators, identified through in silico screening, are effective at inhibiting the enzymatic activity of EYA, but their selectivity and effectiveness in cell culture and in vivo have not yet been characterized. Moreover, metal chelators can present problems as potential therapeutics through the physiological depletion of metal ions and inhibition of other metalloenzymes. Finally, the allosteric inhibitors identified through HTS efforts may provide an additional level of selectivity, but have yet to be thoroughly characterized in vivo. Although certain sub-types of hydrazones (most notably hydroxyphenylhydrazones) have a reputation as pan assay interference compounds, appearing as hits in many high throughput screening assays, not all hydrazones are problematic and thus these compounds should not be ruled out as leads for further optimization and drug development¹¹⁴. Our hydrazone-containing compounds, for example, are clearly specific and not reactive to all proteins, as evidenced by their inactivity against the highly homologous EYA3 protein and other cellular phosphatases.

In conclusion, there is a growing body of evidence detailing the physiological and oncogenic roles of the SIX1/EYA transcriptional complex that emphasize the therapeutic potential of targeting this complex. The SIX1 transcriptional complex impacts multiple aspects of cancer biology, including properties related to both tumorigenesis and metastasis, through the regulation of several important cell cycle regulators, oncogenes, and other tumor promoting molecules. Although the progress made towards developing a clinically-sound small molecule inhibitor is still in its early stages, future efforts in this area may uncover potent and selective inhibitors that will ultimately lead to successful anti-cancer therapies.

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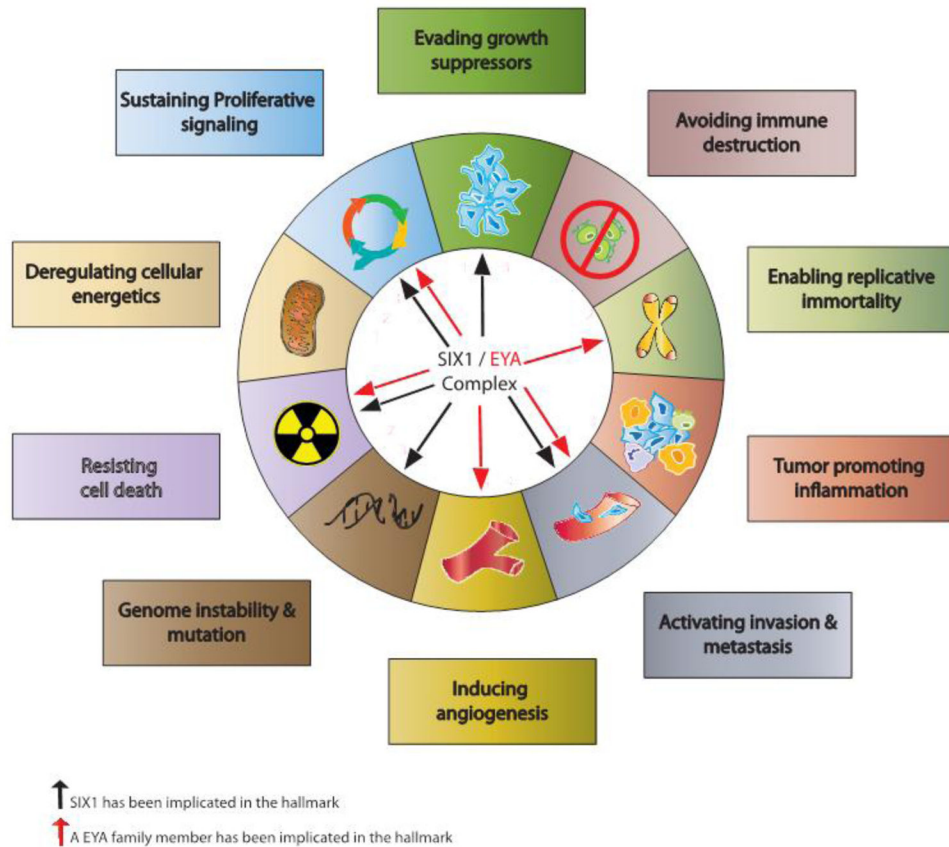


Figure 1. A recreation of the Cancer Hallmarks¹¹⁵, illustrating the known hallmarks in which the SIX1 and EYA family members carry out their tumor-promoting properties. In total, the SIX1/EYA complex has been shown to play a role in four out of the six original hallmarks, one of the two enabling characteristics and one of the two emerging hallmarks, highlighting the importance of targeting this complex as a potential therapeutic.

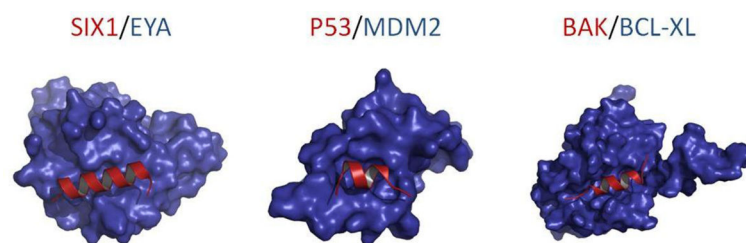


Figure 2. Surface representation illustrating the similarities between the protein interfaces of SIX1/EYA2 and two successfully targeted protein interactions, P53/MDM2 and BAK/BCL-XL. The red ribbon represents the single helix in SIX1, P53, and BAK that interacts with their corresponding protein partners in blue.

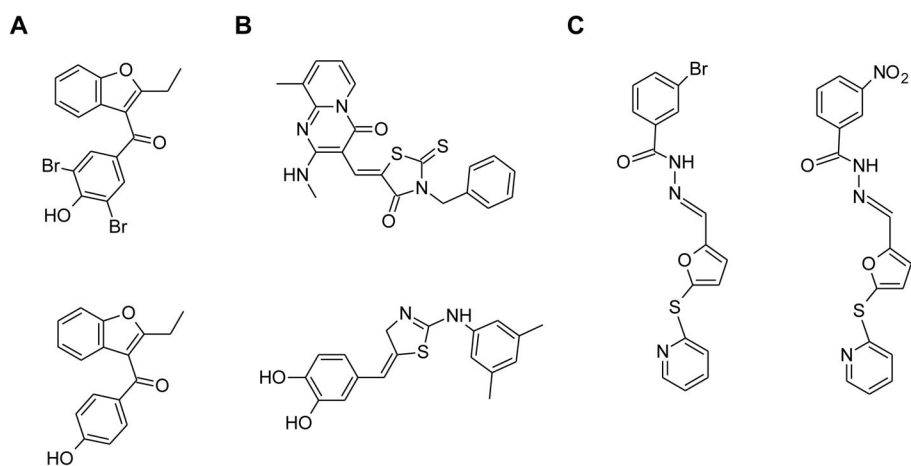


Figure 3. Chemical structures of the 3 series of compounds identified as inhibitors of EYA2 phosphatase activity. **A.** Benzbromarone and benzarone identified through the screening of a small NCI library. **B.** Two representative Mg²⁺ chelators identified through virtual screening. **C.** The top two allosteric N-arylidenebenzohydrazide compounds identified through a large scale HTS.