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The sine oculis homeobox (SIX) family of transcription factors as regulators of development and disease

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Abstract

The sine oculis homeobox (SIX) protein family is a group of evolutionarily conserved transcription factors that are found in diverse organisms that range from flatworms to humans. These factors are expressed within, and play pivotal developmental roles in, cell populations that give rise to the head, retina, ear, nose, brain, kidney, muscle and gonads. Mutations within the fly and mammalian versions of these genes have adverse consequences on the development of these organs/tissues. Several SIX proteins have been shown to directly influence the cell cycle and are present at elevated levels during tumorigenesis and within several cancers. This review aims to highlight aspects of (1) the evolutionary history of the SIX family; (2) the structural differences and similarities amongst the different SIX proteins; (3) the role that these genes play in retinal development; and (4) the influence that these proteins have on cell proliferation and growth.

Keywords

Sine oculis; optix; DSix4; *Drosophila*; SIX; mammals; retina; gonad; mesoderm

SIX proteins: A family history

Mutations within the founding member of the sine oculis homeobox (SIX) family, *sine oculis* (*so*), were first identified and characterized in the fruit fly, *Drosophila melanogaster* at a time when a growing number of mutants affecting the structure, size and pigmentation of the eye were being recovered [1]. Mutations in *so* proved to be particularly interesting as loss-of-function mutants not only had dramatic effects on the compound eye but also, in fact, adversely affected the entire visual system [2–5]. Subsequent molecular efforts identified two additional SIX family members, *optix* and *DSix4* in flies [6,7]. *optix*, like *so*, is expressed and functions in the developing eye, although its role in the retina appears to be distinct from that of *so* [7–9]. *DSix4*, on the other hand, plays no role in the eye but instead functions in several mesoderm derivatives including a subset of somatic muscles, the somatic cells of the gonad and the fat body [10,11]. The three SIX genes that are found in *Drosophila* are thought to have arisen through the duplication of an ancestral SIX gene, an event that occurred prior to the evolution of the Bilateria. Homologs of the *so*, *optix* and *DSix4* proteins have been identified in a wide range of organisms throughout the animal kingdom. A comparison of gene structures and sequence has led to the creation of three subclasses of SIX proteins: each class contains one of the fly genes and their orthologs (Fig. 1) [6]. The remarkable demonstration that *Pax6* (*ey* in flies, *Sey* in mouse, *Pax6* in humans) universally governs retinal development across the animal kingdom was a strong impetus to find vertebrate orthologs of the other genes involved in fly retinal specification [12–14]. In the immediate years after cloning *so* from the fruit fly, homologs were quickly identified in a number of vertebrate systems including medaka fish, chickens, frogs, zebrafish, mice and humans [15–34]. The era of high throughput genomics has increased the number of vertebrate systems in which SIX genes have been identified to

nearly 50. Analyses of these genomes indicates that within the vertebrate lineages there has been a further duplication of each SIX gene resulting in the presence of two members of each subclass: *Six1/2* (*sine oculis*), *Six3/6* (*optix*) and *Six4/5* (*DSix4*; Fig. 1, Table 1) [6].

Equally significant have been the efforts to determine the evolutionary origins of the SIX genes and the points at which particular duplications have occurred. Rapid technological advances in genome biology have resulted in an explosion in the number of sequenced and annotated genomes, many of which are of basal invertebrates. These larger scale endeavors have been complemented by more directed efforts to correlate the expression and activity of SIX genes with particular retinal and photoreceptor cell types. Together these efforts have identified SIX proteins in a number of basal organisms such as the nematode, planaria, jellyfish, sponge and flatworm [35–40]. At last count approximately 40 invertebrate genomes have been found to contain members of at least one SIX family subgroup (Table 2). Unfortunately, these efforts have not led to an elucidation of the evolutionary history of the SIX genes other than the fact that their existence predates each of the organisms studied so far. This dates the evolution of this gene family to a time prior to the Bilateria/Cnidaria split nearly 500 million years ago. The lack of a clear evolutionary history can be attributed to several factors including an incomplete genomic record of all extant and relevant invertebrate species, an incomplete and inaccurate annotation of many sequenced genomes and the possibility that the most informative species are already extinct. Despite the lack of a clear sequence record some insights into the history of the SIX family can be extracted from a neighbor-joining tree analysis of extant SIX family members. A preliminary analysis using the SIX genes from 12 Drosophilids indicates that *DSix4* and *optix* are closely related and that *so* may resemble the ancestral SIX gene (R. Datta and J. P. Kumar, unpublished data). A whole genome duplication event in the vertebrate lineage is most likely responsible for the evolution of the Six1–6 genes (Fig. 2).

SIX proteins: Anatomy of a transcription factor

The homeodomain: DNA binding

SIX proteins are transcription factors that are characterized by the presence of two evolutionarily conserved domains (Fig. 1). DNA binding is mediated through the presence of the homeobox nucleic acid recognition domain (HD) [41]. In general HDs are 60 amino acids in length, contain a helix-turn-helix (HTH) motif and are broadly classified based on their close resemblance to the HDs of either Antennapedia (Antp) or Paired (Prd) proteins [42]. Based on overall sequence similarity and the identity of several critical residues (positions 47, 50, 51 and 54) within the recognition helix, SIX HDs most closely resemble those found within the Prd class. Members of this broad category can be further subdivided based on the identity of residue 50, which is critical for binding specificity [43–45]. SIX proteins contain a lysine at this position (K50) thereby placing them within the Orthodenticle (Otd) subgroup (Fig. 3A, arrow). With multiple family members being expressed during development, often in overlapping patterns, the question of how much diversity exists among the transcriptional targets of the SIX proteins has naturally arisen. Does each family member bind and regulate an exclusive set of promoters or is there a cross listing of target genes amongst the various members?

To parse out the contributions of each factor in organogenesis, efforts have been made to identify, compare and contrast the consensus binding sites and the transcriptional targets of several SIX proteins. Initial studies indicated that there is remarkable similarity in the binding sites for mammalian Six1/2/4/5 (TCAGGTTC) [19,46–50]. This consensus site is similar to the sequence that is bound by *Drosophila* So (GTAANYNGANAYC/G) [51,52]. Recent reports using modified bacterial one-hybrid and protein binding microarrays have further refined the consensus binding sites for the SIX proteins in flies and worms, although individual differences exist (TGATAC and GGGTATCA) [53,54]. In contrast, the site bound by Six3

contains the traditional ATTA homeodomain core recognition sequence, thus it is significantly different from the sequence bound by members of the other subgroups [55]. Not unexpectedly, these studies identified several instances in which individual target genes appear to be directly co-regulated by multiple SIX proteins. For instance, Six2, Six4 and Six5 bind to the same site within the ARE element of the *Na, K-ATPase $\alpha 1$ subunit* gene, while Six1, Six4 and Six5 bind to the identical MEF3 site within the *aldolase A* and *myogenin* promoters [19,20,46–49]. Six3, on the other hand, does not appear to bind these promoters. The *rhodospin* gene is the only verified Six3 target to date and it does not appear to be regulated by other SIX proteins [56].

While there is likely to be a degree of functional redundancy between the So and DSix4 subgroups, it is clear that it will be only partial at best. Evidence to support this contention first surfaced during examinations of *Six1* and *Six4* mutant mice. Despite the nearly complete overlap in expression patterns during embryogenesis, the loss of *Six4* has minimal if any effect on early development, while severe developmental abnormalities in the head, ear and kidney exist in mice mutant for *Six1* [15,57–63]. An effort to understand these phenotypes made use of Six1-VP16 and Six4-VP16 transcriptional activators as tools to identify similarities/differences in binding sites and transcriptional targets [64]. The authors of this study found three types of binding sites and those sites are controlled by either Six1 or Six4 as well as genes such as *Slc12a2* that are under the direct regulation of both transcription factors [64].

The data available so far raises several interesting issues. First, it appears that members of individual subgroups can recognize subgroup specific sites. Second, members within a subgroup can bind to a common site. It is not clear yet if members within a subgroup bind to unique and mutually exclusive sets of promoters. Third, members of different subgroups can recognize a common site. While progress has been made, the exact molecular and biochemical rules that govern the specificity of SIX protein-nucleic acid interactions remains relatively unclear. Two somewhat intertwined and unresolved issues will need to be addressed to fully understand how SIX proteins influence critical developmental decisions at the level of gene regulation. At one level it is important to know the nucleic acid sequences that can be bound by each SIX transcription factor, including common as well as factor-specific sites. Part of this effort will have to include identifying a large enough set of transcriptional targets for each SIX protein so that a reliable consensus binding site can be used in genome-wide searches.

At another level, it will be important to determine the differences within the SIX HDs themselves that contribute to the recognition properties of each protein. A close look at the HD sequence reveals a number of interesting features that may shed light on DNA binding, target gene specificity and the evolutionary history of the SIX proteins. There is a high degree of homology amongst the HDs of all three SIX subfamilies; 62% (37/60) of all residues are absolutely conserved in all fly and mouse proteins (Fig. 3A, gray box). The residues that lie within the recognition helix and are critical for DNA binding specificity are conserved (Fig. 3A, asterisks).

While universally conserved residues are likely to play important roles in defining the structure of the HD and to distinguish the SIX K50 motif from other HDs, they cannot help in distinguishing amongst the SIX proteins themselves. For that we can look to the differences that exist within the HDs (and that of the SIX domain – see below) of the different SIX clades. The best candidates are residues whose identity is specific to a particular subgroup. This situation is exemplified by two residues (position 23 and 42), which are occupied by clade-specific amino acids (clade specific, Fig. 3A, green box). The striking subclass specificity at these residues opens up the interesting possibility that they could be involved in distinguishing between the three different SIX proteins subgroups.

Since all SIX genes are derived from a common ancestor it is reasonable to expect that some degree of functional redundancy will exist amongst members of more than one subgroup. In fact there is considerable experimental evidence to support this contention (see above). A subset of any group of shared functions is likely to be under the control of the HD, and should be reflected in the conservation of amino acid identity across two subgroups but not all three. There are seven residues (12%) that are absolutely conserved across the So and DSix4 clades but a different amino acid occupies those positions within the Optix subgroup (Optix specific, Fig. 3A, red box). Likewise five residues (8%) are absolutely conserved within the So and Optix clades but are occupied by a unique amino acid within the DSix4 subgroup (DSix4 specific, Fig. 3A blue box). In stark contrast, there do not appear to be any examples of residues being conserved across the DSix4 and Optix clades but not within the So subgroup. In total, these observations raise the possibility that some shared DNA binding properties, which are exhibited by the So/DSix4 and the So/Optix subgroups, may be mediated by subgroup-specific amino acids within the HD. While this may be an interesting idea, it is not yet clear if the particular differences actually affect the binding properties of the SIX proteins, particularly since many of the residues are outside the recognition helix. It should be noted that the HDs are thought to have very low intrinsic sequence specificity, and such specificity is usually encoded by either a binding partner or within a set of amino acids that lie adjacent to the 3' end of the HD and extend into the C-terminal (CT) domains. In the case of the SIX proteins, two recent reports have demonstrated that a potential CT extension exists within the Optix subgroup and may functionally distinguish this group from the others [8,65]. An analysis of the CT regions of the SIX proteins does not indicate the presence of a new composite DNA binding domain that could confer sequence specificity. Rather, the CT extension may be important for providing structural stability to the third helix of the homeodomain as its presence increases the affinity of the HD for DNA [65].

The SIX domain: Protein-protein interactions

Shortly after the cloning of *so*, three murine homologs were isolated: *Six1*, *Six2* and *Six4* (*AREC3*). A comparison of the four protein sequences revealed the presence of a second evolutionary conserved domain, the SIX domain (SD), just 5' and directly adjacent to the HD [15,16,19,20]. Unlike the homeodomain, there is some disagreement as to the exact length of this domain. Here, the SD is defined as being 146 amino acids in length with an exception being made for the Optix subgroup, which is a 150-residue moiety (Fig. 3B). Initial studies suggested that portions of the SD may contribute to DNA binding and specificity [19,51]. However, a more recent analysis of *Six2* and *Six6* indicates that all DNA binding properties appear to be confined to the HD and in some cases the adjacent CT extension [65]. Instead, the SD appears to be critical for mediating protein-protein interactions.

The first identified, and subsequently most discussed, biochemical contact between a SIX protein and a binding partner is the physical interaction between *Drosophila* So and the transcriptional co-activator Eyes Absent (Eya) [66]. Both genes are expressed in identical patterns within the developing eye and loss-of-function mutations in either gene results in the complete loss of the retina [4,5,67]. However, unlike Ey/Pax6 which induces ectopic eyes at a high frequency within a wide range of non-retinal tissues, Eya alone can do so only at a relatively low frequency, while So reportedly could not induce eye formation at all [66,68, 69]. Co-expression of both genes, on the other hand, led to a synergistic increase in the frequency and location of ectopic eyes. *In vitro* binding and yeast two-hybrid assays localized the point of interaction to the SD of So [66]. The exact contact point within the SD has not been determined. A model emerged in which the So-Eya complex functions as a composite transcription factor to promote eye development with So providing a DNA binding domain and Eya supplying an activation domain [66,70,71]. This interaction has been conserved across the animal kingdom, although it appears to be specific to members of the So and DSix4

subgroups [7,72,73]. These proteins are expressed in a wide range of non-retinal tissues thereby making the formation of the SIX-EYA complex an important step in many developmental contexts. However, the absolute requirement for this complex in retinal specification has been disputed as expression of *So*, on its own, is in fact sufficient to induce ectopic eye formation [8,74]. The discrepancy between Pignoni et al. [66] and Weansner et al. [8] most likely stems from experimental differences related to the induction of ectopic eye formation. In the experiments described by Weansner and colleagues nearly 220 different enhancer-GAL4 driver lines were used to forcibly express *So* in the developing fly. In contrast, Pignoni et al. used only a handful of GAL4 drivers; thus fewer tissues and cell populations were tested for their ability to respond to *So*. Secondly, Weansner reported ectopic eyes using the *dpp*-GAL4 driver, which was used in both studies. The resulting eyes are relatively small and are found only within the developing epithelium. The eyes degenerate prior to adulthood. However, this is the only case in which this occurs. The eyes that are induced with four other GAL4 drivers survive and are seen in the adult.

SIX proteins do not appear to function just as transcriptional activators. In fact, several members, including those of the *So* and *Optix* subgroups, are bound and modulated by the Groucho/TLE family of transcriptional repressors [55,71,74–76]. This interaction is mediated through contact between Gro and the engrailed homology 1 (eh1) domain, which is located within the SD [74,77,78]. SIX proteins can also repress transcription by complexing with members of the DACH family of co-repressors. Such interactions have been reported for both *Six1* and *Six6* [79,80]. The *Six6*-Dach1 interaction is important for repressing *p27/Kip1*, an inhibitor of the cell cycle G1/S transition [80]. Additional evidence for SIX proteins serving as transcriptional repressors comes from expression of X*Optix2* proteins fused to either the VP16 activation motif or the En repressor domain. Expression of the former leads to a smaller eye field, while expression of the latter leads to an enlargement of the retinal field, which is a phenotype seen with the wild-type X*Optix2* protein [26].

Overall, the data suggest that some SIX family members can form composite transcription factors that in some cases (SIX-EYA) activate transcription but in others (SIX-DACH or SIX-GRO) silence expression of downstream targets. Our current understanding is that independent activation and repression complexes are formed since SIX proteins are unable to simultaneously bind Eya and Gro [71]. An open and inviting question is whether these differing complexes are formed within a single cell, and whether both types of complexes can bind to the promoter of an individual transcriptional target gene. The answer is likely to depend upon the developmental context, co-factor concentration, and chromatin state of target gene promoters.

Despite the attention that the SIX proteins have attracted and the importance of the SIX-EYA interaction, very little is known about the subdomain architecture of the SD. A comparison of the SDs from the different subgroups exposes several features that may be worth considering. To start with, 42% (62/146) of residues within the SD are absolutely conserved across the SIX family (Fig. 3B, gray box). While not as impressive a percentage as the HD, it is remarkably high considering the evolutionary age of these transcription factors. Much like the HD, these residues are probably important for intrinsic properties of the SD. They are unlikely to be informative for discovering distinctions amongst the different subgroups. For such differences it is more productive to look for differences amongst the SDs of the various subgroups.

Some of those differences include five residues (3%) that are conserved within the *So* and DSix4 subgroups (*Optix* specific, Fig. 3B, red box). Another two residues (1.3%) are present just within the *So* and *Optix* subgroups (DSix4 specific, Fig. 3B, blue box). One residue (position 32) is occupied by clade-specific residues (Fig. 3B, green box). Finally, there is a single residue (<1%) that is only found within the DSix4 and *Optix* subgroups (*So* specific,

Fig. 3B, orange box). The identity of this particular residue (position 103) is interesting in that it has been implicated in distinguishing between So and Optix functions in *Drosophila* [76, 81]. A valine to aspartic acid replacement in the adjacent amino acid (residue 102) was demonstrated to be responsible for the conversion of wild-type So protein into a dominant negative moiety, So-D (Fig. 3B, arrow) [81]. Interestingly, what turned out to be important for this functional conversion was not the loss of the valine residue but rather the acquisition of an aspartic acid residue. It appears that the presence of the aspartic residue at position 102 rendered So-D functionally more similar to Optix, which has an aspartic acid residue at position 103 (Fig. 3B, orange box; normal So protein does not). This single substitution had the consequence of altering the binding properties of the So SD, thereby rendering it closer to that of Optix for certain, but not all, protein-protein interactions [76]. A final difference worth mentioning is the presence of a 4-amino acid sequence (residues 33–36) that is found only within members of the Optix subgroup (Fig. 3B, yellow box). Interestingly, this 4-amino acid sequence is effectively identical amongst the vertebrate Six3/6 members but differs significantly from Optix. These differences are good candidates for functionally distinguishing one SIX protein from another. As with residue 103, the remaining amino acids that are highlighted here are likely to play a critical role in binding partner selection.

The non-conserved domains: Activation domains

Flanking the conserved DNA binding and protein interactions domains are stretches of amino acids that are relatively unstructured, considerably variable in length and, in contrast to the SDs and HDs, have a very low degree of sequence conservation across all SIX family members. These regions are generically referred to as the N-terminal (NT) and CT segments (Fig. 1). A role, if any, for the NT is somewhat controversial. In one report the deletion of the NT sequence from mammalian Six1 prevented anaphase promoting complex (APC)^{cdh1}-mediated degradation, suggesting that the NT region contains an APC-dependent degradation motif [82]. In contrast, a construct containing the identical modification of *Drosophila* So is able to completely rescue the no-eye defects of *so* loss-of-function mutants. This implies that this region is completely dispensable for protein function [8]. One potential explanation for this apparent discrepancy is that degradation of So protein may not be an essential step during eye development but rather may be important in other developmental contexts. Alternatively, it should be noted that in the former experiment both the NT and the SD region were simultaneously deleted, thus the region of Six1 that is important for proteasome-mediated degradation could actually reside within the SD. In that case, the NT would be functionally dispensable in both flies and vertebrates. Deletion of individual domains would go towards a better understanding of the potential role, if any, that is played by the NT region. The CT regions appear to augment the function of SIX proteins as transcription factors in three significant ways. First, the CT domains of several SIX proteins appear to contain functional activation domains. Individual segments of Six2 and Six4 were fused to the DNA binding domain of the GAL4 transcription factor. These chimeric proteins were assayed for the ability to activate transcription of a reporter construct and only the CT regions retained this function [19,83]. Thus, several SIX proteins appear to be *bona fide* transcription factors in their own right. This feature could be important for explaining a recent report in which So, under certain circumstances, can in fact induce ectopic eyes on its own without requiring the co-distribution of Eya [8]. The discovery of such self-contained activation domains is noteworthy as it suggests that there are instances in which SIX proteins might activate downstream transcriptional targets independently of EYA. It also opens up the possibility that SIX proteins might first activate the expression of EYA genes. Upon activation, SIX-EYA complexes may then form and promote the transcription of downstream transcriptional targets.

In the fly, SIX and EYA proteins are not always restricted to the same distribution pattern [4, 5,84,85]. For example, in the insect retina So and Eya are distributed in identical patterns, while

Optix is only co-expressed with Eya in the most anterior regions of the retina [4,7,67]. Differences in the distribution patterns of several mammalian SIX and EYA homologs have also been reported [15,70]. However, the lack of complete temporal and spatial descriptions of all Six1–6 and Eya1–4 expression patterns as well as the lack of an inclusive list of all possible SIX-EYA complexes has prevented one from concluding that SIX-EYA interactions are absolutely required in all circumstances in vertebrate systems. However, the retention of intrinsic activation domains suggests that there are likely to be instances in vertebrates that mimic those of the fly. Taken together, a model in which the formation of the SIX-EYA complex may not be an obligate step in development could arise. Second, in a subset of SIX proteins (members of the Optix subgroup), the first 7–14 amino acids of the CT segment serve as an extension of the HD and modulate DNA binding properties (Fig. 4). The CT extension does not appear to create a new composite DNA binding site but rather serves to stabilize interactions between the HD and the target recognition site [65]. The authors demonstrated that this sequence is necessary for Six6 binding and is sufficient to augment the DNA binding affinity of Six2. This CT extension is one of four conserved regions that serve to functionally distinguish the Optix subgroup from the So and DSix4 subgroups (Fig. 4) [8]. The exact role played by the other conserved segments in distinguishing the Optix subgroup from the others is still to be determined. It is also unclear if the CT regions of the other subgroups have similar distinguishing features beyond the activation domains. The unstructured nature of the CT coupled with the low degree of conservation may in fact turn out ironically to be a crucial feature for further distinguishing one SIX protein from the other, particularly amongst members of different subgroups (Fig. 4).

Third, the CT segments appear to mediate APC^{cdh1}-dependent protein destruction. Six1 proteins (lacking the CT region) are stabilized even in the presence of high Cdh1 levels. However, unlike the NT segment, the CT portion of Six1 does not directly bind to Cdh1 [82]. While the CT of Six1 does not directly mediate an interaction with Cdh1, it is clearly necessary and may in fact cooperate with the NT region of the protein.

Such interactions between domains are supported by a report that the induction of ectopic eyes by So is dependent upon the presence of intact NT and CT domains. However, it has also been reported that a modified So protein in which the CT tail is deleted functions like the wild-type protein in rescue assays [8]. It may very well be that the cellular contexts surrounding normal and ectopic eye development differ from each other and that these disparate circumstances are engaged by different portions of So.

SIX proteins in retinal development

The *Drosophila* story

An understanding of the role of the SIX proteins in eye development began with the isolation of what would turn out to be mutations in eye-specific enhancers of *sine oculis* [1,86]. These mutants are characterized by a drastic reduction in retinal development with the strongest alleles leading to a complete loss of the eye (Fig. 5A, B) [1,4,87,88]. So is distributed dynamically within the entire visual system as well as at several invagination points throughout the developing embryo. As a consequence, null mutations have wide-ranging defects and flies harboring such alleles die during mid-embryogenesis [4,5,88]. Within the developing eye imaginal disc, which gives rise to the compound eye, *so* is expressed in a swathe of cells just anterior to the advancing morphogenetic furrow (MF) and in all cells, both photo-receptors and undifferentiated cells, posterior to this moving front (Fig. 5C). Forced expression of *so* in non-retinal tissues is sufficient, on its own, to induce ectopic retinal development in limited circumstance (Fig. 5D) [8]. Co-expression with *eya* synergistically increases the range and frequency of ectopic eye formation [66]. The expression pattern, loss-of-function phenotype and forced formation of ectopic eyes places *so* within an elite group of genes that are

collectively referred to as the eye specification or retinal determination (RD) network (Fig. 6) [89].

Subsequent homology based molecular screens isolated *optix* and *DSix4*; of which only *optix* is expressed within the developing eye [6,7,10]. In the retina *optix* expression is restricted to the *so*-positive cells that lie anterior to the morphogenetic furrow (Fig. 5E) [7]. Thus, while *so* continues to be expressed in differentiating photoreceptor cells, *optix* expression is terminated at the furrow, suggesting somewhat differing roles for the two proteins within the eye. Forcibly expressing *optix* can also induce ectopic eye development in non-retinal tissues, thereby placing it also within the RD network (Fig. 6) [7,8]. Loss-of-function *optix* mutations have not been published, but it is expected that such lesions will lead to substantive effects on eye development.

so and *optix* are directly regulated by the Pax6 homolog Eyeless (Ey: Fig. 6) [90]. Binding sites for Ey within the transcriptional units of both genes have been identified *via* bioinformatics and experimentally verified with EMSAs. Each site has been shown to be responsive to Ey expression *in vivo* [86,90]. During normal eye development it is expected that prior to the initiation of the furrow Ey will initiate *so* and *optix* expression. As the furrow initiates at the posterior margin and progresses towards the anterior edge of the epithelium, this regulatory interaction is likely to be maintained in all *ey*-positive cells that lie ahead of the furrow. At the furrow *ey* expression ceases and this is the most likely reason for the simultaneous cessation of *optix* expression. However, *so* expression is still maintained on the other side of the furrow. One likely explanation for this resides in the presence of experimentally verified So binding sites within the last intron of *so* itself, which creates a potential autoregulatory loop that could result in the maintenance of *so* expression independent of Ey activity [52]. Optix binding sites have not been identified and it is unlikely that a similar autoregulatory loop exists as *optix* expression ceases at the furrow.

The lack of information on transcriptional targets of Optix has hampered efforts to understand the role that it plays in the fly retina. However, several efforts have demonstrated that So directly regulates the expression of several genes that are involved in eye specification, the initiation and progression of the morphogenetic furrow and cell fate choices. So activates the transcription of *ey*, thus creating a positive feedback loop between the two genes ahead of the morphogenetic furrow [52]. As the retina initiates pattern formation, So, along with the Ets transcription factor Pointed (Pnt) directly activates the expression of *hedgehog* (*hh*), a known regulator of pattern formation in the eye [52,91–93]. Posterior to the furrow So appears to influence cell fate specification by directly activating *lozenge* (*lz*), which plays a critical role in setting up the pre-pattern of transcription factors that specify individual cell types in the retina [94–96]. Thus, So plays a critical role in many of the major events during early and mid eye development.

A surprise in the vertebrate retina

Based on the evidence that had been accumulating in the *Drosophila* eye, it was expected that the *so* homologs *Six1* and *Six2* would play a significant role at multiple points in the development of the mammalian retina. Much to everyone's surprise neither gene is expressed in the developing mouse eye. Instead both genes are expressed in several mesodermal and neural crest derivatives such as the otic and nasal placodes, subsets of craniofacial muscles, the notochord, developing somites, the branchial arches, the kidney and several muscle, skeletal and connective tissues [15]. Mutations in human *Six1* are a major underlying cause of branchio-oto-renal (BOR) syndrome [97,98]. However, *Six1* lesions are specifically excluded from Branchio-oculo-facial (BOF) syndrome even though both syndromes share many phenotypic features [99]. Interestingly, the evidence points to members of the Optix subgroup, *Six3* and *Six6*, as being the major SIX proteins in the retina.

Six3 and *Six6* are expressed at multiple points during the development of the vertebrate eye. It is first localized to the optic vesicle and optic stalk but then expands to include the neural retina and lens [15,27,29–32,100–103]. Mutations within either gene are the underlying cause of a wide range of disorders in both vertebrate model systems and human patients. Mutations in *Six3* are associated with holoprosencephaly, a disorder characterized by the failure of the forebrain to divide and form bilateral cerebral hemispheres [104–106]. In extreme cases, only a single centrally located eye will form. The role that loss of *Six6* plays in retinal disorders is somewhat controversial. Some reports have associated mutations in human *Six6* with anophthalmia, a disorder that is characterized by the complete failure of ocular tissue to form [31,107]. However, a screening of several patients with anophthalmia and microphthalmia failed to detect any lesions within *Six6* [108,109]. It may be that these disorders represent a group of molecular lesions of which only a subset resides within *Six6*. Alternatively, the mutations may be located in cryptic regulatory elements that were not subjected to sequencing analysis. The misexpression phenotypes of *Six3* and *Six6* also differ from each other. Forced expression of *Six3* induces the formation of ectopic retinal tissue and lens material [110,111]. Ectopic expression of *Six6*, on the other hand, is sufficient to induce retinal tissue but not lens material [26,112]. This difference may reflect the differing roles that these proteins play in normal development. While both genes are expressed in the optic vesicle, neural retina and lens, *Six6* expression is maintained at higher levels within the retina and is completely lost from the lens during later embryonic stages [22,113].

Interestingly, both vertebrate members of the DSix4 group also appear to play a role in the eye, although the role for *Six5* is more clearly understood. *Six5* is first expressed in the embryonic retina and then later in the adult lens [20,114]. Mice deficient for *Six5* are characterized by myotonic dystrophy (DM) of which ocular cataracts is one feature [115,116]. Interestingly, defects in the neural retina do not appear in these mice, which then begs the question of what role *Six5* plays in the retina and whether its function is redundant with the other SIX proteins. A connection between human *Six5* and DM-associated cataracts has proved somewhat tenuous as sequencing of the *Six5* locus in several patients suffering from DM has failed to identify any lesions within the coding regions or 5' and 3' untranslated regions [117]. However, the regulatory regions of *hSix5* were not analyzed, and it is quite possible that a mutation with a regulatory sequence may be responsible for the ocular defects in DM patients. The role of *Six4* in the retina is even less clear. *Six4* is expressed in the retina but to date retinal loss-of-function phenotypes have not been recorded in either mouse model systems or human patients [19,20,32,118].

To grow or not to grow, that is the question

Many tissue determining or patterning genes are known to also regulate the process of tissue growth and programmed cell death. The SIX genes appear to be no exceptions. Eye discs of *so* loss-of-function mutants are only a fraction of the size of their wild-type counterparts (Fig. 7A, B) [4]. This feature is commonly found in all RD network member loss-of-function mutations [12, 67, 119–121]. It is thought that a combination of reduced cell proliferation and increased cell death is responsible for the reduced retinal primordium. Interestingly, retinal mosaic clones of *so* mutants have significantly different phenotypes from discs that are completely mutant for *so*. Depending upon the location of the mutant patch of tissue within the eye field, either the mutant cells themselves or the surrounding wild-type tissue overproliferates (Fig. 7C) [66]. These observations strongly suggest that *So* may function not only in tissue specification but also in tissue growth. A similar conclusion is drawn from vertebrate studies. *Six1*-deficient mice have decreased levels of cell proliferation and increased levels of apoptosis in the mouse otic vesicle [58, 63]. Consistent with this is an earlier observation in which expression of *Xenopus* *Optix2* (*XOptix2*) leads to retinal field enlargement that is dependent upon increased levels of proliferation [26].

The processes of tissue growth and specification/patterning are intricately linked. A tissue needs to grow to a pre-ordained dimension; no more, no less. However, to achieve the correct size it needs to also keep track of how many cells are being specified/patterned. In the case of the fly eye, the final adult structure contains approximately 800 individual unit eyes or ommatidia. Early in development the tissue is mainly concerned with producing large numbers of cells. However, as undifferentiated cells are organized and transformed into ommatidia, cell proliferation levels are reduced and are eventually halted. The final retinal display of 800 unit eyes is thereby created by constantly checking the numbers of still uncommitted cells against the number of ommatidia and adjusting the rates of cell proliferation and apoptosis accordingly. SIX proteins may serve as a nexus point for proliferation and specification in the retina and other non-retinal tissues such as the otic vesicle. But how do SIX transcription factors regulate tissue growth?

Recent observations suggest that SIX proteins influence the cell cycle by directly regulating the transcription of key supervisors of the G2/M transition (Fig. 8A). The first indication came from a study that showed increased levels of human Six1 mRNA in breast cancer tissue [122]. Further analysis indicated that hSix1 is sufficient to push cells past the arrest at the G2/M checkpoint caused by irradiation induced DNA damage. Under normal circumstances this checkpoint is manned in part by a complex consisting of cyclins and cyclin-dependent kinases (cdks). The activity of the cyclin-cdk complex is itself regulated by opposing activities of the Wee1 kinase and the cdc25 phosphatase [123–125]. Two recent reports have indicated that the *Drosophila* cdc25 homolog, *string*, and mammalian *cyclin A* are direct transcriptional targets of So and Six1, respectively [126,127]. Both proteins are known to be critical players at the G2/M transition [128–132].

Additional evidence points to the G1/S transition as another checkpoint that is under control of SIX proteins (Fig. 8A). Six1 activates expression of *c-Myc*, a transcription factor that plays a critical role in pushing cells into S phase, and cyclin D, another major regulator of the G1/S checkpoint [79,133]. Six6, by complexing with the Dach1 co-repressor, inhibits the transcription of the cdk inhibitor (CDKI) *p27/Kip1* [80]. One of the main targets of p27 activity is the cyclin D/E-Cdk2 complex, a key regulator of the G1/S transition [134]. It should be noted that p27/Kip1, like other CDKIs, interacts with several cyclin-cdk complexes [135]. Therefore, other checkpoints, particularly the G2/M transition, may also be influenced by Six6 regulation of *p27/Kip1* expression. Several interesting and obvious questions arise from these results. Do the other SIX family members play roles in regulating the cell cycle? Do SIX proteins activate/repress the transcription of other cell cycle genes? Is the influence of the SIX proteins limited to the G1/S and G2/M transitions or is passage through other checkpoints dependent upon SIX factors?

Cell cycle regulation of SIX proteins

Many regulators of the cell cycle are themselves regulated in a cell cycle-dependent manner and SIX transcription factors are no exception. They are regulated by three distinct mechanisms: at the level of transcription, through post-translational modifications (phosphorylation) and *via* proteasome-mediated degradation (Fig. 8B). An analysis of human Six1 expression in a mammary carcinoma cell line demonstrated that hSix1 expression is silenced during G1 phase, initiates in S phase, continues through G2 but is then halted during mitosis [122]. A microarray analysis has indicated that Six1 expression is regulated by E2F1, a member of the E2F family that serves in transcriptional activation [136]. Among their many roles in the cell, subsets of E2F proteins regulate the entry into the cell cycle as well as passage through the G1/S transition.

It appears that phosphorylation of SIX proteins may be a critical step for progression of the cell cycle. An elegant study by Ford and co-authors [137] has demonstrated that hSix1 is a phosphoprotein that is hyperphosphorylated during the G2/M transition by casein kinase 2. In this report the authors further demonstrated that the relevant CK2 sites reside within the CT region and that elevated phosphorylation levels diminished the ability of hSix1 to actively bind DNA, as measured by hSix1 bound to the MEF3 site of the aldose A promoter in mitotic *Xenopus* extracts. An alignment of fly and mammalian SIX proteins indicates that each protein harbors several putative CK2 sites. It is thus possible that multiple SIX proteins are similarly regulated during the cell cycle. As CK2 is ubiquitously expressed throughout the cell cycle, it opens the possibility that passage through other checkpoints may require similar modifications of other SIX proteins. Alternatively, it may be that all (or many) SIX proteins are hyperphosphorylated at the G2/M transition. An additional issue that may have clinical relevance is whether the phosphorylation state of SIX proteins is linked to the status of a cancer cell.

A recent study has revealed an additional layer of regulation in which SIX proteins levels are regulated in a cell cycle-dependent manner by the ubiquitin-proteasome pathway [82]. The authors show that this degradation is mediated by the APC in which Cdh1 is the active subunit (APC^{cdh1}). A molecular dissection of Six1 demonstrated that, while the full-length protein is degraded in the presence of Cdh1, removal of either the NT or the CT segments stabilizes Six1. The destruction of Six1 is mediated by direct interactions between Cdh1 and portions of the NT segment of Six1. This is interesting since the canonical D-box and KEN box sequences as well as several known non-canonical recognition motifs, all of which are bound by Cdh1, are not found within Six1. The degradation of Six1 appears to occur during the later phases of mitosis, resulting in a complete elimination of Six1 by the M/G1 transition. It will be interesting to determine if APC^{cdh1}-mediated protein destruction is a common method for regulating SIX proteins during the cell cycle, and if this system is somehow misregulated in metastatic tumor cells.

SIX proteins and cancer

The role of SIX proteins in the cell cycle is significant as their misregulation may be important in cancer and tumorigenesis. The first indication that SIX proteins played such a role came from a screen for genes that were regulated in a cell cycle-dependent manner in human mammary carcinoma cells [122]. In this study hSix1 was first identified as a gene that is highly expressed in primary cancers and metastatic lesions of mammary tissue (hSix1 is normally expressed in embryonic but not terminally differentiated breast cells). Other cancerous cell lines and tissue samples that represent chronic myelogenous leukemia, lung carcinoma, colorectal adenocarcinoma, Burkitt's lymphoma, hepatocellular carcinoma, ovarian carcinoma and rhabdomyosarcoma also show elevated levels of expression when measured against comparable normal tissue samples [122,138–140]. Elevated Six1 levels, at least in some cases, appear to be sufficient to induce cell proliferation and tumorigenesis [127,141]. Gene amplification has been identified as one mechanism underlying the increased levels of Six1 in several breast cancer cell lines [142]. The extent to which SIX proteins regulate other types of cancers remains to be determined, but the data gathered so far suggest that this protein family has a significant role to play in cancer biology.

Concluding remarks

The SIX family of transcription factors, originally identified as key determinants of *Drosophila* retinal development, play significant roles in the construction of multiple vertebrate tissues and organs. More recent studies indicate that these proteins also play critical roles in regulating several transition points of the cell cycle. Together, a picture is emerging in which

the SIX proteins serve as nexus points for tissue growth and patterning. This contention is supported by a growing body of evidence indicating that the misregulation of SIX transcription and/or post-translational modifications is an underlying cause for a wide range of primary cancers and metastatic lesions. The next several years promise to be exciting as more information on the roles that SIX proteins play in development, growth and disease emerge.

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Figure 1.

The sine oculis homeo-box (SIX) proteins in *Drosophila* and mammalian systems: Membership and structure. A schematic diagram of the SIX proteins found in flies and mice. Each color represents a subgroup within the SIX family. The first protein in each subgroup represents the fly protein.

Figure 2.

Model for SIX family duplication events. Model describing the path of evolution for the SIX genes that are found within *Drosophila* and vertebrate lineages. It is based on neighbor-joining phylogenetic tree analysis and BLAST searches.

Figure 3.

Sequence comparisons of SIX and homeodomain regions. A ClustalW alignment of the two conserved domains that are found within the SIX proteins of flies and mice. An explanation of the color code is in the bottom right corner of the figure. The asterisks represent in (A) note residues 47, 50, 51 and 54 of the homeobox nucleic acid recognition domain (HD) recognition helix. The arrow in panel A notes that position 50 of the HD is occupied by a lysine residue (K50) thus placing SIX proteins within the Otd subgroup of homeodomain containing proteins. The arrow in (B) denotes a valine residue that when changed to an aspartic acid converts So protein into a moiety with dominant negative activity.

Figure 4.

Sequence comparison of the C-terminal (CT) region of the Optix clade members. A ClustalW alignment of the CT regions of *Drosophila* Optix and murine Six3 and Six6. The gray regions simply denote regions within the CT tails that share some homology across all three proteins.

Figure 5.

SIX genes in fly retinal development: Expression patterns and phenotypes. (A) Adult wild type. (B) Adult *so*[1] loss-of-function mutant. (C) Wild-type eye-antennal imaginal disc stained with an antibody that recognizes *So* protein. The expression is found ahead and behind the MF and within the ocelli. (D) An adult fly in which *so* was expressed in a non-retinal section of the head. The yellow arrow marks the presence of an ectopic eye. (E) Wild-type eye-antennal imaginal disc stained with an antibody that recognizes *Optix* protein. Note that expression is restricted to regions anterior to the furrow and to a region anterior to the medial ocellus. MF, morphogenetic furrow.

Figure 6.

The retinal determination network in *Drosophila*. (A) A schematic of the eye specification cascade. The colored arrows indicate the direction of interactions and the level to which these have been verified. A key to the color code is in the bottom right of the panel. (B) A list of the nuclear factors present within the cascade along with the vertebrate homologs, domain structure and associated mammalian retinal disease

Figure 7.

SIX proteins regulate cell proliferation in the retina. (A–C) Confocal images of eye-antennal imaginal discs. (A) A wild-type disc stained with phalloidin, which marks F-actin. (B) A *so*[1] loss-of-function mutant stained with phalloidin, which marks F-actin. Note the smaller eye disc. (C) An eye-antennal imaginal disc containing a patch of tissue that is homozygous mutant for the *so*[3] null mutant. The arrow marks the clonal area that is overproliferating. The disc is stained with an antibody that recognizes ELAV, an RNA binding protein found in neurons

Figure 8. SIX proteins and the cell cycle. (A) A schematic illustrating the known points at which SIX proteins influence the cell cycle. Note that SIX proteins appear to modulate the G1/S and G2/M transitions. (B) Schematic illustrating the three different ways that SIX gene expression and proteins are modulated. The grayscale code key is in the top left corner of the panel.

Table 1

SIX genes in vertebrate systems.

Genus species	Six1	Six2	Six3	Six4	Six5	Six6	Common name
<i>Ateles geoffroyi</i>	X						spider monkey
<i>Bos taurus</i>	X	X	X	X	X	X	domestic cow
<i>Canis familiaris</i>	X	X	X	X	X	X	dog
<i>Carassius auratus</i>			X				goldfish
<i>Cavia porcellus</i>	X	X		X	X	X	guinea pig
<i>Danio rerio</i>	X	X	X	X		X	zebrafish
<i>Dasyatis novemcinctus</i>	X	X		X		X	armadillo
<i>Echinops telfairi</i>	X	X				X	hedgehog
<i>Equus caballus</i>	X	X		X	X	X	horse
<i>Erinaceus europaeus</i>	X	X		X	X	X	hedgehog
<i>Felis catus</i>					X	X	cat
<i>Gallus gallus</i>	X	X	X	X		X	chicken
<i>Gasterosteus aculeatus</i>	X	X	X	X	X	X	stickleback fish
<i>Gorilla gorilla</i>	X					X	gorilla
<i>Homo sapiens</i>	X	X	X	X	X	X	human
<i>Lagothrix lagotricha</i>	X						wooley monkey
<i>Loxodonta africana</i>	X					X	elephant
<i>Lemur catta</i>	X						righttailed lemur
<i>Macaca mulatta</i>	X	X	X	X	X	X	rhesus monkey
<i>Macaca nemestrina</i>	X						pigtail macaque
<i>Microcebus murinus</i>	X	X		X	X	X	mouse lemur
<i>Monodelphis domestica</i>	X	X	X	X	X	X	opossum
<i>Mus musculus</i>	X	X	X	X	X	X	mouse
<i>Myotis lucifugus</i>	X			X	X	X	brown bat
<i>Ochotona princeps</i>	X	X		X		X	pika
<i>Ornithorhynchus anatinus</i>	X	X		X	X	X	platypus
<i>Oryctolagus cuniculus</i>	X			X	X	X	rabbit
<i>Oryzias latipes</i>	X	X	X	X	X	X	rice fish (medaka)
<i>Otolemur garnettii</i>	X			X	X	X	small eared galago
<i>Pan paniscus</i>	X			X	X	X	bonobo

Genus species	Six1	Six2	Six3	Six4	Six5	Six6	Common name
<i>Pan troglodytes</i>	X	X	X	X	X	X	chimpanzee
<i>Petromyzon marinus</i>	X	X		X			sea lamprey
<i>Pleurodeles waltl</i>			X				newt
<i>Pongo pygmaeus</i>	X	X	X	X	X	X	orangutan
<i>Rattus norvegicus</i>	X	X	X		X	X	rat
<i>Saguinus labiatus</i>	X						red-bellied tamarin
<i>Sorex araneus</i>	X	X		X	X	X	common shrew
<i>Sperm. tridecemlineatus</i>					X	X	ground squirrel
<i>Squalus acanthias</i>		X					spiny dogfish
<i>Takiugu rubripes</i>	X	X	X	X	X	X	pufferfish
<i>Terradon nigrovirdis</i>			X				pufferfish
<i>Tupaia belangeri</i>	X			X		X	tailshrew
<i>Xenopus tropicalis</i>	X	X	X	X		X	frog
<i>Xenopus laevis</i>	X	X	X			X	frog

Table 2

SIX genes within invertebrate systems.

Genus species	Sine oculis Six1/2	Optix Six3/6	DSix4 Six4/5	Common name
<i>Anthopleura elegantissima</i>	X			anemone
<i>Apis mellifera</i>	X	X	X	honey bee
<i>Aurelia aurita</i>	X			jellyfish
<i>Ceanorhabditis elegans</i>	X	X	X	nematode
<i>Chrysaora colorata</i>	X			purple striped jellyfish
<i>Ciona savignyi</i>		X		sea squirt
<i>Cladonema radiatum</i>	X	X	X	root arm medusa
<i>Crassostrea gigas</i>	X			giant oyster
<i>Cyanea capillata</i>	X			lion's mane jellyfish
<i>Drosophila melanogaster</i>	X	X	X	
<i>Euperipatoides kanangrenis</i>		X		onychophoran
<i>Girardia tigrina</i>		X		planarian
<i>Haliciona sp.</i>	X			purple sponge
<i>Leucosolenia eleanor</i>	X			tube ball sponge
<i>Macrostomum lignano</i>	X		X	flatworm
<i>Memiopsis leidyi</i>	X			comb jelly
<i>Nasonia vitripennis</i>	X			jewel wasp
<i>Nutricula tantilla</i>	X			clam
<i>Oikopleura dioica</i>	X	X		tunicate
<i>Platynereis dumerilii</i>	X			marine annelid
<i>Podocoryne carnea</i>	X	X		jellyfish
<i>Porites astreoides</i>	X			mustard hill coral
<i>Pristina longiseta</i>		X		freshwater annelid
<i>Rhabdocalypus dawsoni</i>	X			hexactinellid sponge
<i>Saccoglossus kowalevskii</i>		X		acorn worm
<i>Strigamia maritima</i>		X		centipede
<i>Strongylocentrotus purpuratus</i>		X		purple sea urchin
<i>Tribolium castaneum</i>	X	X	X	red flour beetle
<i>Urechis caupo</i>	X			innkeeper worm